THESE

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Marie HUSSON

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Etude des mécanismes régulateurs de la réponse immunitaire innée de *Drosophila melanogaster*

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Dr. Michèle CROZATIER	Directeur de recherche, Toulouse	Rapporteur externe
Dr. Frédérique PERONNET	Chargée de recherche, Paris	Rapporteur externe
Pr. Serge POTIER	Professeur, Strasbourg	Examinateur interne
Pr. Jean-Marc REICHHART	Professeur, Strasbourg	Directeur de thèse

« Le matin, le soir, avant et après son travail, à chaque instant, s'arrêtant même au milieu de ses jeux, il fixait les yeux sur l'homme de la montagne et plus il le regardait et plus il l'aimait, et plus il l'aimait et plus il lui ressemblait.»

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Abréviations

Ago : Argonaute
AMP : Anti-Microbial Peptide
AP-1 : Activator Protein-1
ARNdb : ARN double brin
ARNi : ARN interférant
ARNsb : ARN simple brin
Bsk : Basket
CRP : C-Reactive Protein
CrPV : Cricket Paralysis virus
DAF : Decay Accelerating Factor
DAP-PGN : *meso*-diaminopymélic acid type Peptidoglycan
DAPT : N-[N-(3,5- difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester
Dcr : Dicer
DCV : Drosophila C Virus
DIAP : Drosophila Inhibitor of Apoptosis
DIF : Dorsal-related Immune Factor
Dome : Domless
DNA : Deoxyribonucleic acid
DREDD : Death Related ced-3/Nedd2-like protein
DUOX : Dual Oxydase
DXV : Drosophila X Virus
20E : 20-hydroxy-ecdysone
endo-siRNA : endogenous siRNA
EB : Enteroblast
EC : Entrocyte
EE : Enteroendocrine
ERK : Extracellular signal-Regulated Kinase
FADD : Fas (TNFRSF6)-associated via Death Domain
FHV : Flock Hous Virus
dFOXO : drosophila Forkhead box sub-group O
GNBP : GlucaN Binding Protein
GSC : Germinal Stem Cell
Hep : Hemipterous
IAP : Inhibitor of Apoptosis
IBM : IAP Binding Motif
IGF-1 : Insulin Growth Factor-1
IMD : Immun Deficiency
Indy : I'm Not Dead Yet
InR : Insulin Receptor
I-kB : Inhibitor of kB
IKK : IkB Kinase
IL : Interleukin
IMD : Immune Deficiency
IP3 : Innositol 1,4,5-triphosphate

ISC : Intestinal Stem Cell
Jak/STAT : Janus Kinase/Sigal Transducer and Activator of Transcription
JH : Juvenile Hormone
JNK : c-Jun N-terminal Kinase / hopscotch
LPS : LipoPolySaccharide
MAPK : Mitogen Activated Protein Kinase
miRNA : micro RNA
mth : methuselah
NAG : *N*-acetyl-glucosamine
NAM : *N*-acetyl-muramique
NF-kB : Nuclear Factor-kB
nt : nucleotide
PAM : Peptide AntiMicrobien
piRNA : piwi interacting RNA
PGRP : PeptidoGlycan Recognition Protein
PGN : Peptidoglycan
PO : Phenoloxydase
PPO : Prophénoloxydase
PRR : Pattern Recognition Receptor
Puc : Puckered
Q- RT- PCR : Quantitative Real Time PCR
RIP : Receptor Interacting Protein
RISC : RNA Inducing Silencing Complex
ROS : Reactive Oxygen Species
RpL32 : Ribosomal protein L32
SPE : Spätzle Processing Enzyme
siRNA : Small interfering RNA
Stwl : Stonewalling
TAB : TAK1 Associated Binding protein
TAK : TGFb-Activated Kinase
TCT : Tracheal Cytotoxin
TEP : ThioEster-related Protein
TG : TransGlutaminase
TLR : Toll Like Receptor
TNF : Tumor Necrosis Factor
TotA : Turandot A
TOR : Target of Rapamycin
TNF : Tumor Necrosis Factor
TRAF : TNF Receptor Associated Factor
SINV : Sindbis virus
Upd : Unpaired
USP : Ultraspiracle
Vir-1 : Virus induced RNA 1
VSV : Vesicular Stomatitis Virus

La défense des animaux contre les infections repose sur une réponse immunitaire innée, immédiate, complétée par une réponse adaptative chez les vertébrés. L'activation des facteurs NF-κB est le principal signe de la réponse immunitaire innée dans tout le règne animal. La plupart des éléments impliqués au niveau des voies d'activations des facteurs NF-κB sont hautement conservés depuis les insectes jusqu'à l'homme. Cette conservation évolutive ainsi qu'un grand nombre d'outils disponibles et en particulier la génétique font de la *Drosophile* un excellent modèle pour l'analyse de la réponse immunitaire innée.

Mon projet a consisté en la caractérisation des mécanismes régulant la réponse immunitaire innée chez *Drosophila melanogaster*. Pour cela ma thèse a été divisée en trois axes, traitant chacun un aspect différent de la réponse immunitaire innée.

Nous avons tout d'abord analysé les mécanismes moléculaires mis à l'œuvre dans la voie de transduction du signal de la voie Imd, similaire à celle en aval du récepteur au TNFα. A cette occasion un site de clivage entre les résidus 30 et 31 de la protéine Imd a été mis en évidence à la fois *in vitro* et *in vivo.* Le clivage d'Imd en cette position permet la liaison de DIAP2 et la polyubiquitination de la protéine Imd pour activer en aval les autres composants de la voie puis *in fine* la transcription des PAM.

Dans un second temps, nous avons montré que le mutant *Methuselah,* dont l'espérance de vie est supérieure de 35% aux drosophiles sauvages, résiste beaucoup mieux aux infections que des mouches sauvages. Afin de mieux comprendre les raisons de cette résistance, nous avons analysé l'influence de cette mutation sur l'activation de la réponse immunitaire. De manière surprenante, nous avons observé un sauvetage de la mutation *Imd*. Il semblerait que celui ci soit dû au rétablissement de l'expression des PAM après infection.

Et pour finir, toujours dans le registre de la régulation de l'immunité innée chez la drosophile, nous avons étudié le rôle des voies impliqués dans la biosynthèse des petits ARN non codant. Il a été établi que leur absence affecte la survie des *Drosophiles* infectées par voie naturelle, principalement au niveau de l'intestin. C'est la première fois que l'on attribue une fonction à une classe de petits ARN non codant endogènes, dits endo-siARN.

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Introduction générale sur l'immunité innée de *Drosophila melanogaster*

I. *DROSOPHILA MELAGONASTER :* UN MODÈLE D'ÉTUDE DE LA RÉPONSE IMMUNITAIRE INNEE

B. Un outil de la génétique moderne

Cela fait exactement 100 ans que le Dr. Thomas Morgan publia le résultat de ses recherches sur l'hérédité commencées en 1906. En Mai 1910, il découvre un mâle mutant aux yeux blancs dans son élevage de *Drosophila melanogaster*. Un mois plus tard, après plusieurs croisements, il réalisa que la première génération de drosophile issue d'un mâle mutant aux yeux blancs et d'une femelle sauvage aux yeux rouges possède les yeux rouges. Dans la seconde génération, la moitié des mâles possèdent les yeux blancs. Il en déduit rapidement que le gène responsable de la couleur des yeux est un gène au caractère récessif. Ce gène est porté par l'un des gonosomes car parmi les descendants d'un croisement entre une femelle mutante aux yeux blancs et un mâle aux yeux rouges, seuls les males héritent du caractère « yeux blancs ». Les travaux de T. Morgan auront non seulement confirmé la théorie de G. Mendel mais aussi mis en évidence que les gènes sont portés par des chromosomes et forment ainsi la base physique de l'hérédité.

Drosophila melanogaster s'est très vite imposé comme un outil incontournable de la recherche en génétique. Plus connue sous la dénomination de « mouche à vinaigre », la drosophile, mesurant 3 mm de long, est facile à reproduire en laboratoire. A 25°C le développement est d'environ 10 jours entre la ponte de l'œuf et l'émergence de l'adulte fertile. Son génome est réparti sur quatre paires de chromosomes et seules les femelles sont douées de recombinaison méiotique. Un grand nombre d'outils génétiques ont également été développés pour étudier la biologie de la drosophile (les chromosomes balanceurs, les transposons, la transgénèse, l'ARN interférant). Le génome de la drosophile séquencé en 1998 contient environ 14 800 gènes. 80% du génome de la drosophile ne code pas pour des protéines et deux tiers de ces séquences régulent l'expression des gènes (Halligan DL et

Keightley 2006). Ajoutons à cela que 50% des séquences protéiques de la drosophile possèdent un homologue chez les mammifères. Ceci fait de la drosophile un bon modèle d'étude génétique pour différentes maladies humaines comme les maladies d'ordre neuro-dégénératif tels que la maladie de Parkinson, Huntington et la maladie d'Alzheimer. La drosophile est également utilisée pour étudier les mécanismes impliqués au cours du vieillissement, du stress oxydatif, du diabète et de l'immunité innée.

C. Une immunité innée conservée

Sous la pression sélective imposée par divers pathogènes, les organismes multicellulaires ont développé différents mécanismes de défense activés par l'infection et ayant la capacité de les protéger. Au cours de l'évolution deux systèmes immunitaires de lutte contre les agents infectieux ont été sélectionnés : l'immunité innée et l'immunité adaptative. L'immunité adaptative présente deux caractéristiques essentielles, à savoir la spécificité, fondée sur un large répertoire de molécules de reconnaissance qui résultent d'un réarrangement somatique (Immunoglobulines, récepteur des cellules T) mais aussi la mémoire par la sélection clonale de lymphocytes spécifiques. L'immunité adaptive apparait il y a plus de 640 millions d'années avec l'ancêtre des poissons cartilagineux (Douzery *et al*., 2004) et n'existe que chez les vertébrés. L'immunité innée est phylogénétiquement plus ancienne et existe vraisemblablement chez tous les métazoaires. Chez les mammifères, elle constitue la première ligne de défense contre les infections. Les mécanismes de reconnaissance de l'immunité innée font intervenir des récepteurs capables de reconnaître des molécules aux motifs structuraux conservés, présents sur la paroi des procaryotes ou des champignons, mais absents des cellules hôtes. Charles Janeway propose, dès 1989, que de tels récepteurs soient appelés *pattern recognition receptors* (PRR) et suggère qu'ils soient impliqués dans le déclenchement de la réponse innée (Medzhitov *et al* ., 1997). Chez les mammifères, plusieurs PRR ont été

identifiés: par exemple CD14, le récepteur du LPS (Ulevitch *et al*., 1997), les collectines (Epstein *et al* ., 1996) et des récepteurs à large spectre de reconnaissance comme le scavenger receptor (Pearson, 1996). Ces récepteurs constituent une forme primitive de reconnaissance du non-soi. Plusieurs travaux suggèrent l'existence de tels récepteurs chez les insectes. Ainsi, une protéine du lépidoptère Bombyx mori, capable de se fixer aux parois des bactéries à Gram négatif a récemment été isolée et a été nommée GNBP (gram negative binding protein). Cette molécule est inductible après stimulation bacté- rienne et présente des similitudes de séquence partielles avec CD14 (Lee *et al*., 1996). D'autres protéines présentant des similitudes structurales ou fonctionnelles avec des récepteurs de mammifères (scavenger receptor, CD36) ont été décrites chez la drosophile mais aucune preuve n'a été apportée quant au rôle de ces récepteurs dans le déclenchement de la réponse anti-microbienne de la drosophile (Franc *et al*., 1996 ; Pearson *et al*., 1995).

A la fin des années 90, Bruno Lemaitre démontre que le récepteur Toll de la drosophile, jusqu'alors connu pour son rôle au cours de l'embryogénèse est également très important dans la défense anti-fongique et anti-bactérienne de type Gram-positif de la drosophile (Lemaitre *et al*., 1997 ; Rutschmann *et al*., 2002). Cette découverte majeure a été suivie chez les mammifères par l'identification des « Toll Like Receptors » (TLR) et leur rôle dans l'activation de la réponse immunitaire innée. Egalement conservée, la voie IMD découverte un an plus tôt chez la drosophile (Lemaitre *et al*., 1996) est apparentée à la voie du récepteur du TNF alpha chez les mammifères (Silverman *et al*., 2001) mais est aussi impliquée dans la défense contre les bactéries à Gram-négatif (Georgel *et al*., 2001).

Les résultats obtenus chez la drosophile indiquent des similitudes frappantes entre les mécanismes de défense antimicrobienne de la drosophile et des mammifères. Ils soulignent ainsi l'universalité et l'origine évolutive très ancienne de ces réactions immunitaires. Ainsi, la drosophile apparaît comme un modèle prometteur pour dévoiler les mécanismes qui règlent la réponse immunitaire innée chez l'homme.

II. ORGANISATION DE LA RÉPONSE IMMUNITAIRE

A. La barrière épithéliale

L'épithélium digestif, respiratoire et reproducteur de la drosophile est en interaction permanente avec différents types de pathogènes. Chez la drosophile, l'intestin et les trachées représentent des portes d'entrée aux infections. Cependant, le tractus digestif représente la source principale d'infection microbienne. La première ligne de défense antimicrobienne est une barrière physique appelée *matrice peritrophique* et sécrétée par le proventriculus (King DG 1988). Principalement composée de chitine et de protéines, son rôle est similaire au mucus des vertébrés (Terra 2001). Cette matrice tapisse le tube digestif et le protège du passage des microorganismes vers l'hémolymphe. De plus, la lumière intestinale est un environnement très hostile à la multiplication de microorganismes. Ceci est principalement dû à la sécrétion de lyzozymes par les cellules intestinales (Daffre *et al*., 1994 ; Hultmark, 1996) ainsi qu'à la production de peptides antimicrobiens (PAM) et de ROS (Reactive Oxygen Species) (Ha *et al*., 2005) qui représente une seconde ligne très efficace protection contre les infections microbiennes.

B. L'hémolymphe

Les microorganismes qui arrivent à passer la barrière épithéliale entrent dans une cavité interne appelée l'hemocoele, contenant l'hémolymphe. L'hémolymphe ne possède pas de pigments respiratoires. Le système circulatoire de la drosophile est un système ouvert, extrêmement simple. La respiration est réalisée par le système trachéal. L'hémolymphe est pompée vers l'avant au travers d'un élément simple, le vaisseau dorsal (Hertel et Pass 2002). Chez les insectes, l'hémolymphe distribue métabolites, hormones, eau et déchets aux organes appropriés. Elle contient également les cellules sanguines ou hémocytes. C'est également

dans l'hémolymphe que les pathogènes sont reconnus et combattus par la réponse cellulaire ou humorale.

1. L'hématopoïèse

Chez la drosophile, l'hématopoïèse se divise en deux temps. Il est possible de distinguer une première vague hématopoïétique ayant lieu au niveau du mésoderme pro-encéphalique puis une seconde vague hématopoïétique au cours du développement larvaire au niveau du ganglion lymphatique.

La phase embryonnaire de l'hématopoïèse génère un nombre fixe d'hémocytes matures dont 700 plasmatocytes et 30 cellules à crystaux. Ces hémocytes migrent de leur point d'origine et se répandent dans l'embryon. Contrairement aux hémocytes, les cellules à cristaux ne migrent pas et restent localisées au niveau du proventriculus.

La seconde vague hématopoïétique à lieu à la fin du stade embryonnaire, au moment même où le ganglion lymphatique se forme au niveau du mésoderme thoracique dorsal de l'embryon. Au stade 3 du développement larvaire, le ganglion lymphatique est composé de deux à sept lobes répartit le long du vaisseau dorsal correspondant au tube cardiaque dans le système circulatoire de la drosophile. De la fin de l'embryogenèse au second stade larvaire le ganglion lymphatique se compose d'une seule paire de lobe appelée lobes antérieurs ou primaires. En début du troisième stade larvaire (L3) se forme les lobes postérieurs, dits aussi secondaires. Les lobes secondaires sont des réservoirs de prohémocytes immatures. Les lobes primaires contiennent une zone médullaire (ZM) contenant les progéniteurs hématopoïétiques (prohémocytes), une zone corticale (ZC) contenant des hémocytes (plasmatocytes et cellules à cristaux) déjà différentiées et le centre de signalisation postérieure (CSP) (Fig.1B). La différenciation de hémocytes dans le ganglion lymphatique est tout d'abord observée au niveau des lobes primaires au début du stade L3 (Lebestky *et al*., 2000; Jung *et al*., 2005). Dans des larves seines, peu d'hémocytes se différencient au niveau des lobes secondaires.

Cependant, lorsque les larves sont infectées par la guêpe *Leptopilina boulardii* les hémocytes présents au niveau des lobes postérieurs se différencient en lamellocytes. Suite à la métamorphose, la plupart des pro-hémocytes se différencient en plasmatocytes, le ganglion se désintègre et déversent leur contenu dans l'hémolymphe (Crozatier et Meister M, 2007) (Lanot *et al.*, 2001).

La voie JAK/STAT a récemment été décrite pour son rôle dans l'homéostasie des hémocytes. En effet, l'activation de cette voie préserve le caractère prohémocytaire des cellules de la ZM (Krzemien *et al.*, 2007). Ainsi, la différenciation des hémocytes est liée à la perte de l'activité de la voie JAK/STAT dans la zone médullaire. La maintenance de la voie JAK/STAT dans la zone médullaire dépend de l'activité du CSP.

Le CSP a été initialement décrit comme un petit groupe de cellules au niveau du ganglion postérieur connu pour activer la voie Notch. Basé sur le rôle de la voie Notch dans la formation des cellules à cristaux, il a été proposé que ce petit groupe de cellules basées au niveau du ganglion postérieur agissent sur les cellules pour induire leur différenciation en cellules à cristaux (Lebestky *et al.*, 2003).

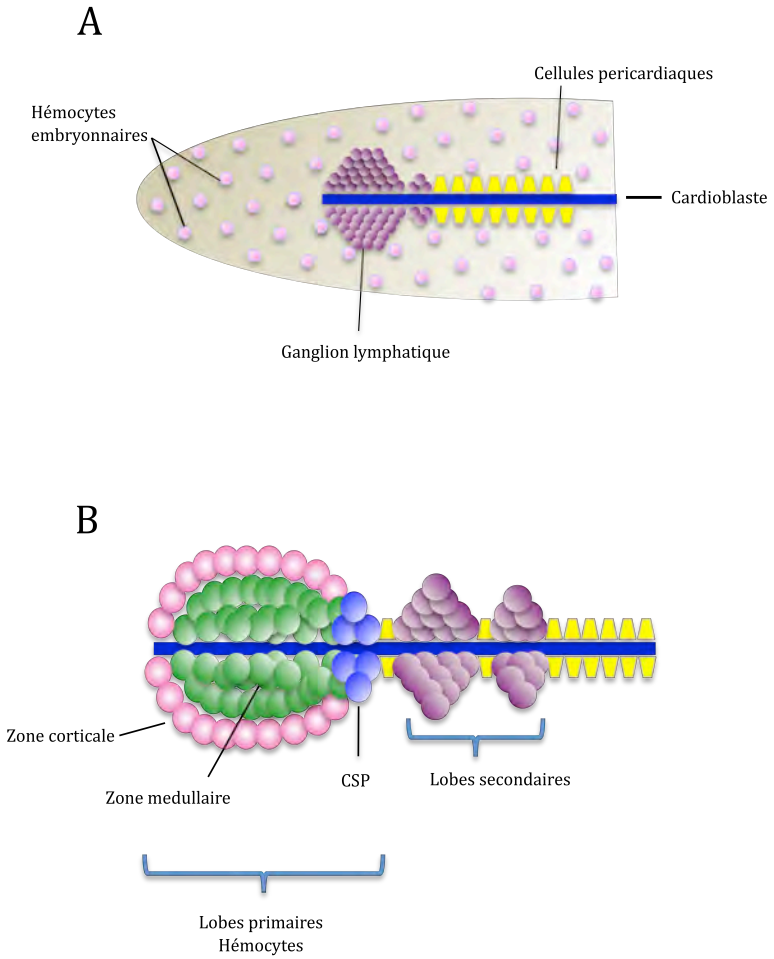


Figure 1. A. Formation du ganglion lymphatique. Chez l'embryon, les hémocytes (rose) sont présent tout au long de l'embryogenèse et persistent au stade larvaire. En parallèle les cellules du mésoderme se différencient en en cellules pericardiaques (jaunes) et en cardioblaste (bleu), future cellules du cœur et du ganglion lymphatique (prune). B. Au cours du premier et du second stade larvaire, le ganglion lymphatique subissent une expansion par la prolifération cellulaire. Ce n'est qu'à la fin du troisième stade larvaire que le lobe primaire se divise en trois zones : une zone corticale situé à l'extérieur du ganglion (rose), la zone médullaire au niveau interne (vert) et un centre de signalisation postérieur (bleu) (CSP). En condition normale, les hémocytes restent dans le ganglion lymphatique tout le long du stade larvaire.

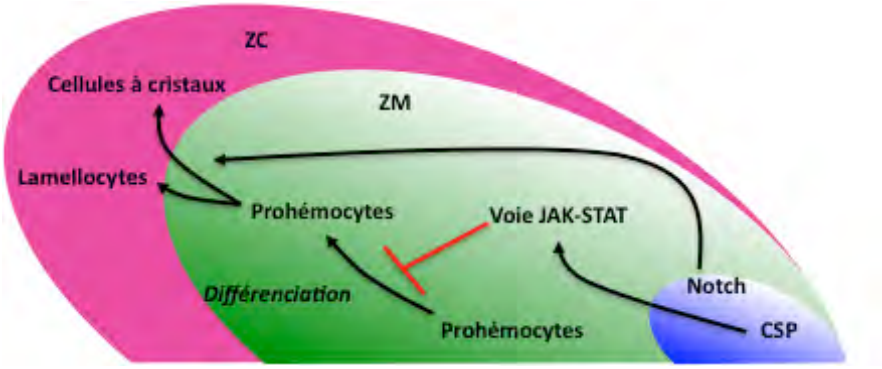


Figure 2. Représentation schématique du ganglion lymphatique au stade larvaire 3 (lobe antérieur/primaire). Trois zones sont représentées, le CSP (bleu), la zone médullaire (ZM) contenant des prohémocytes (vert) et la zone corticale (ZC) contenant les hémocytes (rose). Au cours du développement le CSP participe au maintient de la voie JAK-STAT dans la ZM en maintenant le stock de prohémocytes. L'expression de Notch se situe au niveau du CSP.

Comme nous venons de le mentionner, chez la larve de drosophile, l'hématopoïèse dépend de deux types cellulaires situés au niveau du ganglion lymphatique, les cellules hématopoïétiques primordiales qui sont à l'origine de trois types d'hémocytes et les cellules du CSP. Les cellules du CSP se divisent rarement, restent associées en cluster et agissent comme une niche contrôlant l'homéostasie des hémocytes au cours du stade L3. Les cellules hématopoïétiques primordiales se divisent activement pour générer un large groupe de progéniteurs avant que les hémocytes entre différenciation and début de stade L3. Krzemien et ses collaborateurs ont récemment proposé que les pro-hémocytes larvaires soient régulés comme une population plutôt que des cellules souches individuelle. Il a également été mis en évidence qu'un groupe de cellules non différenciée en mitose soit mélangées avec des hémocytes différenciés désignés comme des intermédiaires de progéniteurs et participant à l'augmentation du nombre d'hémocytes au stade L3 (Krzemien *et al.*, 2010).

2. Différentes classes d'hémocytes

Chez la drosophile, trois classes d'hémocytes participent à la réponse cellulaire : les plasmatocytes, les cellules à cristaux et les lamellocytes. Les plasmatocytes, majoritaires, sont comparables aux monocytes ou macrophages et ont pour fonction la phagocytose. Au stade embryonnaire et pupal, ils phagocytent les corps apoptotiques formés durant le processus de développement et de métamorphose. Au cours du stade larvaire et du stade adulte, les plasmatocytes participent à la réponse immunitaire par la phagocytose. Par ailleurs, les larves ne possédant pas de plasmatocytes, sont incapables d'activer la synthèse de PAM après une infection par voie orale (Charroux *et al.*, 2009 ; Shia *et al.*, 2009). Cependant, chez l'adulte, l'absence de plasmatocytes affecte sévèrement la survie des mouches en condition infectieuse alors que la synthèse de PAM n'est pas affectée. Cela prouve que chez la drosophile adulte la synthèse de PAM n'est pas dépendante des plasmatocytes, contrairement à la larve (Charroux *et al.*, 2009 ; Defaye *et al.*, 2009). Les cellules à cristaux ne représentent que 5% des hémocytes chez l'embryon et la larve. Comme leur nom l'indique, elles contiennent des cristaux de pro-phénoloxydase (forme inactive de la phénoloxydase) impliquée dans la cicatrisation des plaies. Elles disparaitront en début de métamorphose. Lorsqu'un corps étranger, trop gros pour être phagocyté pénètre l'organisme de la larve, les pro-hémocytes se différentient en lamellocytes pour encapsuler le corps étranger.

III. LA REPONSE ANTIMICROBIENNE

A. La réponse humorale systémique

Les insectes possèdent des mécanismes de défense très efficaces contre les infections qui ont vraisemblablement contribué à leur succès évolutif (Hoffmann et Reichhart, 1996). Ces mécanismes sont fondés sur des réactions cellulaires et humorales. Les premières impliquent

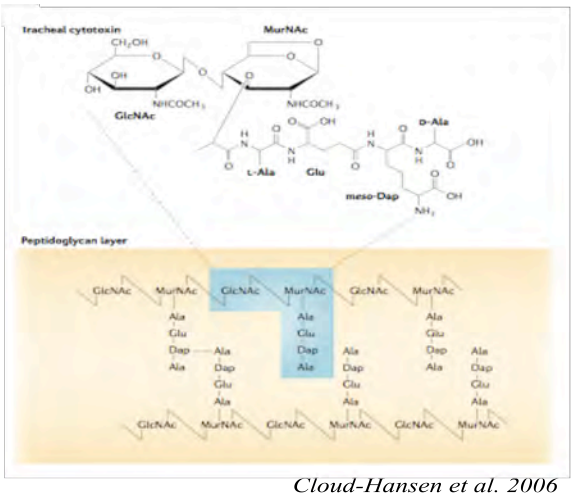
les hémocytes qui participent à l'encapsulation ou à la phagocytose des particules étrangères. Le volet humoral consiste, d'une part, en l'activation de cascades protéolytiques (coagulation et mélanisation) et, d'autre part, en la synthèse de peptides antimicrobiens (PAM). Les études menées chez la drosophile ont démontrées que l'expression des PAMs est dépendante de deux facteurs de transcriptions, DIF et Relish, tous deux appartenant à la famille de facteurs NF-kB et respectivement activés par la voie Toll et la voie Imd. A ce jour, les voies Toll et Imd sont les seules cascades intracellulaires connues pour être activées par des ligands microbiens.

1. La reconnaissance microbienne

Les mouches infectées par différentes classes de micro-organismes synthétisent des PAM (Lemaitre *et al*., 1997). Certains sont induits par les bactéries à Gram positif (Défensine (Dimarcq *et al*., 1994) Drosomycine (Lemaitre *et al*., 1997)), d'autres par les bactéries à Gram négatif (Diptéricine (Wicker *et al*., 1993), Drosocine (Bulet *et al*., 1993), Attacin (Asling *et al*., 1995)) ou encore par des champignons entomopathogènes (Drosomycine (Fehlbaum *et al*., 1994) et Metchnikowin (Levashina *et al*., 1995)). La reconnaissance microbienne nécessite un contact direct entre une protéine de l'hôte appelée PRR (*Pattern Recognition Receptor*) et une molécule d'origine microbienne. Il existe deux sortes de PRR, les protéines de reconnaissance des peptidoglycanes (PGN) dites PGRP (*PeptidoGlycan Recognition Protein*) et les protéines de liaison aux glucanes aussi appelées GNBP (*GlucaN Binding Proteins*).

Les PGRP

La famille des PGRP contient 13 membres. Ils possèdent un domaine PGRP commun, similaire à l'amidase de type II de bactériophage. La plupart des PGRP ont actuellement perdus l'activité amidase mais ont conservés la capacité à se lier au peptidoglycane (PGN) et servent de senseurs microbiens (Royet *et al*., 2007). Les PGRP sont classés en fonction de leur taille. Les protéines courtes, PGRP-SA, -SB, -SC et –SD sont des protéines sécrétées alors que les protéines longues, PGRP-LA, -LB, -LC, -LD, LE et –LF sont des récepteurs transmembranaires (Mengin-Leucreulx *et al*., 2005). Les trois PGRP SA, SD et LC sont des récepteurs non enzymatiques (Werner al., 2000) contrairement au PGRP-SC et PGRP-LB qui possèdent une activité enzymatique (Zaidman-Remy *et al*., 2006 ; Bischoff *et al*., 2006). Les PGRP reconnaissent un polymère de glucopeptide nommé le PGN. Ce dernier est un constituant des parois bactériennes. Il est formé d'une partie glucidique et d'une partie peptidique. La partie glucidique est un polymère de β(1-4) *N*-acétyl-glucosamine (NAG) et d'acide *N*-acétyl-muramique (NAM) reliés l'un à l'autre par des chaines peptidiques de trois acides aminé. Si les PGN sont présents dans la paroi de toutes les bactéries, ils diffèrent cependant de par la composition peptidique de leur chaine. La plupart des bactéries à Gram-positif ont en troisième position une lysine (Lys-type PGN) qui est remplacée par un acide diaminopimélique chez les bactéries à Gram-négatif et les bacilles (DAP- type PGN). La voie Imd est activée par le DAP- type PGN, alors que la voie Toll est activée par le Lys-type PGN (Leulier *et al*., 2003). D'autres études ont montrée que à la fois le DAP-type PGN monomérique et polymérique sont capables d'activer la voie Imd. Le TCT (Fig.3) (Tracheal cytotoxin) est un fragment monomérique de PGN relaché lors de la division cellulaire de cette bactérie. Il a été isolé pour la première fois dans le surnageant d'une culture de *Bordetella pertussis* (Cookson *et al*., 1989). Il s'agit de la plus petite structure capable d'activer la voie IMD (Kaneko *et al*., 2004).



Cloud-Hansen et al. 2006

Figure 3. Structure du peptidoglycane (PGN) et du TCT (tracheal cytotoxin). Chez les bactéries à Gram-négatif, le PGN est composé d'une chaine de Peptidoglycan N-acétylglucosamine (GlcNAc) et N-acetylmuramic acid (MurNAc) connectés l'un à l'autre par une chaine d'acides aminés alanine (Ala), acide glutamique (Glu) et un acide diaminopimélique (Dap). Le TCT dont la formule est la suivante, N-acetylglucosaminyl-1,6-anhydro-N- acetylmuramyl-L-alanyl-D-glutamyl-meso-diaminopimelyl-D-alanine, est une sous unité du PGN monomérique (921 dalton) de bactérie à Gram négatif de 921 Dalton.

Les glucanes

Les GNBP constituent la seconde famille des PRR. Leur séquence est similaire au glucanases d'origine bactérienne et est probablement un cas de transfert horizontal (Ferrandon *et al*. 2004; Lee *et al*. 1996). Cependant, les GNBP ne montrent aucune activité enzymatique et sont capables de se fixer aux β-1,3-glucans de champignons, au LPS ainsi qu'à l'acide lipoteicoïque de bactérie (Kim *et al*. 2000). Les drosophiles adultes sont aussi capables de reconnaître certains facteurs de virulence fongiques et bactériens. La reconnaissance de tels facteurs passe par l'activation de la protéase Perséphone (Gottar *et al*. 2006 ; El Chamy *et al*., 2009).

a) Reconnaissance des bactéries à Gram-négatif par PGRP-LC/LE

PGRP-LC est le récepteur majeur de la voie IMD (Gottar *et al*., 2002) et existe sous la forme de trois isoformes (LCa, LCx, LCy) dont le domaine intracellulaire est le même avec un domaine extracellulaire différent. PGRP-LCx reconnaît le PGN polymérique alors que PGRP-LCa et –LCx reconnaissent conjointement le PGN monomérique (Kaneko *et al*., 2004). PGRP-LE code pour un PGRP à la fois extracellulaire et intracellulaire mais ne possède pas de domaine transmembranaire. Un fragment de PGRP-LE correspondant au domaine PGRP seul agit à l'extérieur de la cellule et potentialise la reconnaissance du PGN par PGRP-LC. La forme complète de PGRP-LE agit comme un récepteur intracellulaire du PGN monomérique (Kaneko *et al*., 2006).

b) Reconnaissance des champignons et des bactéries à Gram-positif

Chez la drosophile, deux GNBP appartiennent aux PRR, GNBP-1 et -3. GNBP-1 active la voie Toll conjointement avec PGRP-SA lors d'une infection à bactérie Gram-positif (Gobert *et al*. 2003). La mutation perte de fonction *GNBP1* réduit la production de PAM et rend les mouches susceptibles aux infections par les bactéries à Gram-positif. Il a également été rapporté qu'une autre mutation perte de fonction dans le gène GNBP3 rend les mouches susceptibles aux infections fongiques (Gottar *et al*., 2006).

2. Voies d'activation de la réponse humorale

a) La voie Toll activée par les bactéries à Gram-positif et les champignons

La voie Toll (Fig. 4), conservée au cours de l'évolution, joue un rôle important dans le développement de l'axe dorso-ventral de l'embryon de drosophile. Les différences majeures entre les voies embryonnaires et immunitaires résident dans le facteur de transcription impliqué et dans la signalisation extracellulaire. Chez l'embryon, l'activation de la voie Toll mène à la translocation nucléaire de Dorsal alors que dans le cas d'une infection microbienne c'est le facteur de transcription DIF (*Dorsal Immun related factor*) qui rejoint le noyau (Ip *et al*., 1993).

Le récepteur membranaire Toll est activé par le clivage de la pro-cytokine Spätzle et sa fixation au récepteur. La pro-cytokine Spätzle est clivée par l'enzyme SPE (Spätzle processing enzyme). En amont de SPE se trouve deux nouvelles protéases, Grass (El Chamy *et al*., 2008) et ModSP (Buchon *et al*., 2009c). Cette cascade protéolytique est activée en amont par les PRR sécrétés (PGRP-SA, PGRP-SD, GNBP-1 et GNBP-3). Nous pouvons distinguer trois voies d'activation de SPE. PGRP-SA (Michel *et al*., 20001), PGRP-SD (Bishoff *et al*., 2004) et GNBP-1 (Gobert *et al* ., 2003)(Wang *et al*., 2006) participent à la reconnaissance des bactéries à Gram-positif. GNBP3 se lie aux levures après avoir reconnu de longues chaines beta-1,3-glucans de leur parois (Gottar *et al*., 2006). Il a également été montré que des protéases d'origines fongiques sont capables d'activer la protéine zymogène Perséphone (Ligoxygakis *et al*. 2002) menant à l'activation de l'enzyme SPE et *in fine* à l'activation de la voie Toll. Cet agent pathogène n'est pas directement reconnu par la nature des composant de sa paroi microbienne mais plutôt par ses facteurs de virulence (Gottar *et al*. 2006). Il a été récemment observé que l'activation de la voie Toll via Perséphone peut être mimée par l'injection de protéases exogènes. Suggérant que Perséphone est activée en réponse à un signal de danger (El Chamy *et al*. 2008). Necrotic, est une serpine inhibitrice de

Persephone et régule la voie Toll. L'abscence de Necrotic est suffisant pour activer de manière constitutive la voie Toll (Levashina *et al*., 1999).

Une fois clivée, la protéine Spätzle mature se lie au récepteur Toll et induit sa dimérisation (Hu *et al*., 2004). Il s'en suit une activation des différents membres intra-cellulaires de la voie : DmMyD88 et Tube (molécules adaptatrices), la kinase Pelle, Cactus et DIF pour la réponse anti-microbienne ou Dorsal au cours du développement embryonnaire (Belvin *et al*., 1996). Les trois protéines dMyD88, Tube et Pelle se lient l'une à l'autre grâce à un domaine d'interaction protéine-protéine *Death Domain* (DD). Par un mécanisme pour l'instant encore inconnu Cactus est phosphorylé puis dégradé par le protéasome (Spencer *et al*., 1999). Cela permet la libération et la translocation nucléaire du facteur NF-κB DIF qui était séquestré par Cactus dans le cytoplasme (Belvin et Anderson, 1996; Meng *et al*., 1999) (Lemaitre *et al*., 1995).

A l'exception de Cactus, la suppression d'un des membres de la voie Toll entraine une réduction de l'expression de différents PAM spécifiques de la voie Toll. (Lemaitre *et al*., 1996) (Rutschmann *et al*., 2002). L'absence de Dorsal peut être complémentée par l'expression de DIF (Stein *et al*., 1998) . Par contre il est impossible d'induire l'expression de PAM en surexprimant Dorsal dans un mutant Dif (Meng *et al*., 1999). Ceci démontre bien le caractère embryonnaire du facteur de transcription Dorsal.

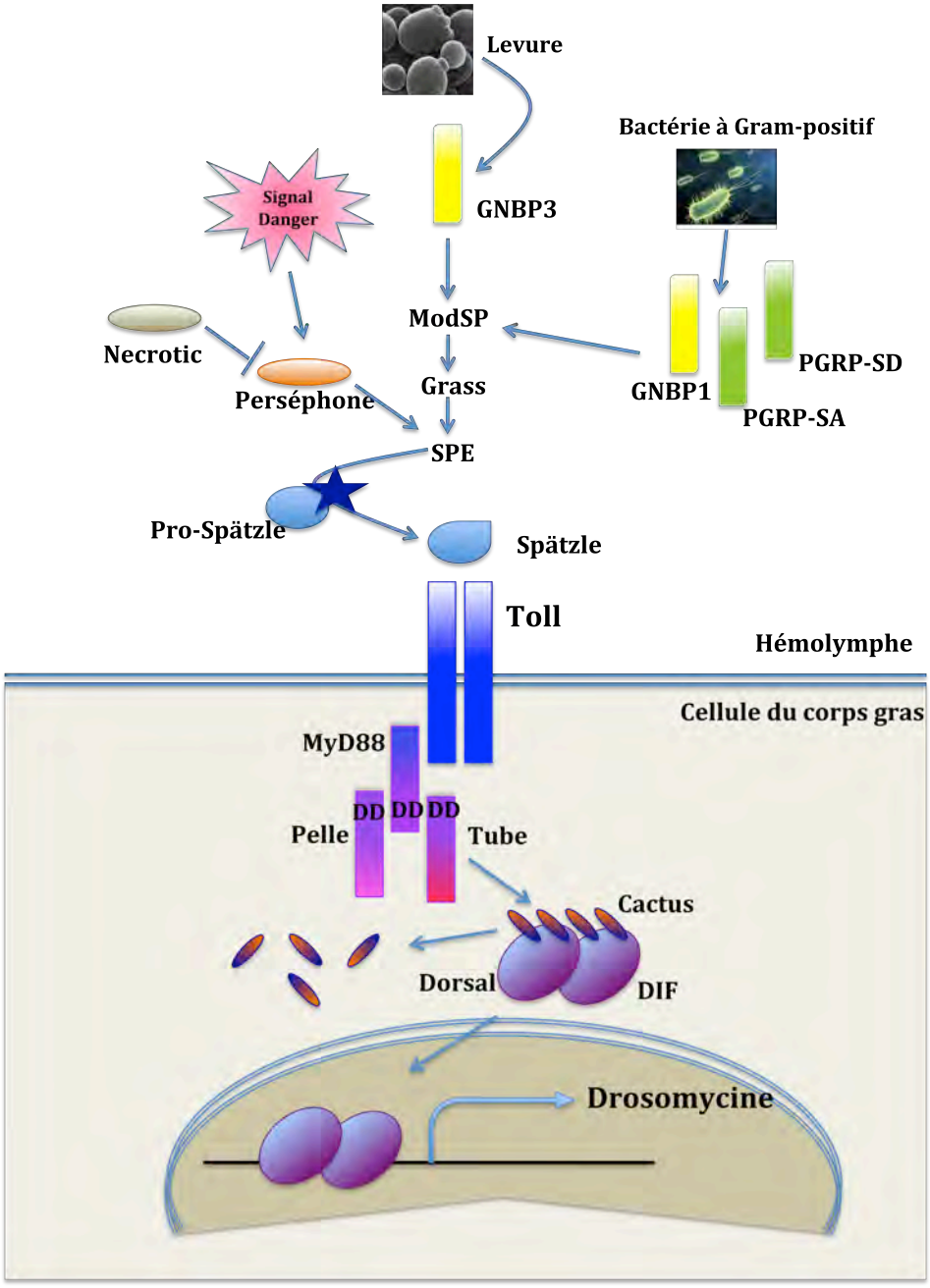


Figure 4. La voie Toll

b) La voie IMD activée par les bactéries à Gram-Négatif et les bacilles

Comme nous venons de le voir précédemment, l'activation de la voie Toll se fait par la fixation d'un ligand préalablement maturé par une cascade protéolytique, déclenchée par la reconnaissance du PGN ou des Glucans. Ce mode d'activation permet d'amplifier le signal de départ. Par contre l'activation de la voie IMD (Fig. 5) est totalement différente. Elle implique une reconnaissance directe du PGN avec son récepteur PGRP-LC.

Une mutation nommée *imd* (immune deficiency) a mené à la découverte de la voie Imd. La mutation du gène Imd affecte l'expression de plusieurs PAM sans vraiment affecter l'expression de drosomycine (Lemaitre *et al*., 1995) (Corbo et Levine 1996) (Georgel *et al*., 2001). Les mouches mutantes pour *imd* sont également sensibles aux bactéries à Gram-négatif.

La protéine Imd occupe une place centrale dans la réponse contre les bactéries à Gram-négatif. Imd est une protéine à *Death domain* similaire à la protéine RIP chez les mammifères (Georgel *et al*., 2001). Imd participe à la fois à la médiation du signal transmembraire de PGRP-LC (Choe KM, 2005) et intracellulaire du récepteur PGRP-LE non sécrété. Par l'intermédiaire de leurs Death Domain, IMD, dmFADD (homologue de la protéine Factor Associated Death Domain des mammifères) et DREDD (homologue de la caspase-8) interagissent (Leulier *et al*., 2000; Naitza *et al*., 2002). Ce complexe IMD, dmFADD et DREDD permet ainsi l'activation de la MAP kinase TAK1 (Transforming growth factorb-Activated Kinase 1). Plusieurs études suggèrent que l'ubiquitination joue un rôle important dans la voie de signalisation IMD. L'activation de TAK1 nécessite la participation des enzymes ubiquitinases Bendless et dUEV1a (homologues de Ubc13 et UEV1a). Mais jusqu'à présent aucune dégradation protéasome dépendante n'a pu être observée (Zhou *et al*., 2005). Le complexe Bendless/UEV1a se trouve positionné entre Imd et TAK1 (Zhou R, 2005). Après avoir mentionné l'importance du phénomène d'ubiquitination dans la voie IMD, il est

intéressant de voir que dUSP36, une ubiquitine protéase de drosophile, régule négativement l'activation de la voie IMD. La surexpression de dUSP36 entraine l'inhibition des voie IMD et JNK. A l'inverse, sa mutation entraine une activation constitutive de ces voies (Thevenon *et al*., 2009). Récement, DIAP2 (*Drosophila* inhibitor of apoptosis) a été décrit comme étant un composant essentiel dans la voie IMD (Gesellchen *et al*., 2005 ; Huh *et al*., 2007 ; Kleino *et al*., 2005 and Leulier *et al*., 2006). La protéine DIAP2 possède en N-terminal trois domaines BIR impliqués dans l'interaction avec les protéines à motif IBM (IAP-Binding Motif) (Wu *et al*., 2000). De plus, DIAP2 possède également en C-terminal un domaine *RING finger* qui lui confère une activité E3-ligase (Vaux et Silke, 2005). Le domaine *RING finger* est indispensable pour son rôle dans l'immunité (Huh *et al*., 2007).

La protéine TAK1, associée à TAB2 (TAK1 Associated Binding protein 2), constitue le point de branchement de la voie IMD vers l'activation de la voie JNK. Pour l'activation de la voie IMD, TAK1 et TAB2 forment un complexe permettant la phosphorylation de IKK (IKappa-Kinase) (Vidal *et al*., 2001; Zhuang *et al*., 2006). IKK est constitué de 2 sous-unités régulatrices IKKγ (key) et de 2 sous-unités catalytiques IKKβ (ird5) (par analogie avec le complexe IKK des vertébrés) (Lu *et al*., 2001; Rutschmann *et al*., 2000). Une fois activé, le complexe phosphoryle le facteur de transcription NF-kB Relish, similaire aux protéines p100 et P105 des mammifères. Relish phosphorylé est clivé, ce qui permet la séparation du domaine inhibiteur ankyrine et du domaine Rel. Le domaine Rel de liaison à l'ADN est ainsi libéré et passe dans le noyau où il active la transcription des gènes cibles (Stöven *et al*., 2000; Stöven *et al*., 2003). Même si cela n'a pas été directement démontré, il est très probable que la coupure endoprotéolytique de Relish soit effectuée par la caspase DREDD (Stöven *et al*., 2003; Erturk-Hasdemir *et al*., 2009).

La voie IMD tout comme la voie Toll est régulée négativement. Ces dernières années, plusieurs protéines ont été décrites comme étant des régulateurs négatif de la voie IMD. Ainsi, une mutation perte de fonction pour les PGRP-SC et –LB entraine une activation de la voie

IMD. Car c'est en dégradant le PGN que ces enzymes réduisent l'activation de la voie immunitaire (Zaidman-Remy *et al*., 2006 ; Bischoff *et al* ., 2006). PGRP-LF, une protéine membranaire non-enzymatique, prévient l'activation constitutive de la voie IMD. En absence de PGRP-LF les voie IMD et JNK sont activées de manière permanente sans qu'aucune infection ne soit nécessaire (Persson *et al*., 2007 ; Maillet *et al*., 2008). Caspar qui correspond à l'homologue de la protéine FAF1 (Fas Associeted Factor 1) est impliquée dans la voie du TNF chez les mammifères, inhibe la clivage de Relish par la caspase DREDD (Kim *et al*., 2006). La surexpression de Pirk réduit la synthèse de PAM après activation de a voie IMD et l'ARNi dirigé contre lui, augmente la production de PAM après infection (Kleino *et al*., 2008). Pims est une autre protéine inhibitrice, décrite comme étant associée avec PGRP-LCx et Imd (Lhocine *et al*., 2008). Rudra est une protéine qui immunoprécipite avec les récepteurs PGRP-LC, -LE et la protéine Imd. Une surexpression de Rudra inhibe l'activation de la voie IMD alors que pour le mutant *rudra* les mouches présentent une suractivation de la voie IMD après infection, ce qui a pour effet d'augmenter leur survie face à l'infection. (Aggarwal *et al*., 2008).

L'induction de la diptéricine, considérée comme un témoin de l'activation de la voie Imd, peut être activée par la surexpression des composants intra-cytoplasmiques de la voie Imd. D'autre part, il a été montré que la surexpression de cette voie est létale au stade larvaire, et qu'elle induit la transcription du gène pro-apoptotique *reaper*, qui est une cible de la voie JNK (Georgel *et al*., 2001).

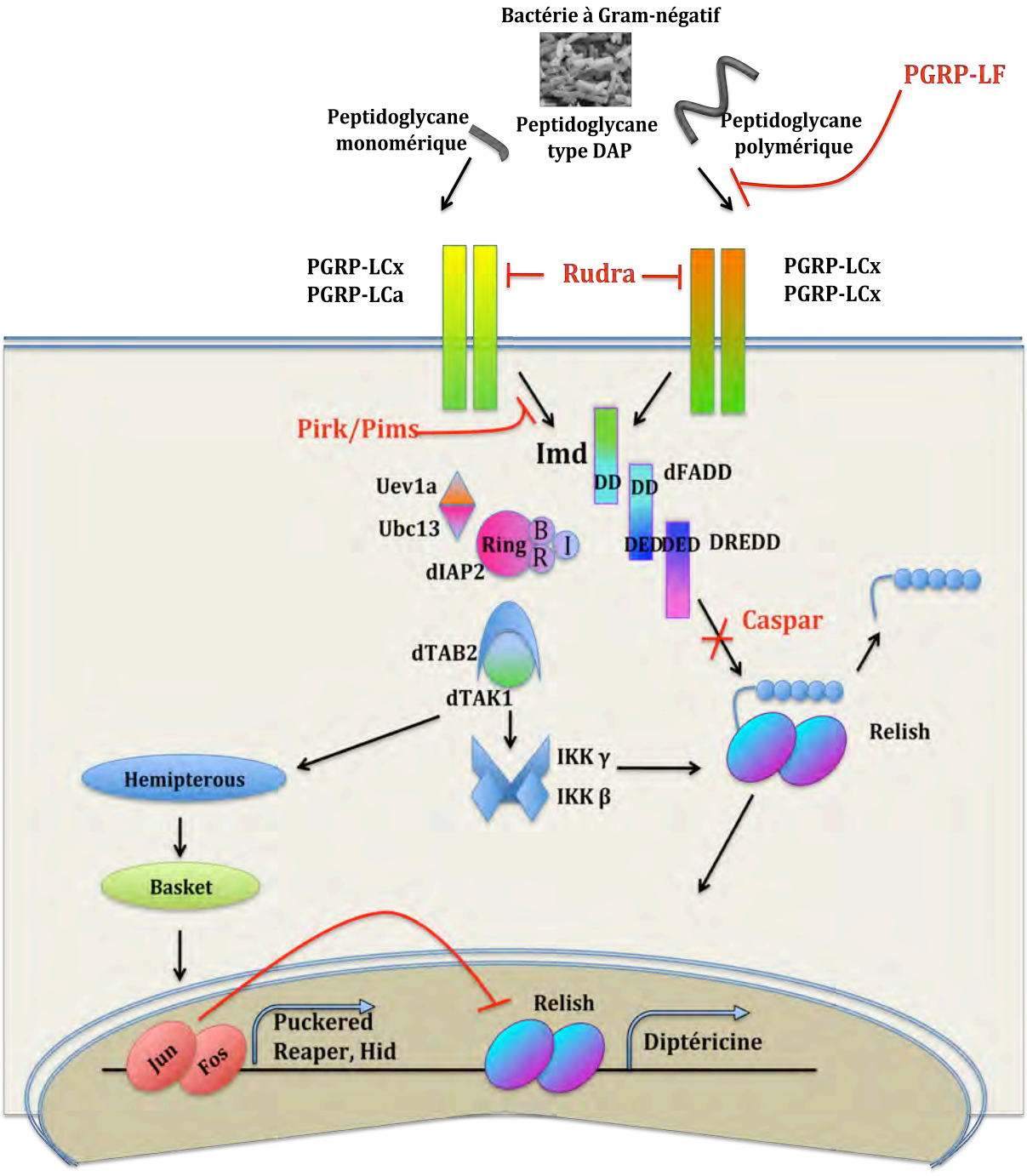


Figure 5 : La voie IMD

c) La voie JNK

La voie de signalisation JNK (Jun N-terminal kinase) participe à la réponse au stress, la migration cellulaire, l'apoptose, et la réponse immunitaire chez la drosophile et les mammifères (Sluss *et al.*, 1996; Leppa et Bohmann, 1999; Stronach et Perrimon, 1999; Boutros *et al.*, 2002; Huang *et al.*, 2009). Dans la réponse immunitaire, la voie JNK semble jouer un rôle précoce et transitoire, en permettant l'activation rapide des gènes liés aux processus de cicatrisation, de réarrangement du cytosquelette et de réponse au stress (Boutros *et al.*, 2002; Galko et Krasnow, 2004).

Il existe une régulation mutuelle entre les voies JNK et IMD. Plusieurs études ont permis de mettre en évidence que TAK1 est capable d'activer la voie JNK au cours du développement et de la réponse immunitaire (Mihaly *et al.*, 2001 ; Delaney et Mlodzik, 2006) mais aussi après stimulation des cellules S2 par le PGN (Silverman *et al.*, 2003). En effet, TAK1 active la JNKK hemipterous qui active à son tour la JNK Basket (Glise *et al*., 1995; Sluss *et al*., 1996). JNK permet alors la phosphorylation des facteurs de transcription AP-1, d-Jun (JRA pour Jun- Related Antigen) et d-fos (Kayak) (Kockel *et al*., 2001). Ces facteurs de transcription permettent la régulation de nombreux gènes cibles parmi lesquels on trouve *puckered*, qui code pour une phosphatase permettant l'inhibition de la JNK Basket. La voie JNK régule également la transcription des gènes *reaper* et *hid* qui codent pour deux protéines pro-apoptotiques (Varfolomeev et Ashkenazi, 2004).

L'activation finale de Relish en fin de cascade de la voie Imd entraine la destruction de TAK1 par le protéasome et fini par réduire l'activation de la voie JNK (Park *et al.*, 2004). Cela permet vraisemblablement à la voie Imd de réguler l'aspect transitoire de l'activation de la voie JNK au cours de la réponse immunitaire (Park *et al.*, 2004).

D'autre part, la voie JNK semble elle aussi contrôler la voie Imd. En effet, il a été démontré que la voie JNK permet l'activation des PAM et coopère avec la voie Imd dans leur contrôle

transcriptionnel (Delaney *et al.*, 2006 ; Kallio *et al.*, 2005). De manière surprenante, une étude montre que, le facteur de transcription AP-1 de la voie JNK peut agir comme antagoniste de Relish dans sa fixation sur le promoteur de certaines de ces cibles (Kim *et al.*, 2005).

d) La voie JAK-STAT

Chez la drosophile, comme chez les mammifères, la voie JAK-STAT participe à la lutte antivirale à travers la production de différentes molécules. La voie JAK-STAT a tout d'abord été étudiée pour son rôle dans la segmentation au cours de l'embryogenèse (Binari et Perrimon 1994). Plus tard il a été montré que la voie JAK-STAT contrôle d'autres aspect du développement, tels que la formation de l'œil, la détermination sexuelle, la spermatogenèse, l'oogenèse et la morphogenèse des trachées et de l'intestin (Hombria *et al.*, 2002). Pour l'instant, quatre molécules ont été identifiées comme faisant parti de cette voie, le ligand appartenant à la famille *unpaired* (*upd*), *domless* le récepteur transmembranaire (*dome*), *Janus kinase/ hopscotch* (JAK) et le facteur de transcription STAT92E. Le récepteur domless est membre de la famille des récepteurs de cytokine de type I connu chez les mammifères pour être les récepteurs de nombreuses interleukines (Il) tels que Il-2 à 9, Il-31. L'activation de la voie JAK-STAT par piqûre septique ou par piqûre propre contrôle l'expression des molécules de la famille des Turandots (Agaisse et Perrimon 2004). Ainsi, l'expression puis la sécrétion du ligand upd3 par les hémocytes induit la synthèse de TotA (Turandot A) par le corps gras (Agaisse *et al.*, 2003). La signification biologique de cet événement n'est pas simple à expliquer car TotA peut également être induit par différents stress tels que le choc thermique, la déshydratation, une pression mécanique, l'injection de détergeant, la piqure propre ou la piqure sceptique (Ekengren *et al.*, 2001 ; Agaisse *et al.*, 2003 ; Brun *et al.*, 2006). La régulation transcriptionnelle des gènes Turandot est très complexe et fait également appel à la voie Imd et la voie des MAPK (Mitogen Activated Protein Kinase) (Brun *et al.*, 2006).

Très récemment, Yano *et al*., ont montrés *in vivo* que *Listeria monocytogenes (L.m)* est reconnue par le PGRP-LE intra-cytoplasmique (Yano *et al*., 2008). La reconnaissance de cette bactérie à Gram-positif, entraine le production d'un nouveau PAM, la Listericin. Une étude *in vitro*, réalisée en cellules S2 montre que l'induction de Listericin est non seulement dépendante de PGRP-LE mais aussi de la voie JAK-STAT. De plus une surexpression *in vivo* de ce nouveau peptide a pour effet de protéger les mouches et de réduire la croissance des bactéries à gram-négatif et de *L.m* (Goto *et al*., 2010).

Malgré l'ensemble de ces observations, pour les mutants de la voie JAK-STAT, ni la survie ni le profil d'expression des PAM classiques n'est affectés par les infections bactériennes et fongiques.

B. La réponse humorale locale

1. Les peptides antimicrobiens

La production de PAM par les cellules épithéliales est induite par les bactéries à Gram négatif qui stimulent la voie Imd. A la façon de la réponse systémique, la réponse locale est médiée par le reconnaissance des bactéries à Gram-négatif par le PGRP-LC qui active la voie Imd (Zaidman-Remy *et al*., 2006). Mais pour l'instant nous ne savons pas pourquoi les bactéries commensales ou le PGN ingéré n'induisent pas une activation permanente de la réponse immunitaire. Cependant, il a été envisagé que le PGRP-LB ayant conservé une activité amidase soit capable de dégrader les fragments de PGN en fragment incapable d'activer le PGRP-LC (Zaidman-Remy *et al*., 2006 ; Mellroth *et al*., 2006).

Certains PAM sont exprimés de manière constitutive au niveau du tractus reproductif et des glandes salivaires (Tzou *et al*., 2000). Cette expression constitutive n'est pas régulée par

les voies NF-kB mais plutôt différents facteurs de transcription tissus spécifiques comme par exemple Caudal (Ryu *et al*., 2004 ; Han *et al*., 2004).

A ce jour, aucune implication de la voie Toll dans la réponse locale n'a pu être mise en évidence. De plus nous ne savons pas si les champignons ou le PGN des bactéries à Gram-positif sont capable d'induire la production de PAM.

2. La mélanisation

Les voies Toll et IMD jouent un rôle essentiel dans l'immunité innée en contrôlant l'expression des PAM. Cependant il leur faut plusieurs heures pour induire la synthèse de leurs effecteurs. La mélanisation est une réponse immédiate, induite en quelques minutes après l'infection par une cascade de protéases à sérine menant au clivage de la prophénoloxydase (PPO) en phénoloxydase (PO) (Ashida, 1990). Cette enzyme oxyde des phénols en quinones pour polymériser et former la mélanine. La mélanine enveloppe les micro-organismes afin de les séquestrer au point de l'infection. Les quinones et d'autres réactifs oxygénés (radicaux libres) générés au cours du processus de mélanisation sont toxiques pour les micro-organismes (Nappi *et al*., 1993). De plus, cette réaction intervient dans la coagulation, la cicatrisation la phagocytose et l'expression des PAM (Cerenius *et al*., 2008).

Chez les larves de drosophile, la mélanisation est exclusivement médiée par les cellules à cristaux. Cependant la source de PO chez l'adulte dépourvu de cellules à cristaux est inconnue. Plusieurs études menée chez d'autres insectes indiquent que la mélanisation est induite par une blessure, mais aussi par certains PRR induits par la reconnaissance de molécules microbiennes tels que le PGN, les glucans et le LPS (Ochiai *et al*., 1999 ; Ochiai et Ashida, 2000 ; Ma et Kanost , 2000 ; Lee *et al*., 2004).

En effet, pour plusieurs insectes comme par exemple *Bombyx mori*, il a été montré que la famille des PGRP participe à l'activation de la PO (Yoshida *et al*., 1996 ; Ochiai *et al*., 1999). Chez la drosophile, la surexpression de PGRP-LE entraine la mélanisation. A l'inverse la mutation de PGRP-LE inhibe l'activation de la PO suite à une infection par *Escherichia coli* (Takehana *et al*., 2002 ; Takehana *et al*., 2004). Il en est de même pour le récepteur transmembranaire PGRP-LC dont la surexpression chez l'adulte et la larve entraine une importante mélanisation (Schmitd *et al*., 2008). La voie Toll participe également au processus de mélanisation. Dans le mutant *necrotic*, un inhibiteur de la cascade des sérines protéases est absent. Cela entraine l'activation de la voie Toll mais aussi la mélanisation constitutive (Levashina *et al*., 1999). Chez *Tenebrio Molitor*, le récepteur PGRP-Sa, également impliqué dans la reconnaissance des bactéries à Gram-positif est nécessaire à l'activation de la mélanisation (Park *et al*., 2007).

3. La coagulation

La coagulation est le processus par lequel se forme une matrice insoluble dans l'hémolymphe ou le sang afin de protéger l'organisme des infections suite à une blessure. Chez les vertébrés, le système circulatoire fermé nécessite une régulation très fine de la coagulation. Car la coagulation pourtant vitale, peut également s'avérer mortelle. Chez les insectes, le système circulatoire ouvert, permet à la coagulation d'être plus exhubérante afin de refermer très rapidement la blessure et limiter la perte de fluide, mais aussi afin de circonscrire les microorganismes au lieu de la blessure (Dushay 2009). Chez la drosophile, le coagulum composé de fibres emprisonne rapidement les hémocytes sur le lieu de la blessure. Cependant, la coagulation est indépendante de la phénoloxydase car elle continue d'avoir lieu en absence de PPO (Scherfer *et al*., 2004). Il est cependant admis que la transglutaminase (homologue du facteur XIIIa chez les vertébrés) est impliquée dans la séquestration des

bactéries en participant à la formation de la matrice du caillot (Wang *et al*., 2010). Les protéines Hémolectine et Fondue, produites par les hémocytes, sont impliquées la formation du caillot. Cependant leur absence chez la larve ou l'adulte n'augmente pas la mortalité due aux infections microbiennes (Scherfer *et al*., 2006).

4. Les réactifs oxygénés (ROS : Reactive Oxygen Species)

a) Synthèse des ROS

Chez la drosophile comme chez les mammifères, les micro-organismes présents dans le tube digestif induisent la production par les cellules épithéliales de réactifs oxygénés. Les ROS ont pour rôle de réduire la multiplication des micro-organismes, mais s'ils ne sont pas finement régulés ils peuvent être toxiques pour les cellules hôtes. Les ROS sont produites dans l'intestin par l'oxydase DUOX (dual oxidase) (Ha *et al*., 2005). L'interaction entre la flore intestinale (bactéries commensales) et les cellules épithéliales de l'intestin entraine l'activation d'un récepteur à protéine G appartenant à la famille des protéines Gq. La sous unité alpha de la protéine Gq entraine l'activation de la phospholipase et la production d'innositol 1,4,5-triphosphate (IP3). L'IP3 a pour conséquence la mobilisation de calcium intracellulaire, lequel module positivement l'activité enzymatique de DUOX (Ha *et al*., 2009a). Pour l'instant la nature de la molécule induisant l'activation de DUOX reste inconnue, mais il est certain qu'il ne s'agit pas de PGN. En présence d'une infection, les cellules épithéliales augmentent l'activité de l'oxydase DUOX mais également son taux de transcription. La régulation transcriptionnelle de DUOX, dans l'intestin, est sous le contrôle de la voie p38. Cependant, si l'activation de la voie DUOX est indépendante du PGN, sa transcription est contrôlée à la fois par des voies PGN dépendantes (Voie IMD) et indépendantes (Protéine G), toutes deux convergeant vers la voie p38 via l'activation de la kinase Mekk1 (Ha *et al*., 2009b). Les mouches mutantes pour p38 et Mekk1 sont très

sensibles aux infections bactériennes au niveau de l'intestin (Ha *et al*., 2009b), alors que ces mêmes mutants montrent une résistance tout à fait normale aux infections bactériennes systémiques (Brun *et al*., 2006 ; Craig *et al*., 2004).

b) Les ROS induisent le renouvellement cellulaire de l'intestin

Chez l'homme, les cellules intestinales sont continuellement renouvellées grâce aux cellules souches. Une dérégulation du phénomène de renouvellement cellulaire a été observée dans un certain nombre de maladies digestives et de cancers (Barker *et al*., 2008). L'intestin de drosophile adulte contient lui aussi des cellules souches intestinales multipotentes (ISC). Il a été montré que les ISC se divisent en réponse à des signaux émit par les cellules intestinales voisines (Micchelli *et* Perrimon, 2006 ; *et*Il a également été rapporté chez les lépidoptères, que la toxine bactérienne provenant de *Bacillus thuringiensis* entraine une mort massive des cellules épithéliales intestinales ainsi qu'une augmentation de la division et de la différenciation des cellules souches (Loeb *et al*., 2000). Depuis, la drosophile s'est imposée comme un excellent outil d'étude de l'interaction entre le pathogène et le physiologie des cellules intestinales (Ha *et al*. 2005a,b, 2009a,b; Bischoff *et al*. 2006; Zaidman-Remy *et al*. 2006; Nehme *et al*. 2007; Ryu *et al*. 2008). Des études récentes suggèrent que *Helicobacter pylori* et *Shigella flexneri*, deux bactéries pathogènes du tractus digestif de l'homme, puissent interférer avec le renouvellement de l'épithélium intestinal afin de favoriser leur multipilcation (Iwai *et al*. 2007; Mimuro *et al*. 2007). Chez la drosophile, des travaux récents ont montré que la production de ROS par les cellules intestinales lors d'une infection bactérienne induit la prolifération des cellules souches intestinales (Buchon *et al*., 2009a and 2009b ; Lee, 2009). Ceci témoigne de l'importance des ROS dans le contrôle de la charge bactérienne dans l'intestin de drosophile, mais également de leur rôle dans le renouvellement de l'épithélium intestinal.

C. La réponse cellulaire

1. La phagocytose

Chez les métazoaires, les cellules apoptotiques sont phagocytées. La phagocytose est le processus par lequel différents types de particules (bactéries, levures, ARN double brin, corps apoptotiques) peuvent être internalisées et digérées. L'une des étapes critiques de la phagocytose est la reconnaissance de la cible. Pour cela, le phagocyte reconnaît la particule à l'aide de récepteurs qui reconnaissent certains motifs biochimiques à la surface de la cible. Croquemort (Crq) est l'un d'entre eux (Franc *et al*., 1996). Crq appartient à la famille des récépteurs CD36. Exprimé exclusivement dans les macrophages, il participe à la phagocytose des corps apoptotiques au cours de l'embryogenèse. dSR-CI est un autre récepteur découvert à la surface des hémocytes embryonnaires (Pearson *et al*., 1995). Il permet la reconnaissance et la phagocytose des bactéries à gram négatif et positif (Rämet *et al*., 2001). La protéine à domaine EGF, Eater, est un des récépteurs transmembranaire le mieux décrit dans la littérature. Exprimé spécifiquement sur les plasmatocytes et les pro-hémocytes, Eater est nécessaire à la phagocytose des bactéries à Gram-positif et Gram-négatif *in vivo* (Kocks *et al*., 2005). Un récepteur contenant six motifs EGF LRP (LPS Recognition protein) a été identifié chez le coléoptère *Holotrichia diomphalia.* Le LRP est sécrété dans l'hémolymphe et se fixe sur le LPS des bactéries à Gram-négatif (Ju *et al*., 2006). Le récepteur PGRP-SC1 est requit dans la phagocytose des bactéries à gram positif (Garver *et al*., 2006).

Dscam est un autre récepteur qui se lie aux microorganismes et participe à la phagocytose. Chez la drosophile, le gène Dscam (*Down syndrom cellular adhesion molécule*), code pour un récepteur, membre de la super famille des immunoglobulines. Ce gène occupe un rôle crucial dans le développement du système nerveux (Schmucker *et al*., 2000), mais est également impliqué dans la réponse immunitaire des arthropodes. L'organisation du gène Dscam

comprend différents clusters d'exons variables flanqués d'exons constants. L'épissage alternatif de Dscam combiné aux exons variables et constants est capable de générer 19 008 domaines extracellulaires différents. Des isoformes de Dscam ont été détectées dans l'hémolymphe. L'absence de Dscam au niveau des hémocytes a pour effet de réduire la phagocytose des bactéries (Watson *et al*., 2005).

2. L'opsonisation

Le génome de la drosophile code pour six protéines structurellement proches du complément C3 (Lagueux 2000). Cinq d'entre elles possèdent un motif thioester (TEP1 à TEP5), d'où leur nom de *ThioEster related Proteins* (TEPs). Le sixième (TEP6), ne possèdant pas de motif thioester est appelé *Macroglobulin-complement related* ou Mcr. Les TEPs facilitent la phagocytose des pathogènes à la façon du complément. La transcription de TEP1, 2 et 4 est induite après infection bactérienne chez la larve. Par contre chez l'adulte seul TEP2 et 4 sont induit après piqûre sceptique (Lagueux *et al*., 2000). Au cours de l'infection bactérienne, TEP2, TEP3 et Mcr se fixent respectivement à la surface des bactéries à Gram négatif, Gram positif et *Candida albicans* (Stroschein-Stevenson 2006). Par ailleurs, il a également été montré que les voies Toll et JAK-STAT activées de manière constitutives sont capables d'induire la transcription de TEP1 (Lagueux *et al*., 2000).

3. L'encapsulation

Chez la drosophile, lorsqu'un corps étranger est reconnu et est trop grand pour être phagocyté, les hémocytes l'encapsulent. Cette réaction est observée lorsque la guêpe parasitoïde, *Leptopilina boulardi* pond un œuf dans la larve de drosophile. L'œuf est aussitôt reconnu par les plasmatocytes circulant qui deviennent adhérent et l'encerclent puis se lient

au chorion de l'œuf de guêpe. Des jonctions septées se forment pour isoler le parasite de l'hemocoele. La dernière phase de l'encapsulation requière la fixation des lamellocytes et la mélanisation liée à la dégranulation de cellules à cristaux (Russo *et al*., 1996).

A la manière des cellules souches germinales de drosophiles et de vertébrés, la voie JAK-STAT est maintenu active dans les pro-hemocytes de la glande lymphatique afin d'éviter toute différenciation des pro-hémocytes en lamellocytes (Krzemien *et al*., 2007). C'est la raison pour laquelle une surexpression de *hopscotch* dans les glandes lymphatiques induit leur hypertrophie et la formation de tumeur mélanitique (Harisson *et al*., 1995).

4. L'épithelium intestinal

Comme nous l'avons déjà mentionné, l'une des armes majeures de la lutte antimicrobienne chez la drosophile consiste en la production de PAM par les cellules du corps gras. La barrière épithéliale produit également des PAM (Tzou *et al*., 2000). L'intestin de drosophile assimile une nourriture riche en microorganismes tels que des fruits en décompositions. Cet intestin est protégé par une barrière de chitine appelée matrice péritrophique recouvrant l'épithélium intestinal et protégeant le tractus digestif des microorganismes. En complément de la production de PAM, des réactifs oxygénés sont également produits (Ryu *et al*., 2006).

Depuis peu, des cellules souches intestinales ont été identifiée chez la Drosophile (Micchelli et Perrimon, 2006; Ohlstein et Spradling, 2006). Ces cellules souches sont réparties le long de l'intestin et localisée au niveau de la membrane basale des entérocytes (Fig. 5).

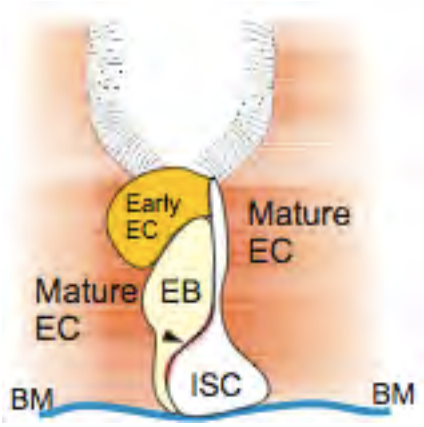


Image extraite de Pearson et al., 2009

Figure 5. Les cellules souches dans l'intestin de Drosophile. L'intestin de Drosophile contient des cellules souches capables de produire à la fois des entérocytes (EC) et des cellules enteroendocrines (absente sur le schéma) (Micchelli and Perrimon, 2006, Ohlstein and Spradling, 2006). Les cellules souches sont associées à la membrane basale (BM). Les cellules souches sont en contact avec leurs cellules fille, les entéroblastes (EB), un stade intermédiaire avant de se différencier en entérocytes (EC) ou en cellules entéroendocrines. Une forte accumulation de jonction de type Armadillo (marqué en rouge) entre les cellules souches et les entéroblastes suggère que des jonctions adhérentes participe à l'association entre EC et EB. Il semblerait que ce lien permette aux cellules souches intestinales de migrer dans différentes régions de l'intestin (Ohlstein and Spradling, 2006).

L'homéostasie des cellules souches est influencée par différentes voies biologiques (Fig. 6). La voie Delta-Notch joue un rôle dans le détermination de cellules souches (Micchelli et Perrimon, 2006; Ohlstein et Spradling, 2006, 2007). La division des cellules souches est symétrique et donne naissance à deux cellules filles initialement similaires. Cependant, très tôt après la division, une cellule sur deux conserve un taux élevé de ligand Delta. Ceci lui permet de garder son caractère de cellule souche intestinale. Alors que l'autre cellule fille perd rapidement Delta et devient un entéroblaste (Ohlstein et Spradling, 2007). Le ligand Delta active la voie Notch dans les entéroblastes voisins et établie ainsi une asymétrie dans le devenir des cellules (Bray, 2006; Ohlstein et Spradling, 2007). Les entéroblastes sont incapable de se diviser mais sont par contre capable de se différencier en entérocytes ou cellules endocrines produisant des hormones. 90% des entéroblastes se différencient en entérocytes et seulement 10% des entéroblastes se différencient en cellules endocrines (Micchelli et Perrimon, 2006; Ohlstein et Spradling, 2007). La capacité des entéroblastes à se différencier dépend l'activation de la voie Notch, déterminée par le niveau de ligand Delta présent dans les cellules souches originales. Dans l'intestin de Drosophile, les cellules

souches sont les seules à subir une mitose, alors que les entéroblastes subissent une endo-réplication.

La voie JAK/ STAT (Janus Kinase/Signal Transducer and Activator of Transcription) est une voie de signalisation conservée impliquée dans de nombreux processus développementaux (voir pour revue Arbouzova et Zeidler, 2006). Plusieurs évidences suggèrent que la voie JAK/STAT participe à la maintenance des cellules souches germinales et somatiques, et favorisant leur prolifération (Kiger *et al.*, 2001, Tulina et Matunis, 2001, Decotto et Spradling, 2005 ; Singh *et al*., 2007). Des études récentes montrent que la voie JAK/STAT participe au maintient de l'homéostasie de l'intestin de Drosophile après infection bactérienne ou ablation de cellules intestinales (Buchon *et al*., 2009, Jiang *et al*., 2009 and Cronin *et al*., 2009). Collectivement, l'ensemble de ces études supportent le modèle selon lequel, l'activation de la voie JAK/STAT est induite par la réponse à différents stresses et stimule l'activation de cellules souches intestinales. Il a été montré très récemment que la voie JAK/STAT est normalement active à la fois dans les cellules souches intestinales et les cellules filles mais ne l'est pas dans les entérocytes et les cellules entéroendocrines. Ceci suggère un modèle où la voie JAK/STAT participe en parallèle ou en aval de la voie Notch à la fois à la prolifération des cellules souches intestinales mais à la différenciation des cellules filles (Beebe *et al*., 2010).

Le taux de renouvellement des cellules intestinales correspond très probablement à un flux constant en réponse à différents stress provenant de la digestion (acides, enzymes, dommages mécaniques, toxines produites par la flore intestinal). L'ablation de cellules intestinales (entérocytes), l'activation de la voie JNK ou l'infection par *Pseudomonas entomophila* a pour effet de perturber l'épithélium intestinal de la Drosophile et d'induire la division et la différenciation des cellules souches intestinales permettant à l'intestin de se régénérer (Jiang et al., 2009). D'autre études très récentes montrent une réponse similaire lorsque l'intestin de Drosophile est soumis à d'autres stresses tels que le DSS (Amcheslavsky et al., 2009), le

stress oxydatif induit par le paraquat (Biteau et al., 2008) et l'infection par la bactérie *Erwinia carotovora* (Buchon et al., 2009). La voie JNK induit l'expression de gènes cytoprotécteurs chez la Drosophile, entrainant une augmentation de son espérance de vie. Nous savons que la voie JNK est requise pour afin de protéger les cellules du stress oxydatif mais également pour induire la prolifération des cellules souches au niveau de l'intestin. Alors que la voie JNK induit la division des cellules souches intestinales, l'activation de la voie Notch limite leur prolifération en assurant la différenciation des entéroblastes. En condition de stress important ou de stress chronique, la voie JNK induit une forte prolifération des cellules souches sans que les cellules ne se différencient totalement, entrainant ainsi une accumulation d'entéroblastes due une activation ectopique de la voie Notch (Biteau *et al*., 2008).

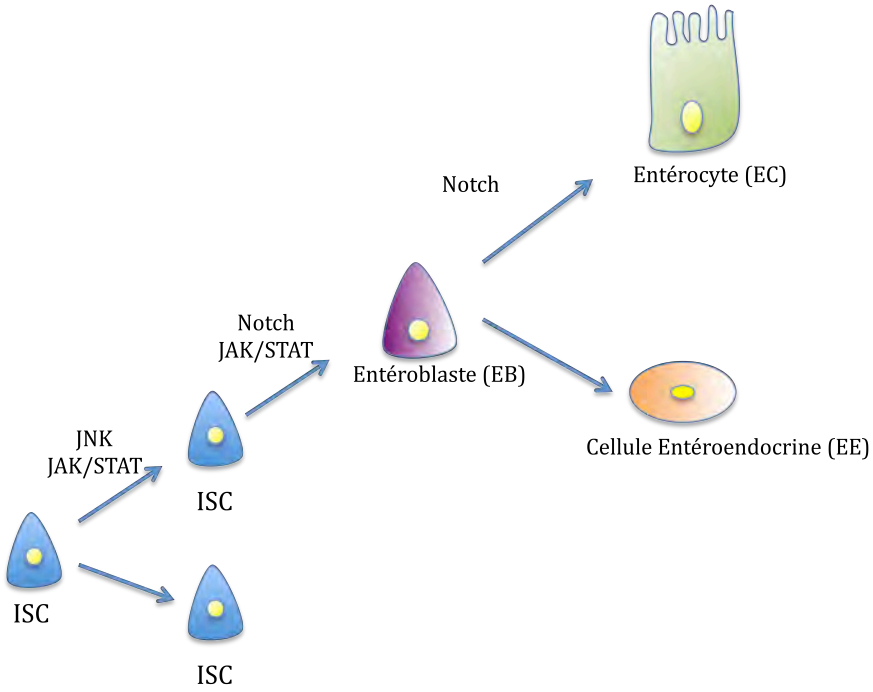


Figure 6. Le récepteur Notch est préférentiellement exprimé par les ISCs, les EB et les entérocytes en cours de maturation. Delta, le ligand de Notch n'est exprimé que par les ISC. L'activation de la voie Notch empêche la différenciation de EB en cellules endocrines (EE). La voie JAK/STAT assure une coordination entre la prolifération des ISC et la compétence des cellules filles à se différencier.

IV. LA RÉPONSE ANTI-VIRALE

A. L'ARN interférant

L'ARN interférant (ARNi) impliqué dans la défense antivirale est une réponse eucaryotique conservée dans le règne végétal et animal. Son mécanisme se base sur la reconnaissance de l'ARN double brin d'origine virale. Une fois reconnu par le complexe Dicer-2/R2D2, l'ARN viral est coupé en petits ARN doubles brins (siARN : Small interfering ARN) et incorporé dans le complexe RISC/Argonaute-2 (Fig. 7). Les siARN servent de guide au complexe RISC/Argonaute-2 afin de reconnaître et dégrader l'ARN viral (Kemp C & Imler JL, 2009). Les mutants *Dcr-2, r2d2, et Ago-2* présentent une susceptibilité aux infections par différents virus à ARN simple brin (ARNsb) comme par exemple le Flock Hous Virus (FHV), Drosophila C Virus, Cricket Paralysis virus (CrPV), Sindbis virus (SINV) (Wang *et al*., 2006 ; Galiana-Arnoux *et al*., 2006) mais aussi par le virus à ARN double brin (ARNdb) DXV(Drosophila X Virus) (Zambon *et al*., 2006). En plus de Dcr-2, R2D2 et Ago-2, il a été découvert récemment une nouvelle molécule participant à la voie de l'ARN interférant. L'absence d'ARS2 dans des cellules ou des mouches adultes les rend susceptible à différentes infections virales (VSV, DCV, SINV et FHV) (Sabin *et al*., 2009). Ars2 se trouverait en amont de Dcr-2 et Ago-2.

L'injection d'ARNdb spécifique d'un virus protège les mouches sauvages contre ce même virus, par contre il est incapable de protéger les mutants *ago2* et *dcr-2* (Saleh *et al*., 2009). Nous savons que Dcr-2 participe à la dégradation de l'ARN double brin viral. Il initie également la transcription de Vago, nouvellement identifiée comme une molécule antivirale et requise pour empêcher la réplication du virus dans les drosophiles (Deddouche *et al*., 2009). Chez la drosophile adulte, Vago est l'un des nombreux gènes induit suite à une infection par virus à ARN.

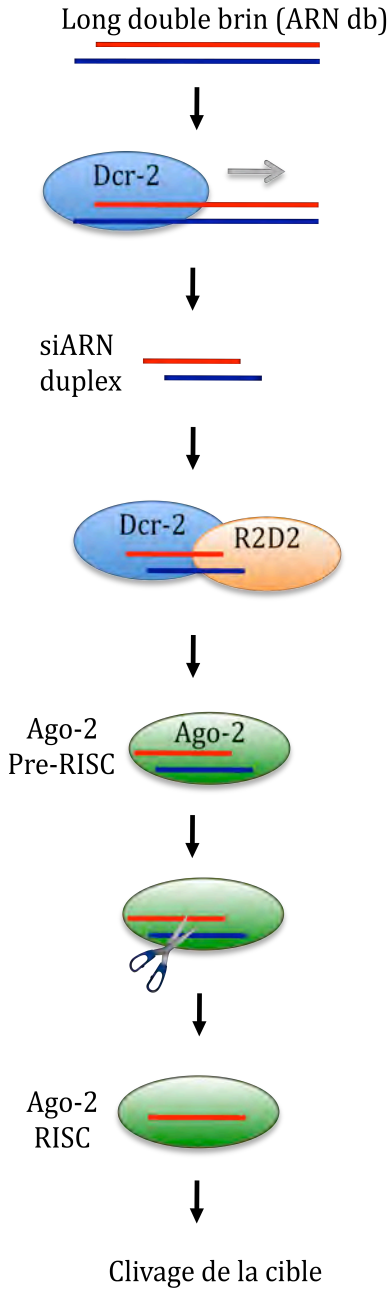


Figure 7. Voie des petits ARN interférent exogènes. ARN double brin (ARNdb) précurseur est maturé par Dicer-2 (Dcr-2) pour générer un duplexe d'ARN simple brin (ARNsb) contenant un brin guide et un brin passager. Dcr-2 et la protéine de liaison à l'ARNsb, R2D2 (chargeant l'ARN au sein du complexe RISC) transfert le duplexe dans la protéine Argonaute 2 (Ago-2). Le brin passager est détruit et le brin guide dirige Ago-2 vers l'ARN cible.

B. La voie JAK-STAT

De manière intéressante, les mutants de la voie JAK-STAT sont sensibles aux infections par le DCV et présentent une charge virale plus importante que des mouches sauvages. L'infection des mouches par le DCV induit l'expression d'un certain nombre de gènes dont la transcription dépend du facteur STAT. Cependant, les drosophiles surexprimant un allèle d'hopscotch (Tumorous lethal) n'induisent pas la surexpression de ces gènes spécifiques de l'infection virale. De plus, le DCV n'induit pas la production de TotM, normalement induit par l'activation de la voie JAK-STAT (Dostert *et al*., 2005). Vir-1 (virus induced RNA1) est un des gènes induit par l'infection par le DCV à condition qu'il y ait réplication virale (Hedges et Johnson 2008). Cependant la sur-expression ectopique de vir-1 dans des mouches transgéniques ou l'utilisation de la technique d'ARNi dirigé contre vir-1 n'affecte pas la résistance des mouches à l'infection virale. Ceci suggère que la voie JAK-STAT participe au control de la réplication virale du DCV, mais n'est pas suffisante à l'induction des gènes antiviraux (Dostert *et al*., 2005).

C. Autophagie

L'autophagie est un mécanisme conservé au cours duquel les protéines cytoplasmiques ou les organelles sont empaquetées de manière non sélective dans des lysosomes pour être dégradées. L'autophagie a été décrite pour être activée au cours du développement par l'ecdysone, mais aussi le stress tels que la restriction calorique et le stress oxydatif. Récemment, l'autophagie a été décrite comme participant à la lutte antivirale contre le VSV. Ce rapport identifie la protéine de surface VSV-G comme étant capable d'initier l'autophagie. Une fois ce processus activé, l'autophagie réduit la réplication virale. Au contraire, une réduction de l'autophagie entraine une augmentation de la réplication virale (Shelly *et al*.,2009).

Caspase-mediated cleavage, IAP binding, and ubiquitination:

linking three mechanisms crucial for *Drosophila* NF-kappaB signaling

Introduction

Chez la drosophile, l'infection induit rapidement la production de PAM par l'activation des voies IMD et Toll, toutes deux activant un facteur de transcription de la famille NF-kB. L'activation de la voie IMD est liée à la reconnaissance de peptidoglycanes (PGN) portant un acide diaminopimelique (DAP) commun à toutes les bactéries à Gram-négatif. La cascade d'évènements en aval du récepteur PGRP-LC implique la protéine adaptatrice Imd, la caspase-8-like DREDD et son adaptateur FADD pour activer la MAP3 kinase TAK1. TAK1 active ensuite le complexe IKK essentiel pour l'activation de Relish. Il a été démontré plus récemment que l'inhibiteur d'apoptose DIAP2 est également impliqué dans cette voie. DIAP2 possède trois domaines BIR en N-terminal, impliqués dans l'interaction avec des protéines à motifs IAP Binding Motif (IBM). De plus, DIAP2 possède un domaine C-terminal en *ring finger* caractéristique des ubiquitines ligases. Cependant les approches génétiques qui ont amené à ces conclusions n'ont pas permis de comprendre les mécanismes moléculaires qui régissent les interactions entre ces molécules et nous avons mené une étude mêlant biochimie et validation génétique pour les analyser.

Nicolas Paquette a ainsi démontré biochimiquement que la stimulation par les peptidoglycanes induit la coupure de la protéine Imd par la caspase DREDD, exposant ainsi en N-terminal un motif IBM. L'ubiquitine ligase DIAP2 se lie au domaine IBM. La liaison de ce dernier induit alors la polyubiquitination K63 de Imd. La description de ce mécanisme crée une connexion entre le clivage caspase-dépendant et l'ubiquitination dans la signalisation NF-kB.

Mon rôle a été de valider *in vivo,* par une approche génétique, le site de coupure d'Imd supposé être le lieu de liaison de DIAP2 à la protéine Imd. Cette coupure pourrait se situer au niveau du motif ^{27}LEKD/A^{31} reconnu comme étant spécifique des caspases. En effet, l'activation de la voie IMD génère un clivage de la protéine Imd et libère une nouvelle extrémité Alanine en N-terminal en position A31. *Imd1,* un allèle hypomorphe d'Imd présente une substitution de l'Alanine 31 par une Valine. De fait, la nouvelle extrémité rend impossible la fixation du domaine BIR (DIAP2) au domaine IBM (Imd), et par conséquent, entraine l'ubiquitination de la protéine Imd. Basé sur ces faits, une construction mutante de Imd où l'Aspartate en position 30 est remplacée par une Alanine (D30A) a été surexprimée en culture cellulaire. Après activation par du peptidoglycane la forme mutée de Imd, au contraire de la forme sauvage, n'est ni coupée ni ubiquitinée, De plus, la surexpression de Imd D30A agit comme un dominant négatif en inhibant l'ubiquitination de Imd. De manière similaire, j'ai pu

montrer qu'en surexprimant le dominant négatif D30A dans des mouches sauvages, l'induction de peptides antimicrobiens est fortement diminuée après infection par *Escherichia coli*.

L'ensemble de ces données *in vitro* et *in vivo* suggère que la coupure de Imd entre les résidus 30 et 31 permet la liaison de DIAP2 et la polyubiquitination de Imd. Ceci active en aval les autres composants de la voie et l'expression *in fine* de peptides anti-microbiens.

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Caspase-Mediated Cleavage, IAP Binding, and Ubiquitination: Linking Three Mechanisms Crucial for *Drosophila* NF-κB Signaling

Nicholas Paquette,[1] Meike Broemer,[2] Kamna Aggarwal,[1] Li Chen,[1] Marie Husson,[3] Deniz Ertürk-Hasdemir,[1] Jean-Marc Reichhart,[3] Pascal Meier,[2] and Neal Silverman[1,*]
[1]Division of Infectious Disease, Department of Medicine, University of Massachusetts Medical School, Worcester, MA 01605, USA
[2]Breakthrough Toby Robins Breast Cancer Research Centre, Institute of Cancer Research, London SW3 6JB, UK
[3]Institut de Biologie Moléculaire et Cellulaire, CNRS UPR 9022, Universite de Strasbourg, Strasbourg Cedex 67084, France
*Correspondence: neal.silverman@umassmed.edu
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SUMMARY

Innate immune responses are critical for the immediate protection against microbial infection. In *Drosophila*, infection leads to the rapid and robust production of antimicrobial peptides through two NF-κB signaling pathways—IMD and Toll. The IMD pathway is triggered by DAP-type peptidoglycan, common to most Gram-negative bacteria. Signaling downstream from the peptidoglycan receptors is thought to involve K63 ubiquitination and caspase-mediated cleavage, but the molecular mechanisms remain obscure. We now show that PGN stimulation causes caspase-mediated cleavage of the *imd* protein, exposing a highly conserved IAP-binding motif (IBM) at its neo-N terminus. A functional IBM is required for the association of cleaved IMD with the ubiquitin E3-ligase DIAP2. Through its association with DIAP2, IMD is rapidly conjugated with K63-linked polyubiquitin chains. These results mechanistically connect caspase-mediated cleavage and K63 ubiquitination in immune-induced NF-κB signaling.

INTRODUCTION

Activation of the *Drosophila* IMD pathway by DAP-type peptidoglycan (PGN) leads to the robust and rapid production of a battery of antimicrobial peptides (AMPs) and other immune-responsive genes (Ferrandon et al., 2007; Lemaitre and Hoffmann, 2007). Two peptidoglycan recognition protein (PGRP) receptors are responsible for the recognition of DAP-type PGN, the cell surface receptor PGRP-LC and the cytosolic receptor PGRP-LE (Kaneko et al., 2006). DAP-type PGN binding causes these receptors to multimerize or cluster (Chang et al., 2006; Lim et al., 2006), triggering signal transduction. IMD signaling culminates in activation of the NF-κB precursor Relish and transcriptional induction of AMP genes.

Currently, the molecular mechanisms linking these PGN-binding receptors and activation of Relish remain unclear. Genetic experiments suggest that the most receptor-proximal component of the pathway is the *imd* protein (Georgel et al., 2001), while the MAP3 kinase TAK1 appears to function downstream (Silverman et al., 2003; Vidal et al., 2001). In turn, TAK1 is required for activation of the *Drosophila* IKK complex, which is essential for the immune-induced cleavage and activation of the NF-κB precursor Relish, the key transcription factor required for immune-responsive AMP gene expression (Silverman et al., 2000). In addition to NF-κB signaling, TAK1 also mediates immune-induced JNK signaling (Silverman et al., 2003).

Other major components in the IMD pathway include the caspase-8-like DREDD and its adaptor FADD. RNAi-based studies suggest that these proteins have two distinct roles in IMD pathway signaling, one relatively early in the cascade and the second further downstream. Using RNAi, DREDD and FADD were shown to be required for immune-induced activation of the IKK complex (Zhou et al., 2005). These data suggested that DREDD and FADD function downstream of IMD but upstream of TAK1; however, it was not established if this upstream role for DREDD involves its protease activity. In its second role, DREDD is thought to proteolytically cleave Relish (Stöven et al., 2003; Erturk-Hasdemir et al., 2009).

In addition to the components outlined above, several studies have suggested that ubiquitination plays a critical role in the IMD signaling cascade. Recently, *Drosophila* inhibitor of apoptosis 2 (DIAP2) was shown to be a crucial component of the IMD pathway (Gesellchen et al., 2005; Huh et al., 2007; Kleino et al., 2005; Leulier et al., 2006). Typical of IAP proteins, DIAP2 has three N-terminal BIR domains, which are involved in interactions with proteins carrying conserved IAP-binding motifs (IBMs) (Wu et al., 2000). In addition, some IAPs, including DIAP2, carry a C-terminal RING finger domain that provides these proteins with ubiquitin E3-ligase activity (Vaux and Silke, 2005). Although it is unclear where in the pathway DIAP2 functions, one study showed that the RING finger is indispensable for its role in the immune response, suggesting it operates as an E3-ubiquitin ligase (Huh et al., 2007). Also, Zhou et al. (2005) showed, using RNAi-based approaches, that the E2-ubiquitin-conjugating enzymes Uev1a and Ubc13 (*bendless*) are critical components of the IMD pathway. Notably, Ubc13 and Uev1a function together in a complex to generate K63-linked polyubiquitin chains. K63-polyubiquitin chains are not linked to proteasomal degradation but instead are thought to play regulatory roles (Chiu et al., 2009; Mukhopadhyay and Riezman, 2007; Xia

et al., 2009). However, no K63-ubiquitinated target protein(s) has been identified in the IMD pathway. Although no connection between DIAP2 and the Bend/Uev1a E2 complex has been established, one attractive scenario is that DIAP2 functions as an E3 together with the Bend-Uev1a E2 complex.

The *imd*^{1} allele is a strong hypomorphic mutation that impairs innate immune responses. Surprisingly, this allele encodes a conservative amino acid substitution, alanine (A) to valine (V) at position 31, and is positioned in a region with no obvious structural motifs (Georgel et al., 2001). The reason for the strong hypomorphic phenotype associated with the A31V substitution remains unclear. In this work, we demonstrate that *imd* protein is rapidly cleaved following PGN stimulation. Cleavage requires the caspase DREDD and occurs at caspase recognition motif $_{27}$LEKD/A$_{31}$, creating a neo-N terminus at A31 that is critical for the immune-induced association of IMD with DIAP2. Substitution of the neo-N terminus with valine, as in *imd*^{1}, disrupts the IMD-DIAP2 interaction. Moreover, once associated with DIAP2, cleaved IMD is rapidly K63-polyubiquitinated. Together, these data resolve a number of outstanding questions in IMD signal transduction and present a clear molecular mechanism linking caspase-mediated cleavage to NF-κB activation.

RESULTS

Signal-Induced Modification of IMD

To determine if the proteolytic activity of DREDD is required upstream in the IMD pathway, the caspase inhibitor zVAD-fmk was utilized. Immune-responsive *Drosophila* S2* cells were treated with zVAD-fmk (or vehicle control) prior to stimulation with *E. coli* PGN. Treatment with caspase inhibitor suppressed the activation of TAK1 kinase, as monitored by in vitro IP-kinase assay. zVAD-fmk also prevented accumulation of phospho-JNK (Figure 1A).

Hypothesizing that IMD signaling results in the DREDD-mediated cleavage of an upstream signaling component, we next examined the fate of endogenous *imd* protein. PGN stimulation of S2* cells resulted in the rapid, signal-dependent modification of IMD (Figure 1B). The faster migrating IMD product did not accumulate in the presence of caspase inhibitor (Figure 1C), suggesting that it represents an IMD cleavage product. Cleavage of IMD appeared very rapidly, within 1 min, and peaked between 10 and 30 min. Treatment of S2* cells with RNAi targeting either DREDD or FADD, prior to stimulation with PGN, resulted in a decrease in IMD cleavage and accumulation of full-length IMD (Figure 1D). Furthermore, overexpression of wild-type DREDD (WT), but not a noncatalytic mutant (CA) or the caspase DRONC, from the copper inducible metallothionein promoter resulted in the cleavage of IMD, independent of immune stimulation (Figure 1E). Together these results suggest that the proteolytic activity of DREDD is required for the cleavage of IMD. Phosphorylation of IMD was also detected after immune stimulation, with the appearance of a slower migrating form, see Figure S1 available online.

DIAP2 Functions between IMD and TAK1

To examine how IMD cleavage might be linked to downstream signaling events such as K63 ubiquitination, we sought to more

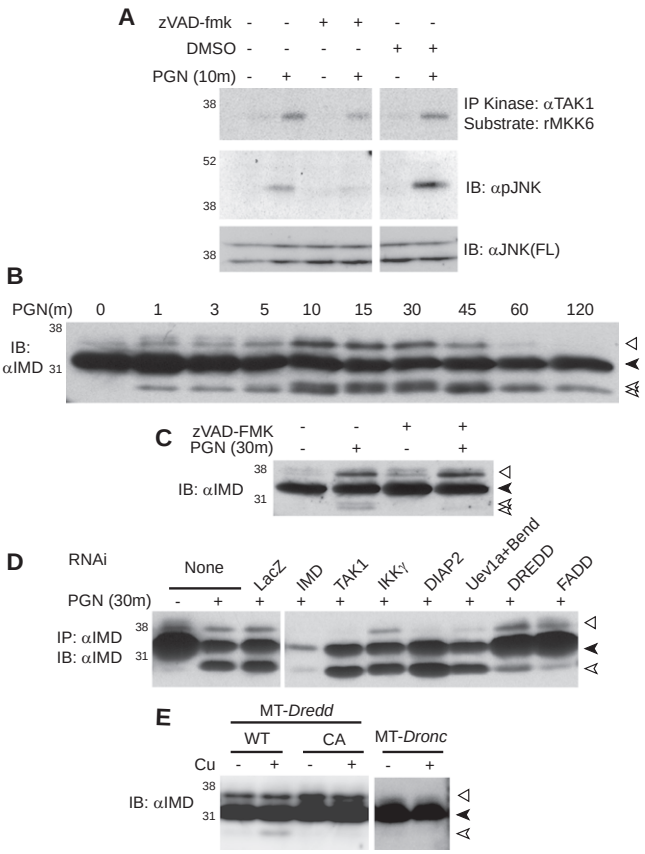

Figure 1. Dredd-Dependent Cleavage of IMD

(A) S2* cells were treated with zVAD-fmk or vehicle control (DMSO) prior to stimulation with PGN for 30 min. TAK1 kinase activity was monitored by TAK1 IP-kinase assay, and JNK phosphorylation was monitored by immunoblotting.

(B) S2* cells were stimulated with PGN from 0 to 120 min, as indicated, and endogenous-IMD was monitored in whole-cell lysates by immunoblotting.

(C) Endogenous IMD was monitored in S2* cell lysates after pretreatment with zVAD-fmk followed by PGN stimulation.

(D) Endogenous IMD was monitored by IP-immunoblotting from S2* lysates after treatment with RNAi targeting various IMD pathway components, as indicated.

(E) Endogenous IMD was monitored in S2* cell lysates after expression of DREDD-WT, DREDD-CA, or DRONC. Caspases were expressed from the metallothionein promoter by addition of copper sulfate for 6 hr. DREDD-CA is a catalytically inactive mutant. All three caspases were robustly expressed (data not shown).

In all cases, ◀ marks unmodified full-length IMD, ◀ highlights phosphorylated IMD, and ◁ marks the cleaved IMD products. See also Figure S1.

carefully characterize the role of DIAP2, a putative ubiquitin E3-ligase, in this pathway. As previously shown, DIAP2 RNAi markedly inhibited the induction of the AMP gene *Diptericin* following immune stimulation with DAP-type PGN, similar to RNAi targeting PGRP-LC or IKKγ, as analyzed by northern blotting (Figure 2A) (Gesellchen et al., 2005; Huh et al., 2007; Kleino et al., 2005; Leulier et al., 2006). Using a stable S2* cell line that expresses *imd* from the metallothionein promoter, AMP gene expression can be induced by the addition of copper (Zhou et al., 2005). DIAP2 RNAi also inhibited *Diptericin* induction in this

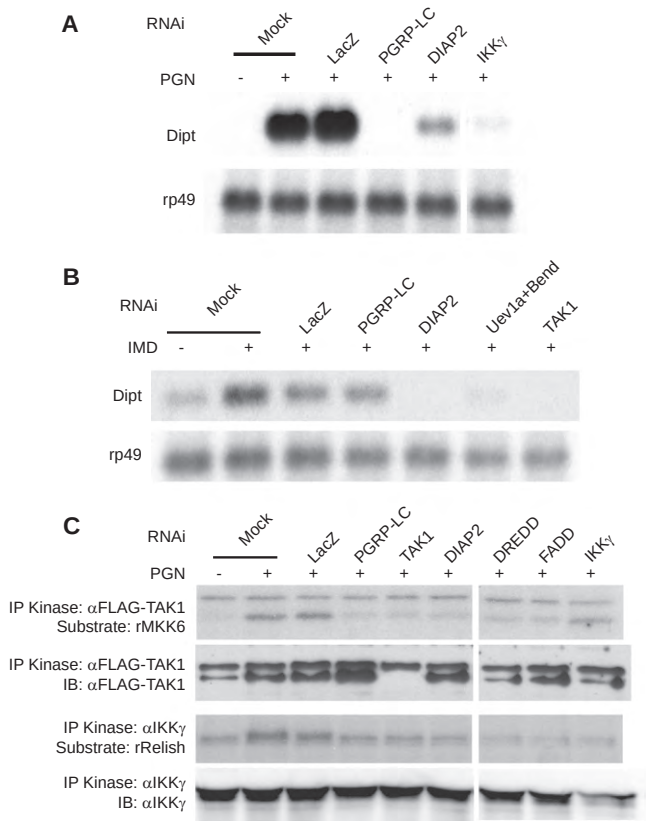

Figure 2. DIAP2 Functions between IMD and TAK1

(A) S2* cells were treated with RNAi against various IMD pathway components, as indicated, prior to stimulation with *E.coli* PGN for 6 hr. Induction of the AMP gene *Diptericin* was monitored by northern blotting. *rp49* levels were monitored as a loading control.

(B) S2* cells stably expressing metallothionein IMD were treated with RNAi against various IMD pathway components, as indicated. Then cells were stimulated with copper sulfate for 6 hr to induce IMD expression. Expression of the AMP gene *Diptericin* and *rp49* were monitored by northern blotting.

(C) The activation of TAK1 or IKK kinases was monitored by IP-kinase assays, after stimulation with *E. coli* PGN for 10 min. Recombinant $MKK6^{K82A}$ and recombinant Relish were used as substrates for TAK1 and IKK, respectively. RNAi was used to target various IMD pathway components, as indicated.

assay (Figure 2B), similar to RNAi targeting Uev1a and Ubc13 or TAK1, as shown previously (Zhou et al., 2005). In contrast, RNAi-mediated knockdown of PGRP-LC did not inhibit IMD-induced signaling. These results suggest that DIAP2, TAK1, Ubc13, and Uev1a, but not PGRP-LC, function downstream of IMD.

In addition, the activation of the two downstream kinases, TAK1 and IKK, was directly assayed by IP–kinase assays. PGN-induced activation of both kinases required DIAP2 (Figure 2C). Together these data argue that DIAP2 functions downstream of IMD but upstream of TAK1, similar to Ubc13 and Uev1a (Zhou et al., 2005).

Cleaved IMD Associates with DIAP2 and Is Ubiquitinated

As IMD is rapidly cleaved following immune stimulation and DIAP2 appears to function immediately downstream, we hypothesized that these two proteins may associate. To that end, endogenous DIAP2 was immunoprecipitated from PGN-stimulated and unstimulated S2* cells. Endogenous IMD coprecipitated with DIAP2 in a signal-dependent fashion (Figure 3A). Strikingly, DIAP2 preferentially associated with cleaved IMD. Overall, the amount of IMD cleavage observed in whole-cell lysates varied, ranging from 5% to 15% of the total, while the cleaved IMD was preferentially (50%–70%) associated with immunoprecipitated DIAP2.

These results, linking cleaved IMD and the E3-ligase DIAP2, suggest that one or both of these proteins may be conjugated with ubiquitin. Using immunoprecipitation followed by immunoblotting for total ubiquitin, IMD was found to be ubiquitinated rapidly and robustly after PGN stimulation of S2* cells (Figure 3B). DIAP2 was also ubiquitinated, but to a lesser extent (Figure 3C, Figure S2A). This modification occurs within 1 min of stimulation, reaching a maximum at 5–10 min, and is lost in approximately 30 min. IMD ubiquitination is stable after boiling in 1% SDS, further arguing that it is directly conjugated (Figure S2B). Analysis of IMD cleavage, DIAP2 association, and ubiquitination from the same samples demonstrates that ubiquitination peaks shortly after cleavage and maximal DIAP2 association (Figure S3).

In order to determine which members of the IMD pathway are required for PGN-induced IMD ubiquitination, S2* cells were treated with RNAi targeting various pathway components and analyzed by IMD immunoprecipitation and ubiquitin immunoblotting. PGRP-LC, IMD, DREDD, FADD, and DIAP2 RNAi reduced IMD ubiquitination. Conversely, targeting downstream components, such as TAK1 or IKKγ, did not robustly affect IMD ubiquitination (Figure 3D). Previously, the E2 complex of Bend and Uev1a was implicated in IMD pathway ubiquitination. RNAi treatment targeting both of these proteins (separately and together) reduced but did not eliminate the ubiquitination of IMD after stimulation (Figure 3E, lanes 6–8), similar to their partial effect on immune-induced *Diptericin* expression (Zhou et al., 2005 and Figure 3E). Recently, it has been suggested that another E2, Ubc5, may also form K63-ubiquitin chains during mammalian NF-κB signaling (Xia et al., 2009; Xu et al., 2009). Therefore, we determined if the *Drosophila* Ubc5 homolog, Effete, was also required for IMD ubiquitination. RNAi targeting Effete, in concert with Bend, or Uev1a (together or separately) were able to completely inhibit ubiquitination of IMD (Figure 3E, lanes 3–5). Similarly, when *Diptericin* induction was analyzed, RNAi targeting Uev1a, Bend, or Effete alone or in pairs only partially inhibited IMD signaling, while treatment with all three RNAis nearly abolished immune-induced *Diptericin* expresion. (Figure 3E, bottom).

Consistent with the above data, pretreatment with the caspase inhibitor zVAD-fmk reduced the amount of ubiquitinated IMD observed (Figure 3F). DIAP2 ubiquitination similarly required these same factors (Figures S2C and S2D). Together, these results indicate that cleaved IMD and DIAP2 associate, triggering their Uev1a/Bend/Ubc5-dependent ubiquitination.

DIAP2 Is the E3 for IMD K63 Ubiquitination In Vivo

Strikingly, infection-induced IMD ubiquitination was readily detectable when IMD was immunoprecipitated from lysates

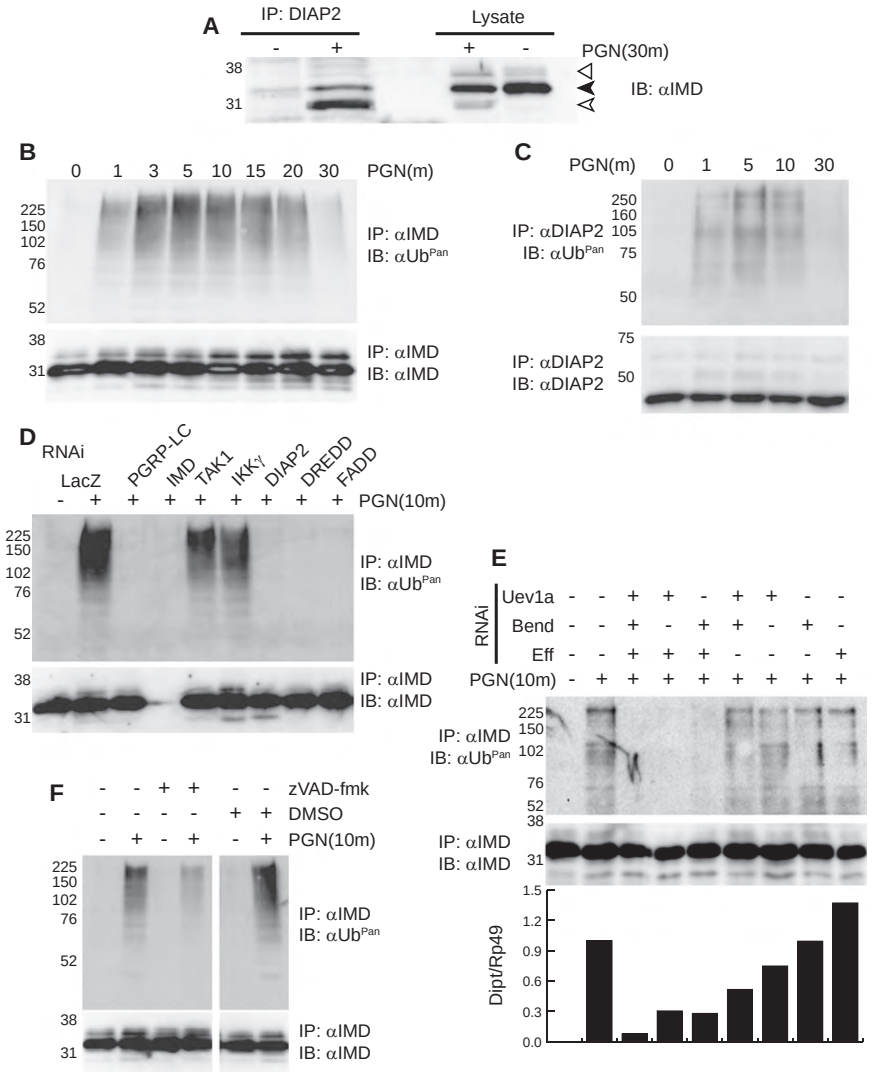

Figure 3. Association of DIAP2 and Cleaved IMD Leads to IMD Ubiquitination

(A) Endogenous DIAP2 was immunoprecipitated from whole-cell lysates prepared from S2* cells before or after a 10 min stimulation with *E. coli* PGN, and associated IMD was monitored by immunoblotting (left lanes). For comparison, levels of full-length and cleaved IMD in whole-cell lysates are shown on the right. ◀ marks unmodified full-length IMD, ◀ highlights phosphorylated IMD, and ◁ marks the cleaved IMD products.

(B) Ubiquitination of endogenous IMD was monitored in lysates from S2* cells after stimulation with *E. coli* PGN for various times (as indicated) by anti-IMD immunoprecipitation followed by immunoblotting with anti-Ub^{Pan} (top). An anti-IMD immunoblot serves as a loading control (bottom).

(C) Ubiquitination of endogenous DIAP2 was monitored in lysates from S2* cells after stimulation with *E. coli* PGN for various times (as indicated) by anti-DIAP2 immunorecipitation followed by immunoblotting with anti-Ub^{Pan}. An anti-DIAP2 immunoblot serves as a loading control (bottom).

(D) Ubiquitination of endogenous IMD in PGN-stimulated S2* cells was monitored after RNAi targeting various pathway members (top). An anti-IMD immunoblot serves as a loading control (bottom).

(E) Ubiquitination of endogenous IMD after PGN stimulation was monitored after treatment with RNAi targeting various E2-ubiquitin-conjugating enzymes (top). An anti-IMD immunoblot serves as a loading control (middle). In parallel, IMD pathway activation was also monitored by northern blotting for the AMP gene *Diptericin*. The ratio of *Diptericin* to *Rp49* message was quantified by phosphoimager and plotted (bottom). The y axis is arbitrary normalized phosphoimager units.

(F) Ubiquitination of endogenous IMD in S2* cells was monitored after treatment with zVAD-fmk or vehicle control (DMSO) (top). An anti-IMD immunoblot serves as a loading control (bottom).

See also Figures S2 and S3.

extracted from adult flies, 30 min after septic infection with *E. coli*. As expected, *diap2* null flies, $diap2^{7c}$, show no IMD ubiquitination following infection with *E. coli* (Figure 4A). Previously, the RING finger of DIAP2 was shown to play an important role in the IMD pathway (Huh et al., 2007). Therefore, we next sought to determine if this domain was required for IMD ubiquitination. Transgenic rescue flies expressing wild-type DIAP2 from the daughterless-gal4/UAS system were able to rescue the $diap2^{7c}$ IMD ubiquitination phenotype. However, expression of a RING finger mutant of DIAP2 ($DIAP2^{C466Y}$) was unable to rescue this phenotype (Figure 4A, right panels). These data demonstrate that IMD is ubiquitinated in a signal-dependent manner in flies and that the DIAP2 RING finger is critical for this ubiquitination. These results strongly suggest that DIAP2 is the E3-ligase involved in IMD modification.

The data implicating Ubc13 (*bendless*), Uev1a, and Effete in this pathway (Zhou et al., 2005; work herein), suggest that IMD may be modified with K63-linked polyubiquitin chains. In order to clarify if IMD is conjugated with K48- or K63-polyubiquitin chains, we took advantage of two monoclonal antibodies that are specific to either K48- or K63-polyubiquitin chains (Newton et al., 2008). After denaturing lysis, these antibodies were used to immunoprecipitate ubiquitin-conjugated proteins from PGN-stimulated S2* cells. Immunoprecipitated samples were then analyzed by immunoblotting for IMD. These experiments demonstrate that IMD is strongly K63 ubiquitinated in a PGN-inducible manner, peaking at approximately 10 min after stimulation (Figure 4B), while negligible IMD was detected in the K48-immunoprecipitated samples. Control probing of these blots, with an antibody that recognizes all ubiquitin forms, showed that the K63- and K48-specific antibodies immunopurified similar amounts of ubiquitin-conjugated material. These data demonstrate that IMD is conjugated with K63-linked polyubiquitin chains.

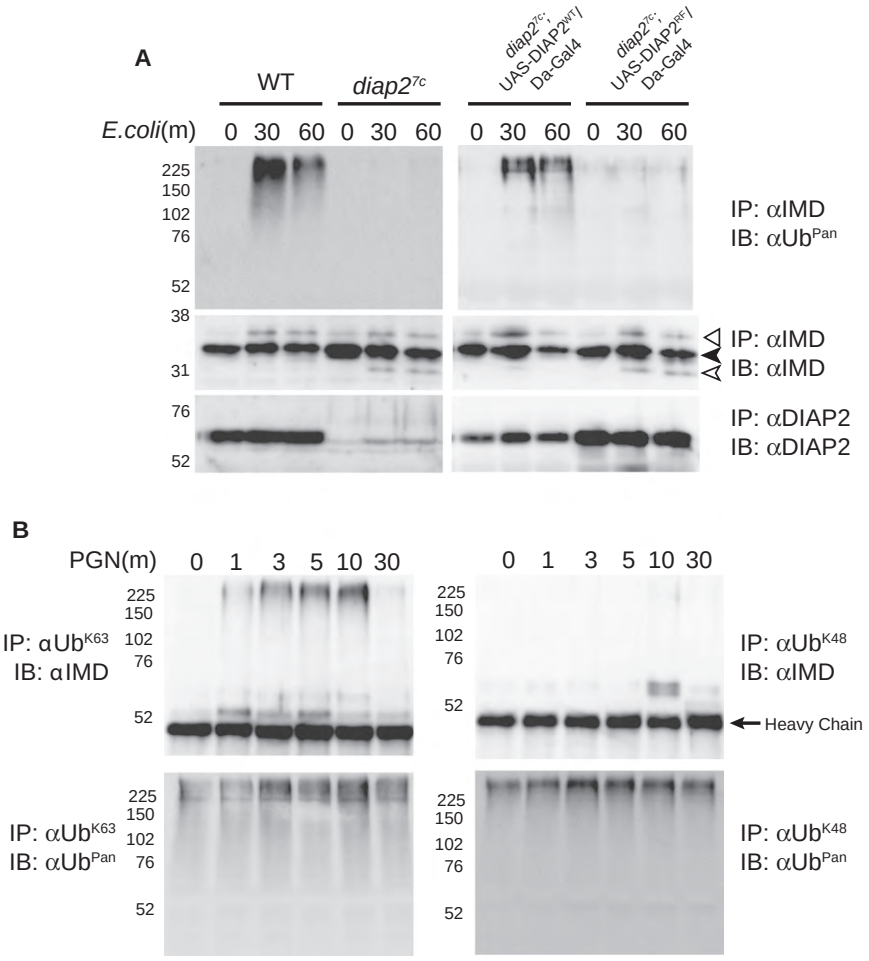

Figure 4. IMD Is K63-Polyubiquitinated

(A) Ubiquitination of endogenous IMD from adult flies was monitored after infection with live *E. coli* (top). An anti-IMD immunoblot serves as a control (middle, ◀ marks unmodified full-length IMD, ◀ highlights phosphorylated IMD, and ◁ marks the cleaved IMD product). IMD ubiquitination was monitored in wild-type (DD1), *diap2*^{7c} (null), and *diap2*^{7c} expressing transgenic wild-type (WT) or RING-finger mutated (RF) DIAP2. DIAP2 immunoprecipitation/immunoblot verifies the presence/absence of DIAP2 protein in the various mutant *Drosophila* lines, as indicated.

(B) Total K63-linked (left) or K48-linked (right) polyubiquitin chains were immunoprecipiated from denatured cell lysates prepared from S2* cells stimulated with PGN for various times, as indicated. The presence of IMD was then monitored by IMD immunoblotting (top). Subsequently, the same membranes were probed for total ubiquitin as a control (bottom).

Noncleavable IMD Prevents Signaling

We next sought to identify the IMD cleavage site and determine how IMD cleavage might be linked to association with DIAP2 and K63 ubiquitination. Based on experiments with either N- or C-terminally epitope-tagged IMD (data not shown) and the size of the cleaved product (~30 kDa), the site of cleavage was tentatively mapped to the N-terminal region of IMD. The involvement of the caspase DREDD further suggested a candidate cleavage site after residue 30, in the motif $_{27}$LEKD/A$_{31}$. Wild-type or D30A mutant versions of IMD, with epitope tags at both the C and N termini, were expressed in S2* cells by stable transfection, using the constitutive actin promoter. Double-tagged wild-type IMD showed the expected cleavage and ubiquitination kinetics after stimulation with PGN, as detected with the C-terminal FLAG tag (Figure 5A). The N-terminal T7 tag did not detect cleaved IMD (data not shown). With the aspartate at the putative cleavage site substituted with alanine (D30A), no PGN-induced cleavage was observed (Figure 5A, lower panel, right lanes), suggesting that caspase-mediated cleavage of IMD occurs at this position. Furthermore, IMD^{D30A} acted as a dominant negative, blocking the PGN-induced ubiquitination of endogenous IMD (Figure 5A, upper panel, right lanes) and blocking downstream IMD signaling as monitored by analysis of *Diptericin* expression (Figure 5B). Similarly, transgenic flies, overexpressing IMD^{D30A}, show a marked inhibition of *Diptericin* induction following *E. coli* challenge (Figure 5C). These data strongly argue that cleavage of IMD occurs between residues 30 and 31 and that this cleavage is required for ubiquitination and activation of downstream target genes.

Using a series of IMD deletion mutants we also analyzed which regions of IMD were required for signal-induced ubiquitination (Figure 5D). As shown previously, overexpression of wild-type IMD drives pathway signaling (Figure 2B). Interestingly, only IMDΔ1 and IMDΔ5 were unable to drive signaling after overexpression (Figure 5E and data not shown). The region removed in the IMDΔ1 construct contains the IMD cleavage site, which is critical for signaling, as shown above, via its involvement in DIAP2 interaction (see below for more details). In order to determine if the region removed in IMDΔ5 functions in ubiquitination, *imd* protein was analyzed in two ways. First, IMD was strongly overexpressed from the metallothionein promoter, at a level strong enough to induce AMP expression (Figure 5F, left). Second, IMD was expressed at lower levels, before cells were stimulated with PGN (Figure 5F, right). In both cases, wild-type IMD shows robust ubiquitination after activation, while IMDΔ5 shows no ubiquitination over background. These data indicated that this region of IMD is crucial for signal-induced ubiquitination and the induction of downstream target genes.

IMD Cleavage Exposes an IAP-Binding Motif

BIR domains, such as those found in DIAP2, preferentially bind to unblocked N-terminal alanines, generated either by removal of the initiating methionine or endoproteolytic cleavage. These

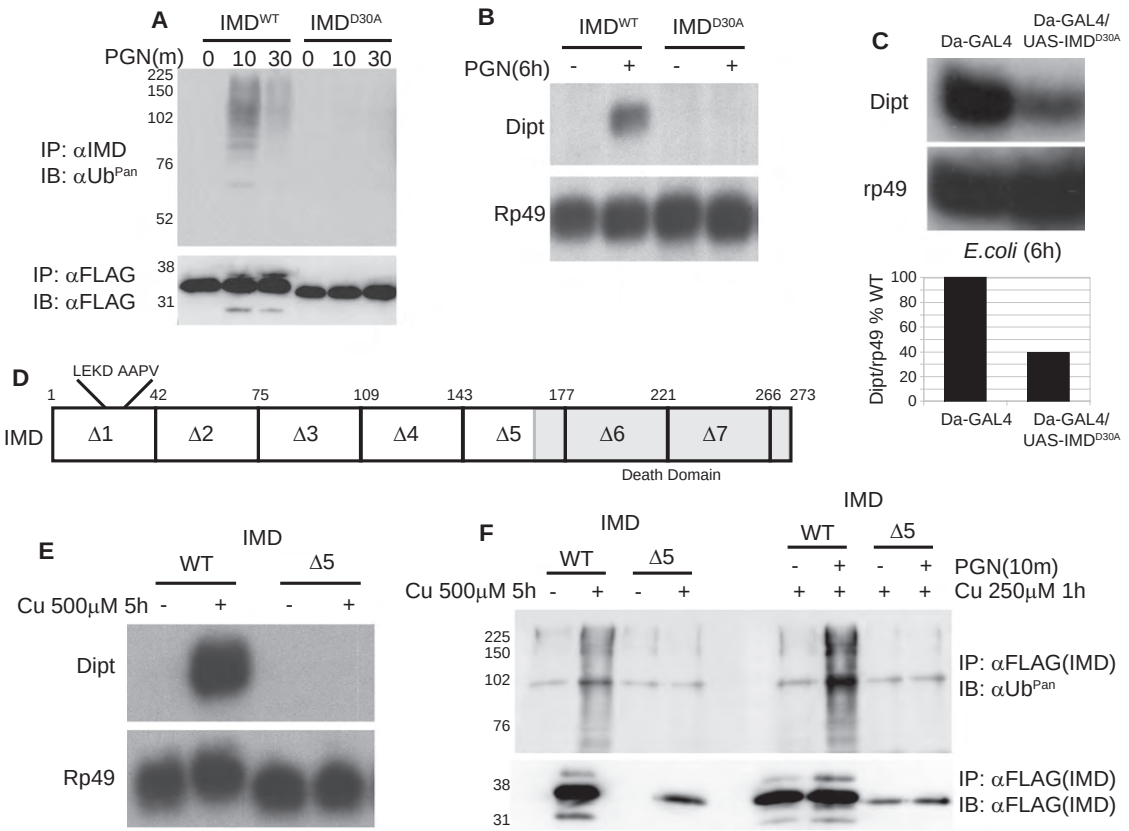

Figure 5. Uncleavable IMD Is a Dominant Negative

(A) Total ubiquitinated IMD was monitored by immunoprecipitation/Ub^{pan} immunoblotting in cells stably expressing either wild-type or mutant (D30A) IMD from the actin promoter (top). The lower anti-FLAG blot monitors cleavage of the wild-type or D30A IMD.

(B) Northern blot analysis of *Diptericin* expression was used to monitor IMD pathway signaling in cells overexpressing wild-type or D30A IMD, before and after stimulation with *E. coli* PGN.

(C) Northern blot analysis of *Diptericin* expression was used to monitor IMD pathway signaling in flies expressing a transgenic copy of UAS-IMD^{D30A} under the control of ubiquitous daughterless-Gal4 driver (top). Quantitation of these data is presented below.

(D) A map of IMD deletions used to analyze signaling activity is shown. The caspase cleavage (LEKD) and IBM (AAPV) domains are indicated. The C-terminal death domain is indicated in gray.

(E) Wild-type IMD or IMDΔ5 was stably expressed in S2* cells from the copper inducible metallothionein promoter. Activation of immune signaling was monitored by northern blotting for *Diptericin*. *Rp49* blot serves as a loading control.

(F) Ubiquitination of wild-type IMD or IMDΔ5 was monitored in two conditions. (Left) IMD protein was expressed at high level by addition of 500 μM copper for 5 hr, identical to that seen in (E). (Right) IMD protein was moderately expressed, with lower copper (250 μM) for only 1 hr, and then cells were stimulated with PGN. In both cases, IMD was then immunoprecipitated with FLAG antibody, and ubiquitination levels were assayed by immunoblotting for ubiquitin. FLAG blot (bottom) serves as a control for the levels of IMD immunoprecipitated.

exposed neo-N-terminal alanines are invariant components of IBMs, which also include a strong preference for proline at position 3 (Shi, 2002, 2004). In particular, the DIAP1 BIR1 domain associates with the neo-N-terminal IBM of the cleaved caspases drICE and DCP-1 (Tenev et al., 2005). Likewise, cleavage of IMD at D30 exposes a putative IBM sequence with an initial alanine and a proline at position 3 (AAPV). Moreover, both the proposed caspase cleavage site and this IBM are highly conserved in the *imd* protein from 12 *Drosophila* species and the *Anopheles* mosquito (Figure 6A, highlighted in blue and red, respectively).

Interestingly, the *imd*^{1} allele substitutes alanine 31 with valine, altering the key residue of the IBM. Although a fairly conservative change, this substitution generates a strong hypomorphic phenotype through unknown mechanisms (Georgel et al., 2001). To test whether cleaved IMD exposes a bona fide IBM, we assessed whether DIAP2 interacted with cleaved wild-type IMD (A31) or cleaved mutant IMD (V31). To this end, we used the ubiquitin fusion technique to express IMD$^{31–273}$ (Varshavsky, 2000), which generates IMD with an unblocked N-terminal IBM (see Figure S4). Wild-type A31-IMD$^{31–273}$ robustly interacted with DIAP2 (Figure 6B, lane marked AAPV). However, V31-IMD$^{31–273}$, equivalent to the cleavage product expected from the *imd*^{1} allele, showed markedly reduced binding to DIAP2 (Figure 6B, lane marked VAPV). Also, in transient transfections, full-length IMD was spontaneously cleaved at a low level (Figure 6B, lower left panels), and only the cleaved product associated with cotransfected DIAP2, consistent with the data examining the endogenous proteins.

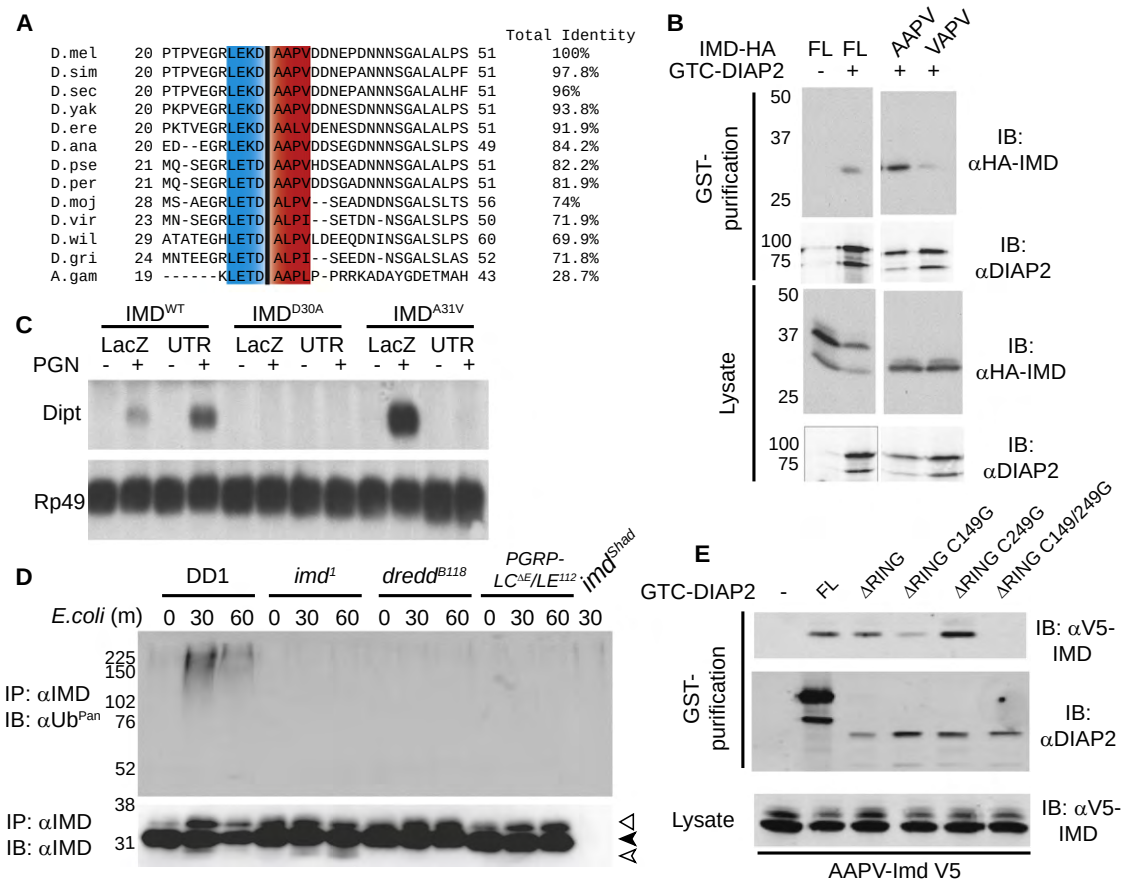

Figure 6. IMD A31 Is Required for DIAP2 Association

(A) An alignment of IMD from 12 *Drosophila* species and the *Anopheles* mosquito shows conservation in the caspase cleavage site (blue) and IBM (red).

(B) The binding of wild-type or mutant cleaved IMD proteins to DIAP2 was monitored using the ubiquitin fusion technique. HA-tagged full-length (FL), A31-IMD$^{31–273}$ (AAPV), or V31-IMD$^{31–273}$ (VAPV) was coexpressed in cells along with GST-DIAP2. GST purification and immunoblotting for anti-HA were used to monitor IMD/DIAP2 association.

(C) Northern blot analysis of *Diptericin* expression was used to monitor IMD signaling in cells expressing wild-type, D30A, or A31V IMD after treatment with RNAi to lacZ or *imd* 3′UTR.

(D) Ubiquitination of endogenous IMD from wild-type (DD1) and various mutant adult flies was motioned after infection with live *E. coli* via IMD immunoprecipitation followed by Ub^{Pan} immunoblotting (top). IMD immunoblot is shown as a loading control (bottom). ◀ marks unmodified full-length IMD, ◀ highlights phosphorylated IMD, and ◁ marks the cleaved IMD product.

(E) Association of cleaved IMD and DIAP2 mutants was monitored via GST-coprecpitation, as in (B).

See also Figure S4.

In order to verify the importance of the neo-N-terminal alanine on IMD pathway signaling, we overexpressed IMD-A31V in S2* cells from the actin promoter. Cells were treated with RNAi against the 3′UTR region of IMD, to remove the endogenous wild-type protein, or lacZ (as a control) prior to stimulation with PGN. As expected, expression of IMD-D30A shows a strong dominant-negative phenotype, consistent with previous data. On the other hand, cells expressing IMD-A31V and treated with the control lacZ RNAi showed a strong *Diptericin* induction. Treatment with IMD 3′UTR RNAi, which selectively degrades endogenous, but not ectopically expressed IMD, resulted in a near complete inhibition of *Diptericin* induction, showing that IMD-A31V is not sufficient for signaling in cells, as observed in flies (Figure 6C).

In order to probe whether or not these same mechanisms are involved in immune signaling in the whole animal, IMD cleavage and ubiquitination were also probed in the *imd*^{1} (A31V) strain. In lysates prepared from these flies, cleaved IMD was readily detected following *E. coli* infection, but ubiquitinated IMD was completely absent (Figure 6D). In fact, the cleaved IMD product accumulates in *imd*^{1} animals, relative to the wild-type control, presumably because cleaved IMD is rapidly and efficiently ubiquitinated in wild-type but not *imd*^{1} animals. Likewise, cleaved IMD is more easily detected in the *diap2* mutant strain (Figure 4A). On the other hand, neither cleaved nor ubiquitinated IMD was detectable in either *dredd* or *PGRP-LE*;*PGRP-LC* mutant animals, consistent with the results from cell culture.

In order to determine which domains of DIAP2 are required for the binding of cleaved IMD, a series of DIAP2 mutations were generated. A C149G mutation of BIR2, predicted to abrogate its IBM binding (Ribeiro et al., 2007), reduced IMD$^{31–273}$

association. Under the same conditions, mutation of the BIR3 domain (C249G) did not affect the interaction with IMD$^{31–273}$. When both BIR2 and BIR3 domains were altered (C149/249G), IMD$^{31–273}$ completely failed to interact (Figure 6E). As expected, the RING finger domain did not contribute to IMD binding, since its deletion had no noticeable effect on IMD$^{31–273}$ binding. Together these results indicate that cleaved IMD carries a bona fide IBM at its neo-N terminus, which preferentially binds to the BIR2 of DIAP2, and to some extent also binds the BIR3 domain. The alanine at the N terminus of the IMD cleavage product is critical for this binding, while valine at this position, as in *imd*^{1}, weakens the interaction.

DISCUSSION

In previous work, we demonstrated that the caspase-8-like protease DREDD and its binding partner FADD are required upstream in the IMD pathway, at a position similar to Ubc13 and Uev1a (Zhou et al., 2005). However, it was not clear from these studies if the protease activity of DREDD is also required in this role upstream in the IMD pathway. Here we show that upon immune stimulation the *imd* protein is rapidly cleaved in a DREDD- and FADD-dependent manner. In fact, expression of DREDD, without immune stimulation, was sufficient to cause IMD cleavage. A caspase recognition site was identified in IMD, with cleavage predicted to occur after aspartate 30. Substitution of this residue with alanine prevents signal-induced cleavage and creates a dominant-negative allele of *imd*. This putative cleavage site in IMD ($_{27}$LEKD/A$_{31}$) is similar to the Relish cleavage site ($_{542}$LQHD/G$_{546}$), consistent with the notion that both proteins are cleaved by the same protease. Likewise, when IMD cleavage was blocked by caspase inhibitors, IMD was no longer ubiquitinated. Alignment of *imd* protein sequences from 12 *Drosophila* species and the *Anopheles* mosquito showed that the cleavage site is highly conserved (LEKD or LETD in all cases). These findings strongly argue that IMD cleavage after position 30 is mediated by DREDD and that this cleavage is critical for further downstream signaling events.

Cleavage of IMD exposes a highly conserved IBM (Figure 6A), which then binds the BIR2/3 domains of DIAP2. In the context of programmed cell-death regulation, these IBM motifs are best defined by their neo-N terminal alanine as well as the proline at position 3, both of which are also present in cleaved IMD, supporting the notion that IMD includes an IBM starting at position 31. The notion that IMD carries an IBM also provides a molecular explanation for the hypomorphic phenotype observed in the *imd*^{1} mutant, which carries a valine substitution for this alanine at position 1 of cleaved IMD. Although several IAP proteins have been implicated in mammalian innate immune/NF-κB signaling (Deveraux and Reed, 1999; Verhagen et al., 2001; Bertrand et al., 2008, 2009), the significance of their associated BIR domains, as well as their possible binding to proteins with exposed IBMs, has remained largely unexplored. We show here that the BIR/IBM association plays a crucial role in innate immune NF-κB signaling in *Drosophila*. These findings present a unique role for the BIR-IBM interaction module outside of the cell-death arena.

Furthermore, characterization of signaling in the *imd*^{1}, *diap2*, *dredd*, and *PGRP-LC/LE* mutant flies provides critical in vivo

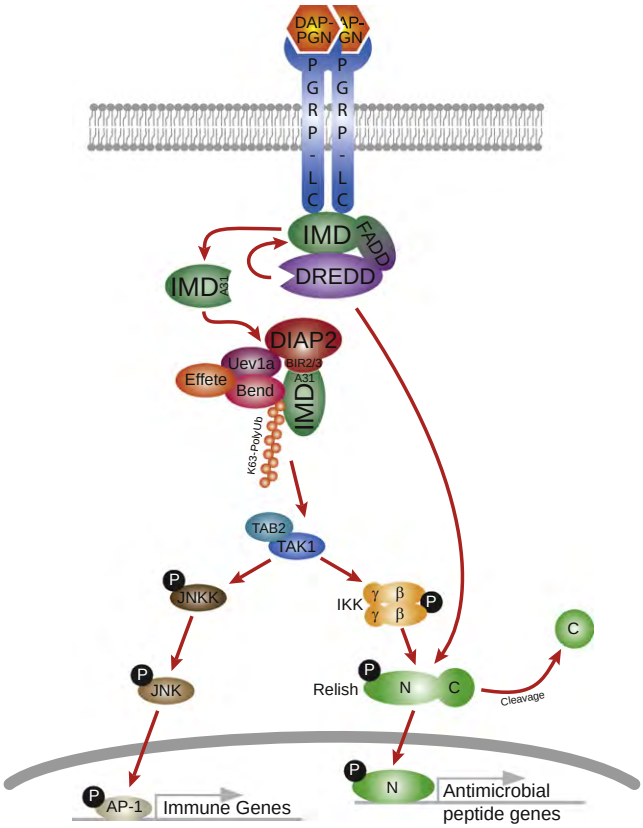

Figure 7. IMD Pathway Model

DAP-type PGN binding causes multimerization or clustering of PGRP receptors. This likely recruits the adaptor proteins IMD, FADD, and the caspase DREDD. Once in proximity, DREDD cleaves IMD, generating an exposed neo-N-terminal A31 residue. This neo-N terminus then binds the E3-ligase DIAP2 via its BIR2/3 domains. In conjuction with the E2-ubiquitin-conjugating enzymes UEV1a, Bendless (Ubc13), and Effete (Ubc5), IMD (and to a lesser degree DIAP2) are then K63-polyubiquitinated. These polyubiquitin chains then induce the activation of downstream kinases, ultimately leading to the phosphorylation and activation of Relish and induction of downstream targets, like the AMP genes.

verification of the cell-culture data and leads to the model presented in Figure 7. In particular, the molecular mechanism we propose suggests that immune stimulation leads to the DREDD-dependent cleavage of IMD, perhaps by recruiting IMD, FADD, and DREDD to a receptor complex. Consistent with this aspect of the model, *dredd* mutants and receptor mutants failed to cleave (or ubiquitinate IMD) following infection. Once cleaved, the exposed IBM of IMD interacts with BIR2 and BIR3 of DIAP2. Currently, we do not know precisely where in the cell the IMD/DIAP2 association occurs. In Figure 7, this interaction is diagrammed as occurring away from the receptor proximal complex, but this is only for illustrative purposes. Once associated with DIAP2, cleaved IMD is rapidly K63 ubiquitinated. As the RING-mutated version of *diap2* failed to support IMD ubiquitination in flies, DIAP2 likely functions as the E3 for this reaction. Furthermore, the *imd*^{1} allele, which fails to interact with DIAP2 because of a mutation in the IBM, demonstrates

the critical nature of the IMD-DIAP2 interaction for innate immune signaling. Consistent with the notion that cleavage precedes ubiquitination, mutants that fail to generate ubiquitinated IMD (i.e., *diap2* and *imd*^{1}) actually accumulate more cleaved IMD than is observed in wild-type flies. Presumably, in wild-type animals, cleaved IMD is efficiently ubiquitinated and thus is difficult to detect in our assays. On the other hand, *dredd* mutants or mutants lacking the key immunoreceptors (*PGRP-LC/LE*) failed to cleave and ubiquitinate IMD, consistent with our cell-culture data.

Previous work has suggested that ubiquitination plays a critical role in IMD signaling in the *Drosophila* immune response (Gesellchen et al., 2005; Huh et al., 2007; Kleino et al., 2005; Leulier et al., 2006; Zhou et al., 2005). However, the molecular target(s) of ubiquitination and the mechanisms of its activation have remained elusive. As discussed above, the data presented here indicate that DIAP2 functions as the E3-ligase in the IMD pathway, a function usually attributed to the TRAF or, more recently, cIAP proteins in mammalian NF-κB signaling pathways (Bertrand et al., 2009). The E2 complex of Bend and Uev1a also appears to be involved in IMD ubiquitination. RNAi targeting of these K63-ubiquitinating enzymes reproducibly decreases IMD ubiquitination and the induction of target genes; however, the degree of inhibition is variable and never complete (data herein and Zhou et al., 2005). We now show that a third E2 enzyme, Effete, the *Drosophila* Ubc5 homolog, also plays a vital role in ubiquitination of IMD. RNAi treatment targeting Effete, in concert with Uev1a and/or Bendless reproducibly eliminated IMD ubiquitination and the induction of *Diptericin*.

Several lines of evidence argue that IMD is the critical target for K63 ubiqutination in this pathway. First, IMD is by far the most robustly modified component that we have identified, and the only one in which modifications can be detected in whole animals. Second, the protein produced as a result of the *imd*^{1} mutation, which does not signal, is also not ubiquitinated. Third, we present a deletion mutant, IMDΔ5, which is not ubiquitinated and fails to signal. Finally, Thevenon et al. (2009) recently identified the *Drosophila* ubiquitin-specific protease, USP36, as a negative regulator of IMD ubiquitination. Functionally, USP36 is able to remove K63-polyubiquitin chains from IMD, promoting K48-mediated polyubiquitination and degradation of IMD. Consistent with our model, animals which overexpress USP36 show decreased levels of IMD ubiquitination and reduced IMD pathway activation as monitored by *Diptericin* RNA expression, and are susceptible to bacterial infection. Together, these data strongly argue that IMD is the critical substrate for K63-polyubiqutination in IMD pathway signaling, although other proteins may also be conjugated to lesser degree (as shown here for DIAP2) and could potentially substitute for IMD as the platform for ubiquitin conjugation. Interestingly, Chen and colleagues recently showed that unanchored K63-polyubiquitin chains (i.e., ubiquitin chains that are not conjugated to a target substrate) are sufficient to activate the mammalian TAK1 and IKK kinase complexes. Furthermore, they show that unanchored polyubiquitin chains are produced after stimulation of HEK cells with IL-1β (Xia et al., 2009). Thus, the presence (or absence) of K63-polyubiquitin chains may be more important than their conjugation substrate.

K63-polyubiquitin chains are likely to serve as scaffolds to recruit the key kinases TAK1 and IKK in the IMD pathway. Both of these kinases include regulatory subunits with highly conserved K63-polyubiquitin binding domains. *Drosophila* TAB2, which complexes with TAK1, and the IKKγ subunit are predicted to contain conserved K63-polyubiquitin-binding domains (Ea et al., 2006; Kleino et al., 2005; Zhou et al., 2005; Zhuang et al., 2006). Thus, we hypothesize that K63-polyubiquitin chains will recruit both the TAB2/TAK1 complex and the IKK complex, creating a local environment for optimal kinase activation and signal transduction; however, this aspect of our model is still speculative.

Although mammalian caspase-8 and FADD are best known for their role in apoptosis, a growing body of literature indicates that these factors, along with RIP1 (which has some homology to IMD), also function in RIG-I signaling to NF-κB (Balachandran et al., 2004; Takahashi et al., 2006). In addition, caspase-8 has been implicated in NF-κB signaling in B cell, T cell, and LPS signaling (Bidere et al., 2006; Chun et al., 2002; Lemmers et al., 2007; Salmena et al., 2003; Su et al., 2005; Sun et al., 2008). Cells, from mice or humans, lacking caspase-8 have defects in immune activation, cytokine production, and nuclear translocation of NF-κB p50/p65 (Chun et al., 2002; Lemmers et al., 2007). Furthermore, recent evidence also shows that during mammalian NOD signaling the RIP2 protein is ubiquitinated in a cIAP1/2-dependent manner (Bertrand et al., 2009). Given that *Drosophila* homologs of RIP1, FADD cIAP1/2, and caspase-8 also function in the IMD pathway, the results presented here may help elucidate the mechanism by which these factors function in these mammalian immune signaling pathways.

EXPERIMENTAL PROCEDURES

Fly Stocks

All fly strains, except the IMD^{D30A} line, were previously published (Supplemental Experimental Procedures). The UAS-IMD^{D30A} line was generated by standard *P* element-mediated transformation.

RNAi

dsRNA used in this work was produced using T7 RiboMAX Express Large Scale RNA Production System (Promega). S2* cells were treated with RNAi delivered by calcium phosphate transfection; see the Supplemental Experimental Procedures for details.

RNA Analysis

Total RNA was isolated with the TRIzol reagent (Invitrogen), and gene expression was analyzed by northern blotting, as previously described (Silverman et al., 2000).

IMD Antibody

For the production of anti-IMD polyclonal antibodies, the full-length IMD coding sequence was cloned in the *E. coli* expression vector pDS56/RBII, 6xhis. His-tagged-IMD was expressed in M15 *E. coli* (strain M15), purified by Ni chromatography, and used to immunize rabbits.

TAK Antibody

For the production of the anti-TAK polyclonal antibody, the peptide KSDGRERLTVTDTKP was generated and used to immunize rabbits (Open Biosystems, Inc.). Serum was then pooled and affinity purified.

Protein and Immunoprecipitation Assays

Following stimulation, S2* cells were lysed in standard lysis buffer (see the Supplemental Experimental Procedures). For total protein analysis, 50–100 μg of total lysate was immunoblotted. For coimmunoprecipitation analysis, 200–600 μg of total cell lysate, or 600–900 μg of fly lysate, was subjected to precipitation with appropriate agarose crosslinked antibody or with antibody and protein A agarose (Sigma).

K48- and K63-Polyubiquitin Immunoprecipitation Assays

K48- and K63-polyubiquitin immunoprecipitation assays were performed as described previously (Newton et al., 2008) with minor modification for *Drosophila* cells (Supplemental Experimental Procedures).

Kinase Assays

The kinase activity of IKK and TAK1 was assayed by immunoprecipitating kinases using antisera to TAK1, IKKγ, or anti-FLAG (Sigma), as indicated. Standard kinase assay conditions were then used to monitor activity; see the Supplemental Experimental Procedures for details.

Caspase Inhibitor Treatment

S2* cells were split 0.5 × 10^6 cells per ml, and 20 hr later 1 μM 20-hydroxyecdysone was added. Twenty-four hours later, 100 μM caspase inhibitor zVAD-fmk, dissolved in DMSO, was then added to cells for 30 min or 4 hr prior to stimulation with *E. coli* PGN.

IMD/DIAP2 Pull-Down

S2 cells were transfected with pAc myc-Imd-HA or the pMT DHFR-HA-Ub-Imd (31-273) constructs with or without pMT-DIAP2-GTC. IMD/DIAP2 expression was induced by the addition of $CuSO_4$ and cells were lysed (see the Supplemental Experimental Procedures). DIAP2 was then purified using GSH-Sepharose (GE Healthcare), eluted, and immunoblotted as indicated.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.molcel.2009.12.036.

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Supplemental Information

Caspase-Mediated Cleavage, IAP Binding, and Ubiquitination: Linking Three Mechanisms Crucial for *Drosophila* NF-κB Signaling

Nicholas Paquette, Meike Broemer, Kamna Aggarwal, Li Chen, Marie Husson, Deniz Ertürk-Hasdemir, Jean-Marc Reichhart, Pascal Meier, and Neal Silverman

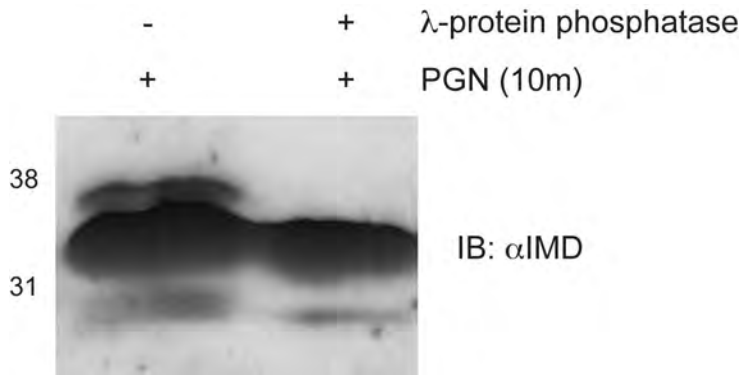

Figure S1. IMD is phosphorylated, related to Figure 1

A slower migrating form of endogenous IMD was also detectable following immune stimulation. λ phosphatase treatment of whole cell lysates resolved this higher molecular weight species, indicating that it is a phosphorylated isoform of IMD. Interestingly, RNAi-mediated knockdown of TAK1, DIAP2 or Uev1a and Ubc13 resulted in reduced levels of phospho-IMD (Figure 1D), suggesting that TAK1 may be responsible for phosphorylating IMD in some sort of regulatory loop. This phosphorylation event may regulate signaling, but requires further study.

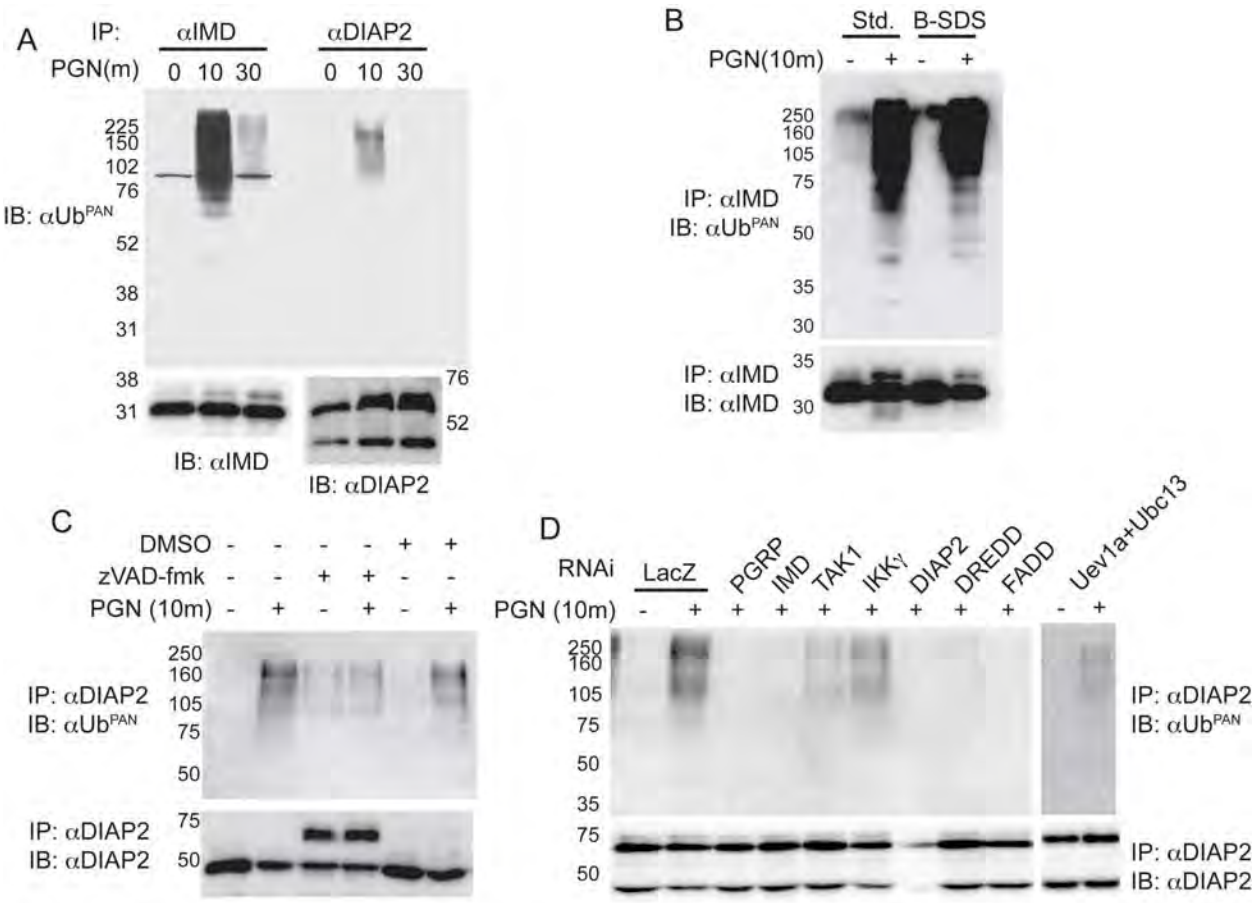

Figure S2. DIAP2 is ubiquitinated, related to Figure 3

(A) 200 µg or 600µg of S2* cell lysates were immunoprecipiated for endogenous IMD or DIAP2, respectively. Samples were then run side-by-side and immunoblotted with endogenous ubiquitin antibody. IMD and DIAP2 blots are also shown as a loading control, lower panels.

(B) Endogenous IMD was immunoprecipitated from standard S2* lysate (Std.) or lysates that had been boiled with 1% SDS (B-SDS). Samples were then immunoblotted for endogenous ubiquitin. IMD blots are shown as a loading control, below.

(C) PGN-induced ubiquitination of endogenous DIAP2 in lysates from S2* cells was monitored after pretreatment with zVAD-fmk, or vehicle control (DMSO). An anti-DIAP2 immunoblot serves as a loading control.

(D) PGN-induced ubiquitination of endogenous DIAP2 in lysates from S2* cells treated with RNAi to various IMD pathway components. An anti-DIAP2 blot serves as a loading control.

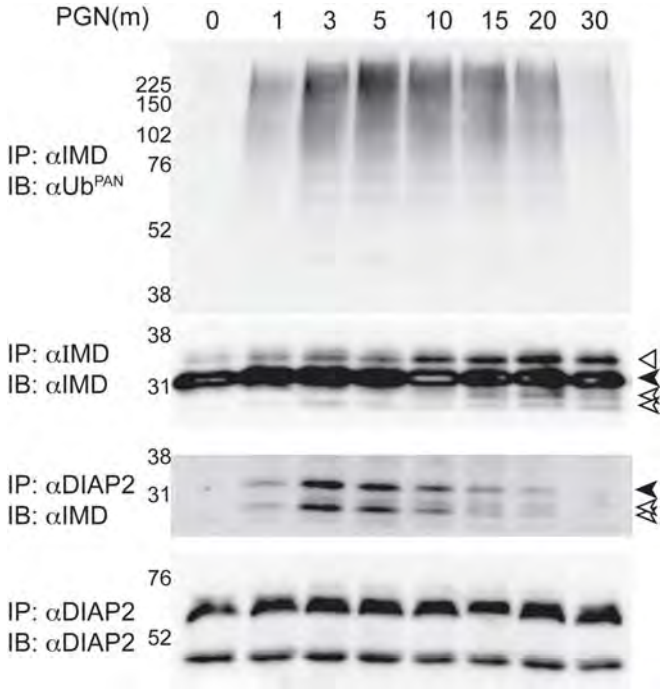

Figure S3. Coincident IMD ubiquitination, cleavage and DIAP2 association, related to Figure 3

Lysates from S2* cells stimulated with PGN for various times (as indicated) were immunoprecipitated for endogenous IMD or DIAP2. Immunoprecipiated IMD samples were then immunoblotted for endogenous ubiquitin and endogenous IMD (top two panels, same as in Figure 3B). The same lysates were also immunoprecipitated with DIAP2 and were probed for endogenous IMD and DIAP2 (bottom two panels). ◀ marks unmodified full length IMD, ◁ highlights phosphorylated IMD,and ◀ marks the cleaved-IMD products.

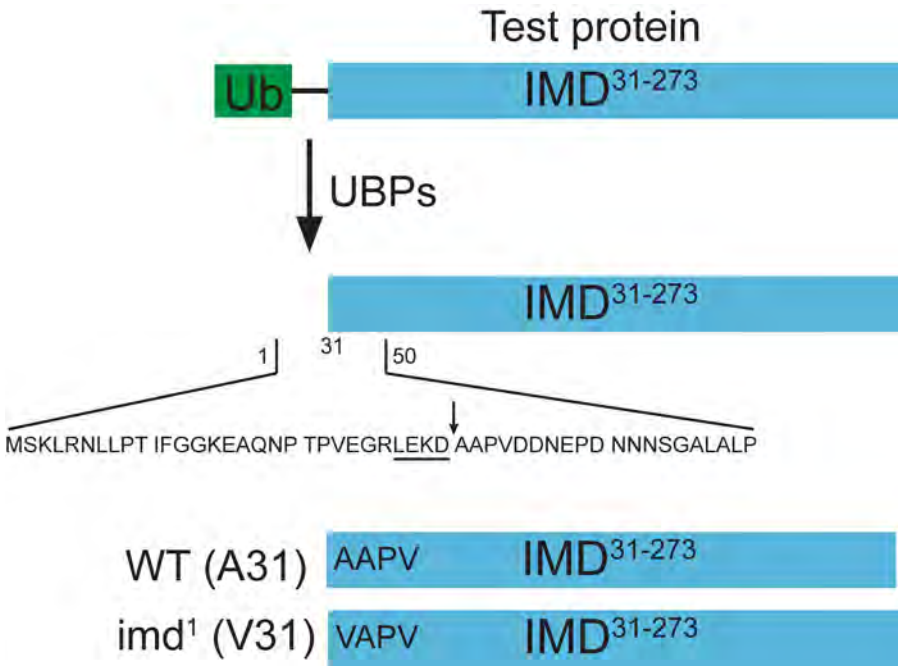

Figure S4. Ubiquitin fusion technique, related to Figure 6

imd protein from residues 31-273 was expressed with the N-terminal ubiquitin fusion technique. Once translated, ubiquitin-specific proteases (UBPs) remove the ubiquitin moiety. The remaining target protein is left with an exposed neo-N-terminal residue. Two such proteins were expressed in this fashion, A31-IMD$^{31\text{-}273}$ (WT) and V31-IMD$^{31\text{-}273}$ (*imd*^{1}).

Supplemental Experimental Procedures

Fly Stocks

The following fly strains were used in this work. DD1 (labeled as WT in most cases) (Gottar et al., 2002; Rutschmann et al., 2000), *diap2*^{7c} (Leulier et al., 2006), *diap2*^{7c}; UAS-DIAP2^{C466Y}/UAS-DIAP2^{C466Y} or TM6-Tb (Leulier et al., 2006), *imd*^{1} (Georgel et al., 2001), *imd*^{shadok} (Kaneko et al., 2006), *daughterless*-GAL4/UAS-IMD^{D30A}, *dredd*^{B118} (Leulier et al., 2000), and double PGRP-LC, PGRP-LE mutant flies (*pgrp-le*^{112};+;*pgrp-lc*$^{\Delta E}$)(Takehana et al., 2004)

RNAi

RNAi to IMD pathway components, and LacZ as a control, were produced using T7 RiboMAX Express Large Scale RNA Production System (Promega). S2* cells were split to 0.5x10^{6} cells per ml and allowed to incubate for ~24 hours at 27°C. 2µg/ml RNAi was then delivered by calcium phosphate transfection and cells were allowed to recover for ~24 hours at 27°C. 1µM 20-hydroxyecdysone was then added to the cells for ~24 hours. Finally cells were stimulated with either *E.coli* peptidoglycan (100ng/ml) or induced with copper, for 6 hours, before isolation of RNA or whole cell extraction, for Northern blotting or immunoprecipitation/immunoblot/kinase assays, respectively.

Protein and Immunoprecipitation Assays

Following stimulation with PGN (100ng/ml) or $CuSO_4$ (500µM), S2* cells were lysed in buffer (10% glycerol, 1% Triton X-100, 20mM Tris, 150mM NaCl, 25mM β-glycerolphosphate, 2mM EDTA, 1mM DTT, 1mM Sodium Orthovanadate, 1X Protease Inhibitor Cocktail). For total protein analysis, 50-100µg of total protein were prepared as previously described (Kaneko et al., 2006) and immunoblotted with anti-IMD, anti-pJNK

(Santa Cruz), or anti-JNK(FL) (Santa Cruz). For immunoprecipitations, 200-600µg of total protein extract was immunoprecipitated as previously described (Kaneko et al., 2006) with anti-DIAP2 (Leulier et al., 2006), anti-IMD or anti-FLAG-M5 (Sigma), before immunoblotting with anti-DIAP2, anti-Ubiquitin (Santa Cruz), or anti-IMD. For flies, after being infected with *E. coli 1106* by pricking, 10-15 frozen adult male flies were ground in 500µL of 2X lysis buffer. 600-900µg of total protein were then immunoprecipitated in 2X lysis buffer overnight at 4°C with anti-IMD. Immunoprecipitated IMD was then washed 2 times 500µL with lysis buffer and analyzed as described above.

K48- and K63-polyubiquitin Immunoprecipitation Assays

Following stimulation with PGN (100ng/ml), S2* cells were lysed in buffer (10% glycerol, 1% Triton X-100, 20mM Tris, 150mM NaCl, 25mM β-glycerolphosphate, 2mM EDTA, 1mM DTT, 1mM Sodium Orthovanadate, 1X Protease Inhibitor Cocktail) containing 6M Urea and 2mM NEM. 900µg of total protein extract was then diluted to 3M Urea (using lysis buffer) and immunoprecipitated overnight at room temperature with K48- or K63-polyubiquitin antibody. Samples were then spun at ~14000xg to remove any possible Urea precipitate. Protein A agarose was added and samples were allowed to roll at room temperature for 8-24 hours. Finally samples were washed 3x 500µl in lysis buffer containing 3M Urea and 2mM NEM before separating by SDS-PAGE and immunoblotting for IMD (as above). Membranes were then reprobed for total ubiquitin using anti-ubiquitin antibody (Santa Cruz).

Kinase Assays

Activity of kinases was assayed as previously described (Silverman et al., 2000; Wang et al., 2001). Briefly, cultured S2* cells or S2* cells stably transfected with

inducible metallothionein FLAG-TAK1 were pretreated with 1μM 20-hydroxyecdysone for 24-48 hours. For FLAG-TAK1 assays, cells were pretreated with low copper (100μM $CuSO_4$ for 1 hour). Cells were then stimulated with *E.coli* peptidoglycan (100ng/ml) for 10 minutes or not. Cells were lysed in 100μl/ml of lysis buffer (10% glycerol, 1% Triton X-100, 20mM Tris, 150mM NaCl, 25mM β-glycerolphosphate, 2mM EDTA, 1mM DTT, 1mM Sodium Orthovanadate, 1X Protease Inhibitor Cocktail), and kinases were immunoprecipitated with appropriate antibodies for 2-3 hours at 4°C; 75μg of total protein for FLAG-TAK with anti-FLAG M2 Agarose (Sigma), 75μg of total protein for endogenous TAK1 with anti-TAK1 antisera and 50μg of total protein for IKK with anti-IKKγ. Immunoprecipitated kinases were washed 2 times with 500μL of lysis buffer and 2 times 500μL with kinase reaction buffer (20mM HEPES, 20mM β-glycerolphosphate, 10mM $MgCl_2$, 50mM NaCl, 1mM DTT, 0.1mM Sodium Orthovanadate). Kinases were then added to 10μL of kinase reaction buffer containing 200μM cold ATP, 1μL γ-^{32}P-ATP, and substrate (1ug rMKK6-K82A for TAK KA, or 50ng rRelish for IKK KA). Kinase reactions were incubated at 30°C for 30 minutes, separated by SDS-PAGE, fixed, dried, and autoradiographed.

Cloning

T7/FLAG double tagged IMD was constructed in the pPAC-PL vector at *Kpn*I and *Not*I using standard cloning techniques. Subsequently, Act-T7-IMD D30A-FLAG and Act-T7-IMD A31V-FLAG were produced using QuikChange Site Directed Mutagenesis (Stratagene). AAPV-Imd (31-273) or the mutated form VAPV-Imd (31-273) were expressed using the Ubiquitin-fusion technique (Varshavsky, 2000).

IMD/DIAP2 Pull Down

S2 cells were transfected with pAc myc-Imd-HA or the pMT DHFR-HA-Ub-Imd (31-273) constructs with or without pMT-DIAP2-GTC. Expression was induced with 350μM $CuSO_4$ for 15 h and cells lysed in lysis buffer (50mM Tris, pH 7.5, 150mM NaCl, 1% TX-100, 10% glycerol, 1mM EDTA, Complete Protease Inhibitor Cocktail (Roche), 1 mM DTT). DIAP2-GTC was purified using GSH-Sepharose (GE Healthcare) and complexes eluted with 10mM Glutathione solution (in 50mM Tris-HCl pH 8.0). DIAP2 and interacting IMD proteins were analysed by Western Blot using antibodies against HA (myc-Imd-HA) (Roche), V5 (Imd (31-273) (AbD Serotec) and DIAP2 (Leulier et al., 2006).

Boiling SDS Immunoprecipitations

After stimulation with PGN S2* cells were harvested and spit. Half of the total cell pellet was lysed in standard buffer, as described above. The second half of the pellet was lysed in lysis buffer containing 1% SDS and immediately boiled for 10 minutes. In the case of the boiled SDS lysis, the total SDS in the sample was diluted to below 0.05% before immunoprecipitation. Lysates from both treatments were then immunoprecipiated for IMD using anti-IMD sera, as above.

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Conclusion

Il a été démontré qu'après infection par le PGN, la protéine Imd est clivée par la caspase-3 DREDD au niveau du motif de reconnaissance ^{27}LEKD/A^{31}. Ceci a pour effet d'entrainer la création d'un nouveau domaine, le domaine IBM (IAP-binding motif) en position A31 de l'extrémité N-terminale. Ce domaine est indispensable à la fixation de la E3-ligase DIAP2. En interaction avec UEV1a (E2-ubiquitin-conjugating enzymes), Bendless (Ubc13) et Effete (Ubc5) les protéines Imd et DIAP2 sont rapidement polyubiquitinées en K63. Chez la drosophile, TAB2 se lie à TAK1 et à la sous unité IKKγ et possède un site de liaison aux chaines de polyubiquitine K63. Cette chaine de polyubiquitine induit en aval, le recrutement des complexes TAK1/TAB2 et IKK menant *in fine* à la phosphorylation et l'activation du facteur Relish et la transcription des gènes cibles.

La protéase ubiquitine spécifique USP36 a été récemment identifiée comme un régulateur négatif de l'ubiquitination de la protéine Imd (Thevenon et al., 2009). USP36 est capable d'ôter la chaine de polyubiquitine située en K63 et d'induire la dégradation de la protéine Imd. De plus une surexpression de cette protéase dans la drosophile réduit considérablement l'activation de la voie Imd et augmente la susceptibilité des mouches à l'infection bactérienne. Ces résultats montrent que le phénomène d'ubiquitination en K63 joue un rôle essentiel dans l'activation de la voie Imd. Chez les mammifères, il a été montré que des chaines libres de polyubiquitine K63 (non conjuguées à un substrat) sont capables d'induire l'activation des kinases TAK1 et IKK (Chiu et al., 2009). Ainsi la présence ou l'absence de chaine K63 polyubiquitiné semble plus importante que le substrat lui même.

Les protéines de mammifères RIP1, FADD, cIAP1/2 et la caspase-8 sont impliquées dans des cascades intracellulaires menant à l'activation du facteur NF-kB. Chez la drosophile, les homologues de ces protéines font partie de la voie Imd. Ainsi, l'étude de la voie Imd chez la drosophile est une excellente opportunité pour comprendre les mécanismes qui participent à l'activation de ces mêmes molécules chez les mammifères.

Methuselah :

a regulatory receptor of the Imd pathway

Introduction

L'un des changements les plus dramatiques au cours du vieillissement chez les mammifères est l'altération des fonctions immunitaires et le développement d'une inflammation chronique. Comme pour de nombreux processus physiologiques, la drosophile est un modèle d'étude très précieux des mécanismes du vieillissement. De façon similaire au développement d'une inflammation chronique chez les mammifères, la drosophile présente lors de son vieillissement une augmentation de l'activité des gènes liés à la réponse immunitaire. L'identification de mutations ayant pour effet d'augmenter l'espérance de vie chez la drosophile est très importante si l'on veut tenter de disséquer les mécanismes moléculaires liés au vieillissement. Plusieurs mutations ont déjà été isolées pour leurs effets sur le vieillissement.

Le mutant *methuselah* présente une espérance de vie supérieure de 35% à celle de drosophiles sauvages. Le gène Methuselah code pour un récepteur à protéine G. Cette mutation a un effet non seulement sur l'espérance de vie des drosophiles mais a aussi pour effet d'augmenter leur tolérance à différents stress comme la chaleur, le stress oxydatif et la restriction alimentaire. Mon projet a consisté à analyser l'effet de cette mutation sur la réponse immunitaire de mouches jeunes. Le mutant *methuselah* présente une résistance à l'infection par des bactéries à Gram-négatif comme *Enterobacter cloacae*, mais pas à d'autres types d'infections. Cette résistance n'est pas due à une hypothétique activation constitutive de la voie Imd, qui aurait pu protéger les mouches par la production préventive de peptides antimicrobiens. Cependant, j'ai analysé les relations entre les mutations *methuselah et* $Imd^{Shadock}$ *(*Imd^{Sha}*)*. En temps normal le mutant Imd^{Sha} est incapable d'induire une réponse immunitaire caractérisée par l'expression de PAM. Il est également très sensible aux infections par des bactéries à Gram-négatif. De manière surprenante, les mouches mutantes à la fois pour Imd (*Imd*^{Sha}*)* et pour Methuselah sont aussi résistantes aux infections que les simples mutants *methuselah*. Le sauvetage de la mutation Imd^{Sha} est dû au rétablissement de l'expression des PAM après infection.

Nous avons tenté de d'expliquer comment l'absence du gène *methuselah* permet l'activation de la réponse immunitaire lorsque les mouches portent l'allèle mutante *Shadock* sont mutantes pour la protéines Imd (*Imd*^{Sha}*).*

I. Introduction

Generaly aging is process progressively affecting function of most organs. The immune system is no exception. In mammals the adaptive immune system declines seriously during aging, while innate immunity is overactivated and may lead to chronic inflammation (Salminen *et al.*, 2007). Such an aging related imbalance between adaptive and innate immunity leads to a pro-inflammatory phenotype with an activated innate immunity responses. A common hallmark of human senescence is the enhanced pro-inflammatory status (as measured by chronically elevated concentration of serum C-reactive protein (CRP), as well as tumour necrosis factor (TNF), interleukin (IL)-6 and other markers) (Johnson, 2006). Several studies showed that aging is characterized by an up-regulation of expression of genes related to inflammation, stress resistance and DNA repair (see for review Sahin and DePinho, 2010; Kenyon, 2010). T cells involved in the adaptive immunity lose their capacity to proliferate, consequently leading to cytokines production (Hasler and Zouali 2005). In addition to their inability to proliferate, T cells display a resistance to apoptosis (Spaulding *et al.*, 1999). Moreover, CD4+ T cells also fail to express the CD40L (CD40 Ligand) that can contribute to age-related defective humoral responses, such as reduced antibody production after vaccination (Salerno-Goncalves and Sztein, 2006). Alterations in proportion of different lymphocytes types, signalling present in them, and their function of lymphocytes are supposed to play a role in the inability to distinguish « the self » from « the non-self » during aging (Hasler and Zouali, 2005).

With a lifespan of approximately two months, *Drosophila* is an excellent model for aging studies. Several mutations increasing the lifespan of *Drosophila* have been isolated, for instance InR (Insulin-like Receptor) (Tatar *et al.*, 2001), Chico (insulin receptor substrate, Clancy *et al.*, 2001), Ecdyson Receptor (Simon *et al.*, 2003), Indy (I'm Not Dead Yet) (Rogina *et al.*, 2000), Rpd3 (Rogina *et al.*, 2002) and dFOXO (Hwangbo *et al.*, 2004).

Interestingly, majority of genes known to elongate the life span of *Caenorhabditis elegans, Drosophila melanogaster, Mus musculus* and *Homo sapiens sapiens* are related to glucose metabolism. The first pathway shown to influence aging in animals was the insulin/IGF-1 pathway (Kenyon *et al.*, 2005). The signal transducer of the insulin/insulin-like growth factor signalling (ILS) pathway called forkhead transcription factor FOXO (dFOXO in *D. melanogaster* and DAF-16 in *C. elegans*), may play a pivotal role in adapting metabolism to nutrient conditions (Tullet *et al*., 2008). Activation of FOXO (or inactivation of InR or ablation of Insulin Producing Cells) leads to the extend lifespan (Broughton *et al.*, 2005; Hwangbo *et al.*, 2004; Lin *et al*., 2001; Ogg *et al.*, 1997; Tatar *et al.*, 2001; Wessells *et al.*, 2004). When energy levels are low and ILS is reduced, FOXO enters the nucleus, resulting in enhanced FOXO target gene expression (Hafen *et al.*, 2004). In *C. elegans*, mutations decreasing the activity of DAF-2, that encodes a hormone receptor similar to the insulin and IGF-1 receptors, double the lifespan of the animal (Braeckman *et al.*, 2001). In mice and human, the IGF-1 receptor, upstream regulators and downstream effectors can all extend lifespan (Bartke, 2008; Kappeler *et al.*, 2008; Selman *et al.*, 2008). Human population sutdies shown that mutations known to impair IGF-1 receptor function are overrepresented in a cohort of Ashkenazi Jewish centenarians (Suh *et al.*, 2008). Similarly, DNA variants in the insulin receptor gene are linked to longevity in a Japanese cohort (Kojima *et al.*, 2004).

In *Drosophila*, studies of Chico (Bohni *et al.*, 1999), InR and the Ecdyson Receptor (EcR) mutants suggested that juvenile hormone (JH) and 20-hydroxyecdysone (20E) are secondary pro-aging signals downstream of InR (Tu *et al.*, 2006). The Rpd3 histone deacetylase, likely to be involved in caloric restriction, promote long lifespan (Rogina *et al.*, 2002). Interestingly, the histone deacetylase Sin3A/Rpd3 interacts with the Ecdysteroid Receptor complex (Tsai *et al.*, 1999), suggesting a response to JH and 20E signalling.

ROS (Reactive Oxygen Species) belong to of the principle causes of cell deterioration and tissue damage in the course of aging (Tissenbaum and Guarente, 2002). In humans, oxidative damage of DNA and proteins are implicated in a variety of degenerative diseases: Alzheimer's disease, latheral amyotrophic sclerosis, Parkinson's disease and rheumatoid arthritis (Stadtman *et al.,* 2001). Moreover, the accumulation of genomic mutations caused by ROS leads to cancerogenesis (Beckman and Ames 1998). In order to bypass the effects of ROS, organisms have developed several mechanisms some of which with an effect on longevity. Among signalling pathways activated in response to oxidative stress are the extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) signalling cascades, the phosphoinositide 3-kinase (PI(3)K)/Akt pathway, the nuclear factor (NF)-kB signalling pathway and p53 activation. The activation of these pathways is not only restricted to the oxidative stress response, as they are known to play a key roles in cellular homeostasis (for detail for references see the review : Finkel and Holbrook, 2000). However, the processes leading to activation of these mechanisms are not well understood. It is known that stress induces the activation of a MAP kinases cascade (Davis, 2000; Paul *et al.*, 1997; Stronach and Perrimon, 1999). Different stress activators such as UV and free radicals activate the JNK pathway. It has been shown that overactivation of the JNK pathway protects *Drosophila* against oxidative stress, bacterial infection and also increases its lifespan (Wang *et al.*, 2003; Libert *et al.*, 2008).

The effect of JNK signalling on lifespan is clearly established whereas its role in the NF-kB immune response activation remains controversial. On one hand, hyperactivation of JNK pathway protects against bacterial infections (Libert *et al.*, 2008). In particular, Delaney and his collaborators showed, by overexpression of an inhibitor of the JNK pathway *puckered*, that this pathway is required for the synthesis of the Antimicrobial Peptides (AMP) *diptericin* upon bacterial infection of *D. melanogaster* (Delaney *et al.*, 2006). On the other hand, it has been also shown that activation of the JNK pathway could counteract the NF-kB activation

throught the fixation of the transcription repressor AP1 on the NF-kB target genes promoters consequently inhibiting their transcription. Indeed, AP1 fixation on DNA leads to the recruitment of histone deacetylases to block the transcription of the gene encoding the antibacterial peptide attacin-A (Kim *et al.*, 2005; Kim *et al.*, 2007). Other results obtained in S2 cells (*Drosophila* hemocyte-like cell line) suggest that the JNK pathway is not required for AMP gene induction since neither Hep nor Bsk (JNKK or JNK) RNAi knockdown prevents expression of *diptericin*, *cecropin*, or *attacin* (Silverman *et al.*, 2003).

The innate immune system in old *Drosophila* adults is overactivated has it as been shown previously in mammals (Pletcher *et al.*, 2002; Seroude *et al.*, 2002). So far, we do not know whether this overactivation is the consequence or the cause of aging. In particular, aged flies produce more AMPs (Seroude *et al.*, 2002; Landis *et al.*, 2004), they do not lose their phagocytic activity and yet their survival rate is reduced upon bacterial infection (Ramsden *et al.*, 2008). Interestingly, a recent study indicates that *Drosophila* larvae mutants for the insulin pathway substrate Chico (Insulin substrate) and the Cytohesin Steppke (recently identified in mice), both overexpress AMPs in the abscense of any infection. The authors also observed that AMP upregulation is abolished in *foxo* mutant larvae after starvation and in adults after feeding with a drug negatively regulatoring of the insulin pathway. In contrast, overexpression of FOXO^{TM}, a dominant active form of the protein expressed in the nucleus, results in a strong induction of AMP expression in both, normal fed larvae and adults (Becker *et al.*, 2010). These results highlight the importance of the *Drosophila* insulin pathway in the induction of antimicrobial peptides expression. The results are consistent with previous studies reporting that genetic mutants of the ILS pathway, which have an extended lifespan, such as *chico* mutants in *Drosophila*, show enhanced pathogen resistance (Libert *et al.*, 2008).

The gene *methuselah* (*mth*) is one of the genes discovered as having an effect on the lifespan when mutated (Lin et al., 1998). It encodes a G protein-coupled receptor with a long

N-terminal extracellular chain (West *et al.*, 2001). An insertion of a P-element in the gene leads to a 35% increase in life span. The null allele of *mth* gene display pre-adult lethality in homozygotes suggeting the gene also plays an essential role in development. In mth mutant flies, P-element insertion in the third intron of the gene (Fig.1 : mth^1) reduces the level of its expression by interfering with RNA splicing without eliminating the gene function. This hypomorphic mutation enables flies to resist oxidative stress, high temperatures as well as nutritional restriction (Lin *et al.*, 1998).

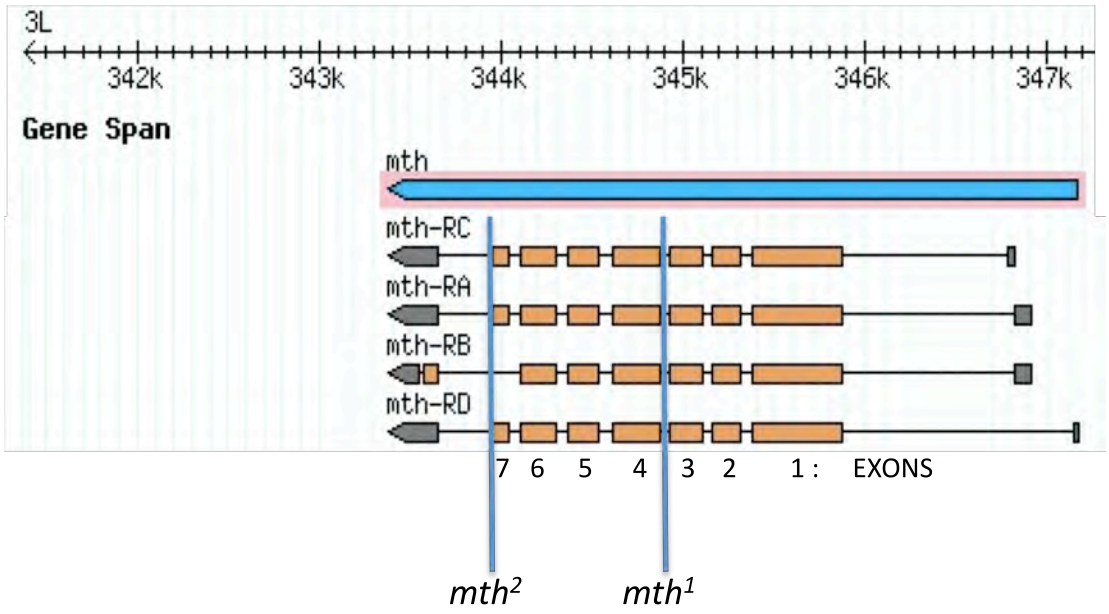


Figure 1. P-element insertions in *methuselah* gene. mth^1 is an insertion between exon 3 and 4 (Lin et al., 1998). mth^2 is an insertion of two nucleotides upstream of exon 7. (modified from http://flybase.org/)

Moreover, normal reduction of germinal stem cells that occurs with aging is not observed in the mutant (Wallenfang *et al.*, 2006). Stunted (Sun) A and B, two endogenous peptides have been identified as ligand for the Methuselah receptor. The phenotypes of mutations in these genes are similar to the phenotype of the *methuselah* mutation (Cvejic *et al.*, 2004). However, the signalling pathway downstream of this receptor has not been indentified. The Methuselah

receptor is also implicated in exocytosis of neurotransmitters at neuromuscular junctions of larvae (Song *et al.*, 2002), and is involved in visiomotor and phototaxic synchronization (Petrosyan *et al.*, 2007).

The *methuselah* mutant is resistant to oxidative stress similarity to mutant of the *puckered* gene, negatively regulating the JNK pathway. JNK pathway is a potential regulator of NF-kB activation of gene expression in particular genes of immune response. In addition, a *puckered* mutation shows an enhanced pathogen resistance (Libert *et al.*, 2008). All these data prompted us to use *methuselah* as an restart point of analysing the interaction between aging, immune response and JNK pathway activation. The results presented here describe the Methuselah receptor as a regulator of the Imd pathway, participating in keeping of the balance between Imd and JNK pathways.

II. Experimental procedures

Microbial Strains

Enterobacter cloacae (a kind gift of H. Monteil), *Escherichia coli* (1106), *Micrococcus luteus* (CIP A270). Strains were grown in LB (Luria Bertani medium) at 37°C.

Drosophila melanogaster Strains and Maintenance

The parental strain *W1118* flies were used as wild-type flies. Mutant flies: *methuselah*^{1} (*mth*^{1}) (Wang *et al.*, 1998), *methuselah*^{2} (*mth*^{2}) *(Bloomington stock number: BL24837), kenny*^{1} (Rutschmann *et al.*, 2000), *Imd*^{1} (Lemaitre, 1995), *Imd* $^{\text{Shadok}}$ (*Imd Shadok*) (Gottar *et al.*, 2002). Fly stocks were raised on standard cornmeal-agar medium at 25°C.

Septic injury and Survival Experiments

Septic injury experiments were performed on 4–6 days old adult flies (10 males and 10 females). Bacterial challenges were performed by challenging adult flies with a thin tungsten needle previously dipped into a concentrated culture of the appropriate bacterial strains. Challenged *Drosophila* were incubated at 29°C (*E. coli*, *E. cloacae, M. luteus).* Surviving flies were counted once a day. Results are expressed as a percentage of infected flies at different time points after infection.

Quantitative Real-Time PCR

RNA was isolated with TriReagent RT (Molecular Research Company) according to the manufacturer's instruction and was analyzed by Q-RT PCR. (BioRad CFX384^{TM} Real Time System, CFX Manager Software). iScript cDNA synthesis Kit (Biorad) has been used for cDNA synthesis. The qPCR MasterMix for SYBR Green I (Eurogenetec) was used for quantitative PCR. Primers used for real-time PCR were as follows:

RpL32	forward, 5’-GACGCTTCAAGGGACAGTATCTG-3’, reverse, 5’-AAACGCGGTTCTGCATGAG-3’.
diptericin	forward, 5’-GCTGCGCAATCGCTTCTACT-3’ reverse 5’-TGGCTTATCCGATGCCCGACGA-3’
attacin	forward 5’-GCAACATGCAGAACACAAGCA-3’ reverse 5’-GCCTCGGTAATGGCGAAA-3’
cecropin	forward 5’-ACGCGTTGGTCAGCACACT-3’ reverse 5’-ACATTGGCGGCTTGTTGAG-3’
drosocin	forward 5’-CACCCATGGCAAAAACGC-3’ reverse 5’-TGAAGTTCACCATCGTTTTCCTG-3’
imd	forward 5’ GGCCATGATGCAGTCACAAG-3’ reverse 5’ CCCAAGTGCGTGGAAACC-3’
dredd	forward 5’-CGCTATTGCAGAAAACATATCCAT-3’ reverse 5’ AGAGCTTCCACCGATCGATATC-3’
relish	forward 5’-CCAAAATGAAAACCTTACCGACAT-3’ reverse 5’-GCCAATTCCAAGGGAGTATGG-3’
tak1	forward 5’-TATCCTCGTACCAGCAGGCC-3’ reverse 5’-CACCTTCGGCGAACTCCAT-3’
puckered	forward 5’-GGCCTACAAGCTGGTGAAAG-3’ reverse 5’-AGTTCAGATTGGGCGAGATG-3’

Gene expression was normalized to the expression of the ‘housekeeping’ ribosomal protein L32 (RpL32)

III. Results

A. Methuselah mutation increases the resistance of *Drosophila* to infection by Gram-negative bacteria *Enterobacter cloacae*

We first tested *methuselah* mutant resistance to infection with different pathogens such as *Metarhizium anisopliae* and *Beauveria bassiana* (entomopathogenic fungi), *Enterococcus faecalis* (Gram-positive bacteria) and *Enterobacter cloacae* (Gram-negative bacteria).

Two different alleles of *Methuselah* have been used. Both of them carry a P-element insertion in the gene. *Lin et. al* published *mth* 1998 in *Drosophila* and mth^2 is a P-element insertion from the Bloomington Stock Center (Fig.1).

None of these mutated alleles showed any phenotype related to the susceptibility to infections by either fungi or Gram-positive bacteria. However both mth^1 and mth^2 mutant flies reexhibited resistance to *E. cloacae* (E.c) infection when compared to wild-type flies (Fig.2). The average half-life of mutant flies after challange with *E. cloacae* was 12 days, compared to 6 days observed for the wild-type flies (w^{1118}). However, there were no differences between the half-life of non-infected mutant flies and wild-type flies. This could be explained by the fact that mated *mth* mutant lose their long-lived phenotype (Baldal *et al.*, 2006).

We then tested whether *methuselah* mutation modulates Imd pathway activation as the Imd pathway is involved in the resistance against Gram-negative bacteria.

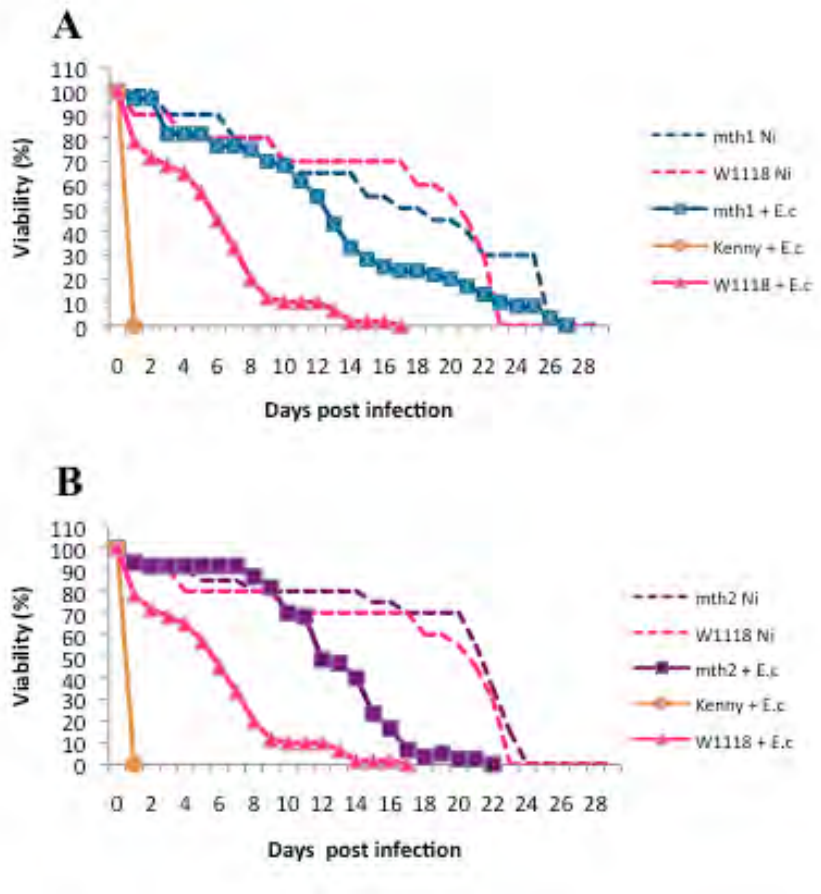


Figure 2. Survival after *E. cloacae* (E.c) infection (n=3) of A *mth*^{1} and B *mth*^{2} mutant flies.W1118 is the parental wild-type strain. *Kenny* (*Key*^{1}) mutant flies for the IMD pathway were used as a control for Gram-negative infection. Both *Methuselah* alleles provide a resistance to *E. cloacae* infection. The phenotype is specific for a Gram-negative bacterium.

B. *Methuselah* mutation suppresses *Imd* ^{Sha} phenotype

In order to see if the observed resistance to bacterial infection is linked to a modulation of the Imd pathway, we constructed double mutant flies using the *mth*^{1}, *mth*^{2}, *imd*^{1} and *imd* ^{Sha} loss of function mutant flies. *Shadok (Sha)* carries a mutation in the C-terminal death domain. The *Imd*^{1} allele is characterized by a substitution of the Alanine 31 by a Valine in the coding sequence. Mutant flies were challenged with *E. cloacae*. Flies carrying the *Imd* ^{Sha} mutation died in one day, whereas *imd* ^{Sha}*;mth* flies survived as long as wild-type flies. Even one copy of the allele *mth* is able to rescue the *imd* ^{Sha} mutant phenotype (Fig.3). Flies carrying the *Imd*^{1} mutation died after one day of infection as the *imd* ^{Sha} mutant flies. However, in the *imd*^{1}*;mth* mutant flies, the *mth* allele was unable to rescue the *imd*^{1} allele phenotype (Fig.3).

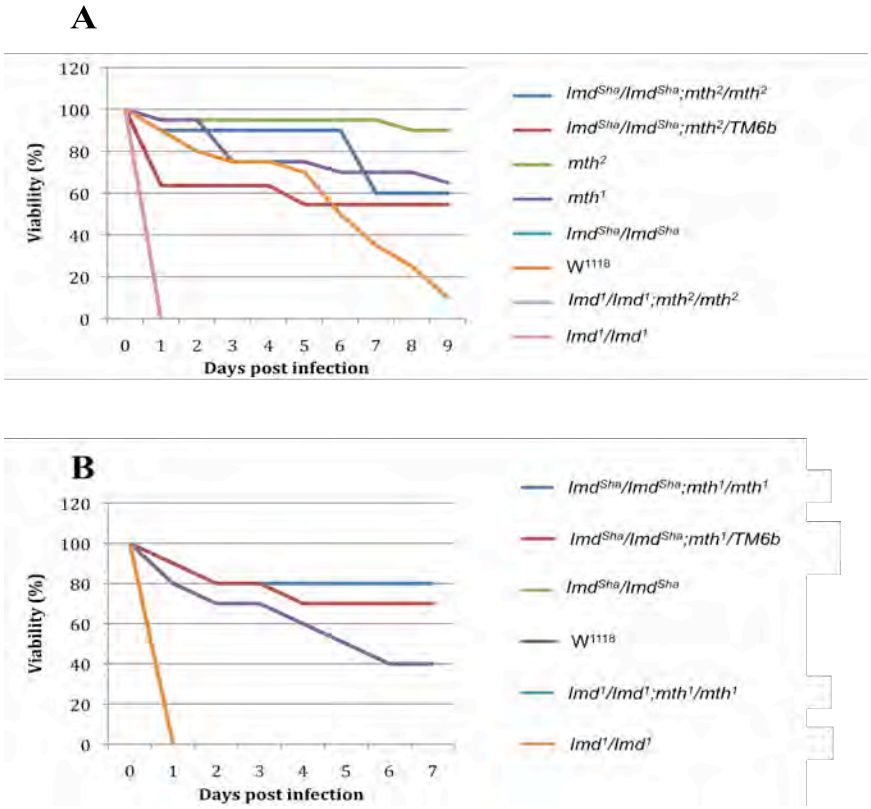


Figure 3. Survival after *E. cloacae* challenge (n=2) : *methuselah* mutations *mth*^{1} (A) and *mth*^{2} (B) suppresse the *imd*^{Sha} mutation phenotype. However the *imd*^{1} mutation remains sensitive to *E. cloacae* infection even in a *methuselah* mutant background. *imd*^{1}*/imd*^{1}, *imd*^{Sha}*/imd*^{Sha}, *imd*^{1}*/imd*^{1}*;mth*^{2}*/mth*^{2} and *imd*^{1}*/imd*^{1}*;mth*^{1}*/mth*^{1} mutant flies died the first day following the septic injury and therefore the curves are superposed.

We next tested the Imd pathway activation in these double mutants at the level of AMP genes expression. In wild-type flies, the Imd pathway activation leads to high expression of *attacin*, *cecropin* and *drosocin* genes after Gram-negative bacteria infections. Accordingly, the induction of these genes is impaired in *Imd* mutants. Most importantly, transcription of these AMPs was upregulated in *imd*^{Sha}*; mth* double mutants as in the wild-type flies after the challenge (Fig.4).

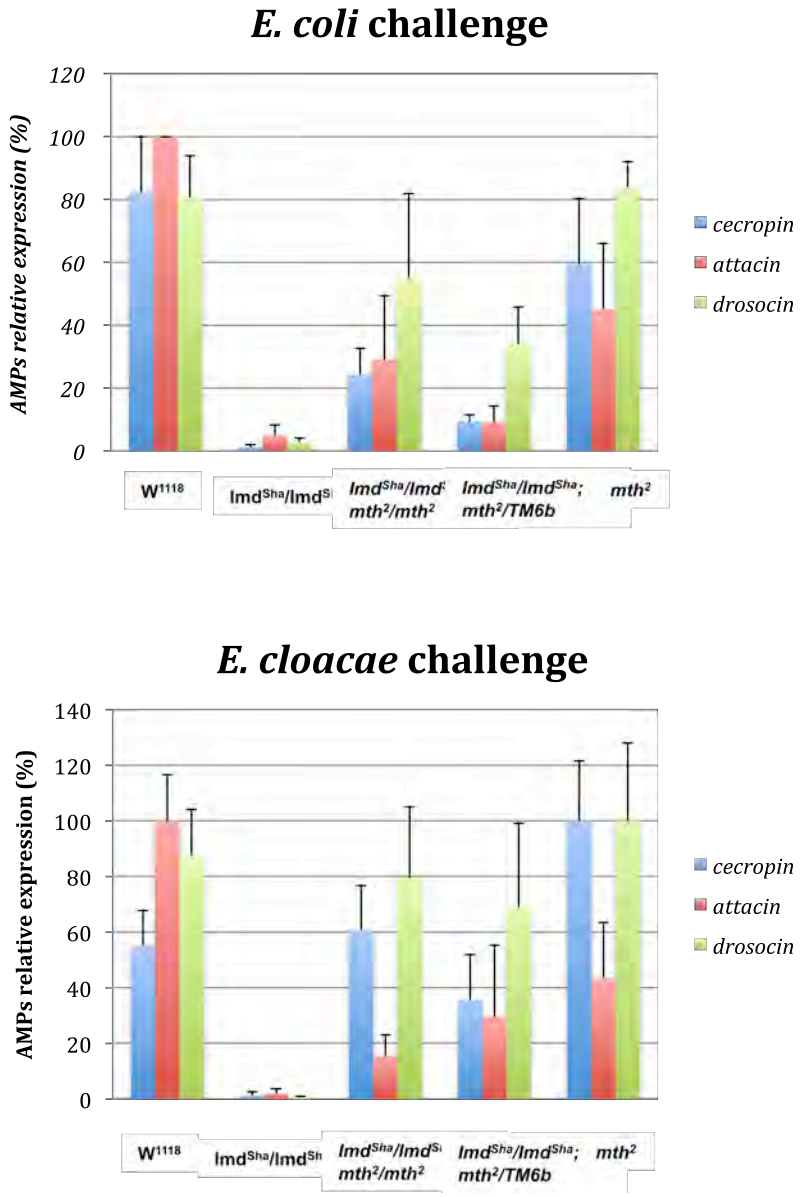


Figure 4. ***Mth*^{2} compensate the *imd*^{Sha} mutation and restores AMP genes transcription (n=3).** Quantification by Q-RT-PCR 6 h post challenge with *E. coli* and *E. cloacae*, respectively.

These results show that, surprisingly, the mutation of *methuselah* gene in *imd*^{Sha} mutant flies leads to activation of the NF-kB dependent transcription.

C. A non-conventional anti-microbial peptides transcriptional pattern in *methuselah* mutants infected by different Gram-negative bacteria

Since *mth* mutant flies are more resistant to infections than wild-type flies and that the *mth* mutant suppresses the *imd*^{Sha} mutant phenotype at the level of both susceptibility to infections and expression of AMP genes, we then analysed the expression of various immune system related genes in *mth* mutants.

Without bacterial challenge AMP genes are not transcribed in absence of infection in *mth* mutants. Therefore, the protective effect observed in *mth* mutants is not due to constitutive activation of immune response.

1. Challenge with *Enterobacter cloacae*

In course of an *E. cloacae* infection, the transcription of the AMPs coding gene (*attacin, cecropin, drosocin* and *diptericin*) is similar in *mth* mutant and wild-type flies (Fig.5). The resistance of *mth* to *E. cloacae* infection is therefore not due to any overexpression or sustained expression of AMPs.

2. Challenge with *Escherichia coli*

We then measured AMPs transcription upon challenge with *Escherichia coli* 1106 *(E. coli)* with septic injury. The mutants do not respond in the same manner to this type of infection. Indeed, for wild-type flies *w*^{1118}, the transcription of *drosocin, diptericin, cecropin* and *attacin*

genes is activated after infection with Gram-negative bacteria. In the wild-type flies, *cecropin* and *attacin* genes expression peaked at 3 h and 6 h, respectively. The peaks of *diptericin* and *drosocin* was at 12 h and 48 h, respectively. In *mth* mutant flies, the expression pattern of AMPs is quit different. *drosocin* and *diptericin* transcripts are almost not induced in *mth*^{2} flies infected with *E. coli* (Fig.6). However, expression of *cecropin* is hyperinduced and transcription of *attacin* is delayed (Fig.6).

After *E. cloacae* infection, the expression pattern of AMPs is identical for wild-type and mutant flies. On the contrary, upon septic injury with *E. coli*, AMPs transcription in *methuselah* flies is abnormal. *methuselah* receptor seems to be required for wild-type activation of the immune response upon *E. coli* infection.

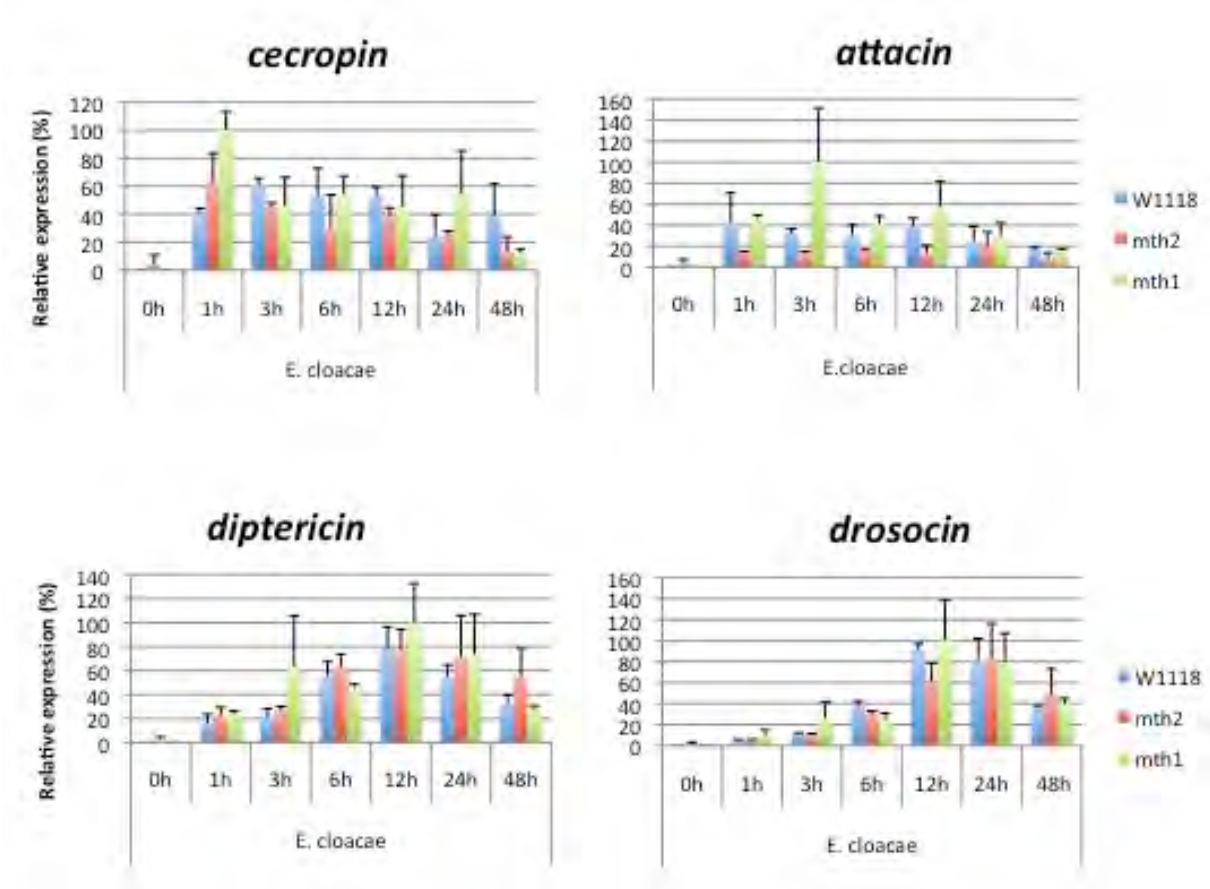


Figure 5. Expression of AMPs genes upon *E. cloacae* infection (n=3). Time course of *drosocin, cecropin* and *diptericin* and *attacin* transcription after septic injury with *E. cloacae* measured by Q-RT-PCR

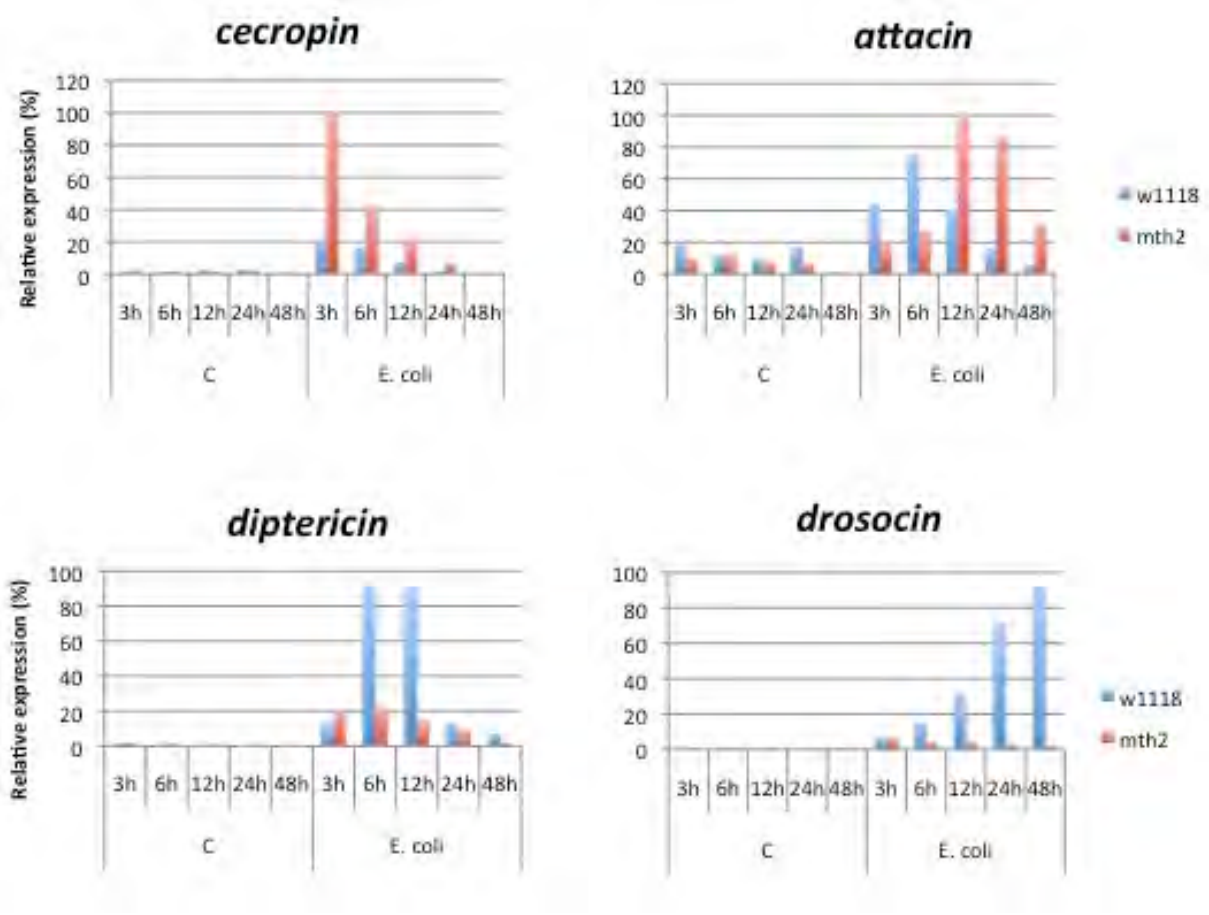


Figure 6. Expression of AMPs genes upon *E. coli* infection (n=2) Time course of *drosocin*, *cecropin* and *diptericin* and *attacin* genes transcription after septic injury with *E. cloacae* measured by Q-RT-PCR. The control (c) represent flies non challenged at different time points.

D. JNK pathway activation in *methuselah* flies

The JNK pathway is linked to the IMD pathway by the TAK1 kinase that activates hemipterous (JNKK) (Chen *et al.*, 2002). The product of hemipterous induces the phosphorylation of Basket (JNK) that, in turn, activates the transcription factor AP-1. This activation of the JNK pathway by this IMD pathway component induces the healing process as well as activation of stress-linked genes (Silverman *et al.*, 2003; Boutros *et al.*, 2002). Among them, the gene *puckered* encodes a MAPK phosphatase responsible for the dephosphorylation and inactivation of Basket (Martin-Blanco *et al.*, 1998). The *puckered* mutants are resistant to oxidative stress (Wang *et al.*, 2003). These mutants are also more resistant to bacterial infections even if the expression of AMPs is unaffected (Libert *et al.*, 2008). Mutants of both genes, *puckered* and *methuselah* have therefore the same phenotype of resistance to the oxidative stress (Lin *et al.*, 1998 ; Wang *et al.*, 2003) and to bacterial infections, as we just showed for *methuselah* mutant. In order to test if *methuselah* could be an inhibitor of the JNK pathway, we measured expression of *puckered*, an effector of JNK pathway in a *methuselah* mutant background.

Similarly, for AMPs gene expression, the obtained results were for septic injury with *E. coli* and *E. cloacae* measured by the level of mRNA for expression. In wild-type flies challenged in this infection model with *E. coli*, the peak of JNK pathway activation is reached 12 h after challenge and 48 h in the case of *methuselah* mutant flies (Fig.7A). Therefore, in *methuselah* mutants this activation is delayed. The same delay was observed for another target of JNK pathway, the gene *punch* (data not shown).

Yet, when *methuselah* mutants were infected with *E. cloacae*, no difference was observed between mutants and wild-type flies (Fig.7B).

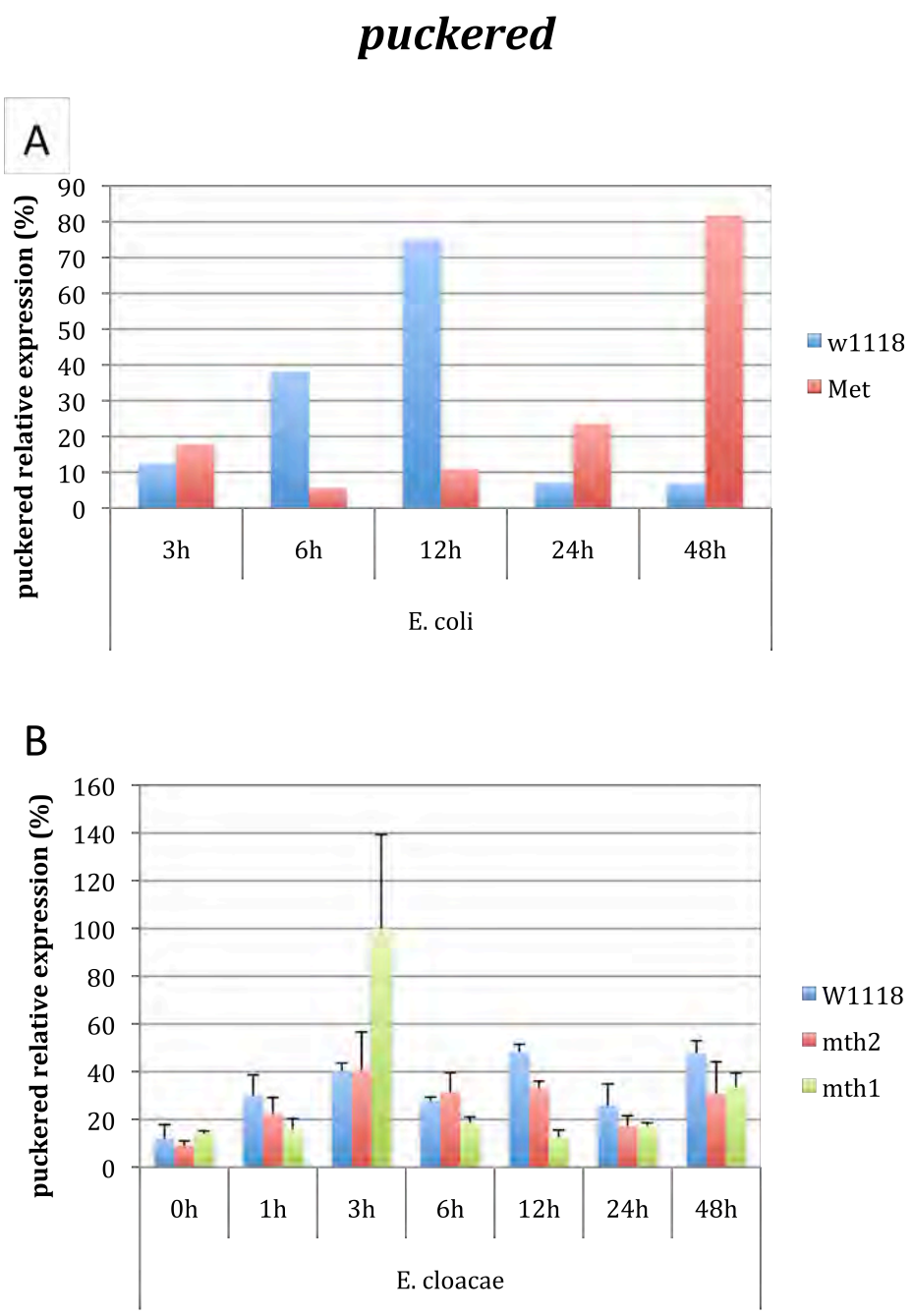


Figure 7. puckered gene transcription in *mth1* and *mth*^{2} mutants. Comparison of JNK pathway activation by the measurement of puckered gene expression in methuselah mutant and wild-type septic injury with *E. coli* (n=2) (A) and *E. cloacae* infection (n=3) (B).

E. Imd and TAK1 are not transcribed in *methuselah* mutant upon *E. coli* infection

In normal conditions, of the IMD cascade pathway leads to the degradation of TAK1 by the proteasome. As a consequence, the Imd pathway will be inactivated (Park *et al.*, 2004). Imd is also degraded upon activation (Thevenon *et al.*, 2009).

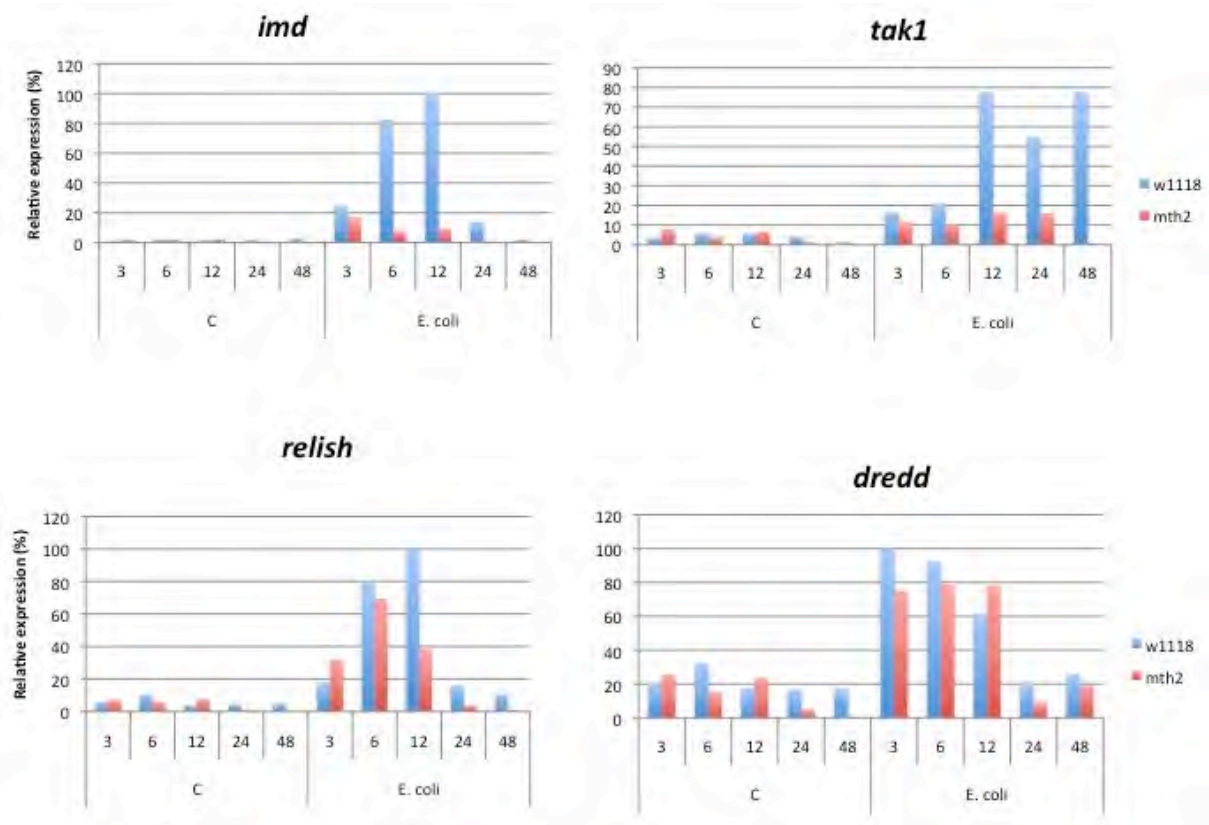


Figure 8. Expression Imd pathway components during infection. Time course (hours) of *imd, tak1 dredd* and *relish* relative genes transcription after septic injury with *E. coli* measured by Q-RT-PCR (n=2). The control (c) represent flies non infected at different time point**.**

The transcription of Imd pathway components such as Imd, TAK1, DREDD and Relish was induced in wild-type flies after infection by *E. coli* (Fig.8), probably in order to replace molecules that have already been used. Whilst the genes Relish and DREDD were transcribed at similar levels upon *E. coli* infection of both the *mth* mutant and wild-type flies, Imd and

TAK1 were not induced in *mth* mutant (Fig.8). These results suggest that Imd and TAK1 proteins might not be activated in *mth* mutant flies upon *E. coli* infection. In order to validate our hypothesis it would be necessary to look at TAK1 phosphorylation and Imd clivage, and also to their degradation.

F. Induction of the *turandot A* gene

Results presented previously in this thesis have shown that mth gene mutants are resistant to oxidative stress (Lin et al., 1998). It is known that the MAP3 kinase Mekk1 is required for resistance to the oxidative stress. In the work by Brun and collaborators it was proven that turandot genes (A and M) show complex regulation pattern responding to signals from JAK-STAT, Imd and MEKK1 pathways. The genes totA and totM itself encode stress response proteins Turandot A and M. As shown on Fig.9, upon septic injury with *E. cloacae* and *E. coli* totA is induced up to the higher level in mth mutant flies than in wild-type flies. This stronger induction is specific for challenge with Gram-negative bacteria as totA expression remains low after septic injury with M. luteus (fig 9). We also measured *totA* expression in imd^{Sha}*;mth2* double mutants (Fig.10). The imd^{Sha} mutant flies are not able to induce *totA* expression upon *E. coli* or *E. cloacae* challenge. However, in a *mth* mutant background, imd^{Sha} mutant flies induce *totA* gene expression.

Those results suggest that the *totA* overexpression observed in *mth* mutant flies upon septic injury is likely specific for Gram-negative bacteria. Based on this results, we hypothesize that this overexpression might be linked to increased Mekk1 activation in *mth* mutant flies.

Brun *et al.*, reported that upon infection with Gram-negative bacteria *totA* expression depends on Relish transcription factor (Brun *et al.*, 2006). Our results show that imd^{Sha} mutant flies

are unable to induce *totA* expression unless the Methuselah receptor is mutated, suggesting an interplay between the Imd pathway and the signalling from Methuselah receptor.

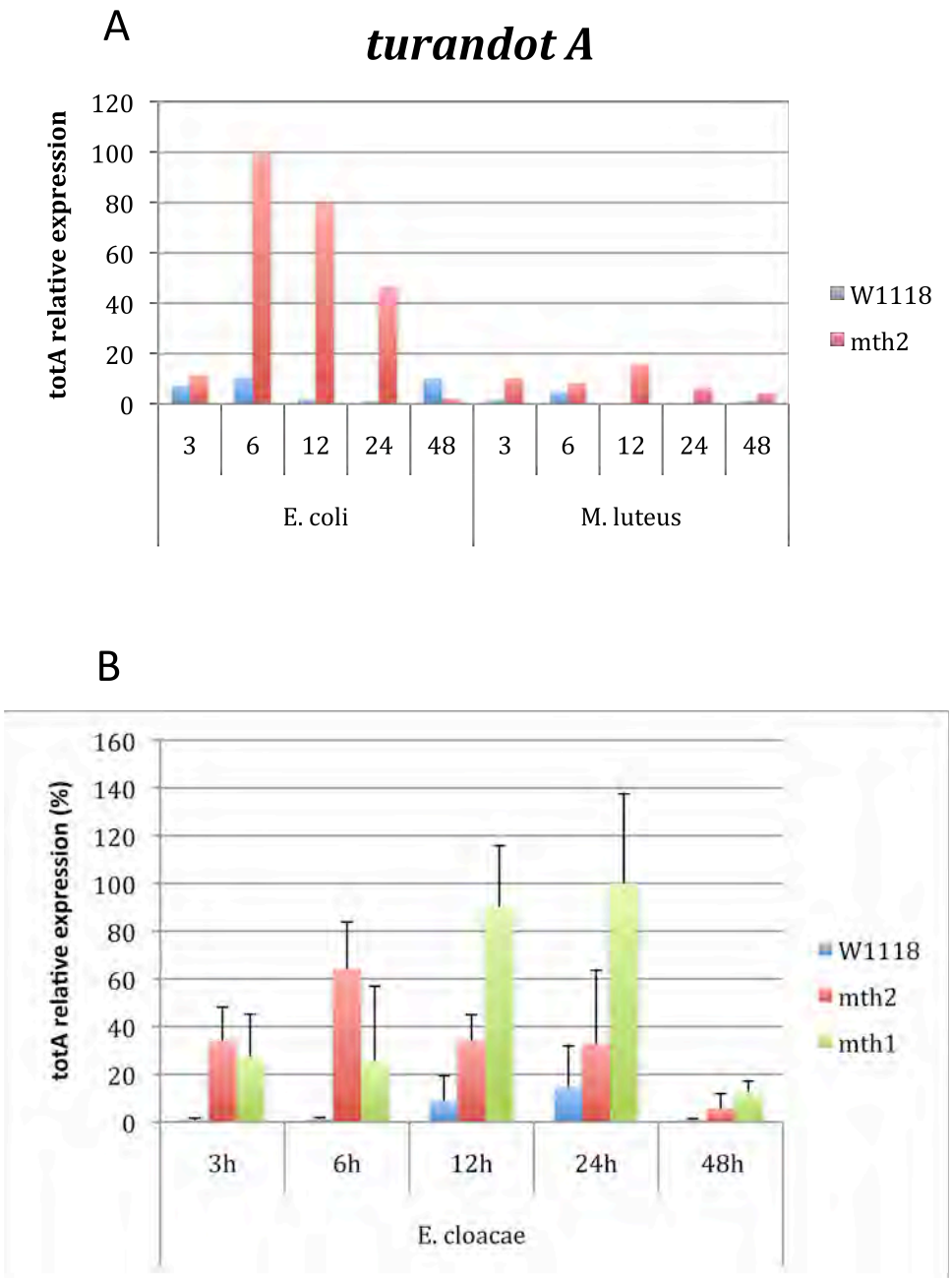


Figure 9. Comparison of relative *turandot A* gene transcription. Time course (hours) in *methuselah* mutant upon septic injury with *E. coli* and *M. luteus* (n=2) (A) and *E. cloacae* (n=3) (B).

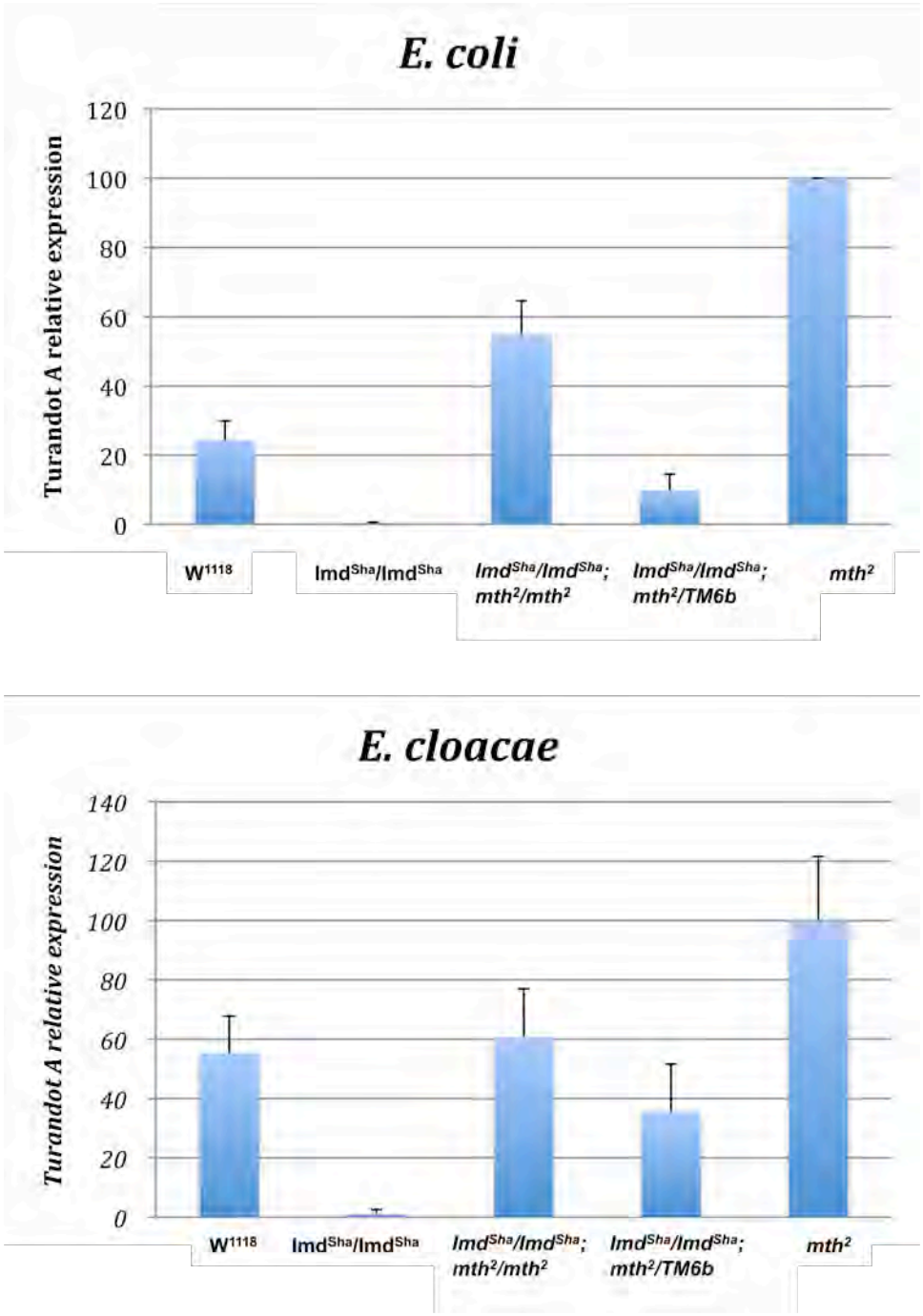


Figure 10. The *turandot A* gene expression level. The samples are taken 6 h after septic injury with *E. coli* and *E. cloacae* infection (n=3).

IV. Discussion

A. *Drosophila* mutants for Methuselah receptor are more resistant upon *E. cloacae* challenge

To identify how the immune system operates in *mth* mutants, we infected mutant flies with different pathogens. Our results show that the *mth* mutant flies are able to resist the challenge by the Gram-negative bacteria *E. cloacae*. However, the transcription of AMP genes (coding for Cecropin, Attacin, Drosocin and Diptericin) is similar in *mth* mutant and wild-type flies. Furthermore, in the absence of infection, AMP genes are not transcribed in *mth* mutants. The data indicate that the resistance of the *mth* mutants to *E. cloacae* infection is not due to constitutive expression of AMPs.

It is known that the hyperactivation of the JNK pathway results in resistance to oxidative stress (Wang *et al.*, 2003) and to bacterial infection (Libert *et al.*, 2008). Therefore we measured the level of transcription of *puckered* (a phosphatase in the JNK pathway) following *E. cloacae* challenge of *mth* mutant flies. Our results did not show any difference between *mth* mutant and wild-type flies in this infection model.

Mth mutants are resistant to oxidative stress (Lin *et al.*, 1998). Amongst others, the MAP kinase Mekk1 is required for resistance to oxidative stress and a stress-related *totA* gene expression. The *totA* gene transcription is induced after exposition of flies to high temperature, mechanical pressure, dehydration, UV irradiation and oxidative agents (Ekengren *et al.*, 2001). The *totA* gene is also transcribed after the clean injury and more strongly after the septic one with *E. coli* (Ekengren and Hultmark 2001; Brun *et al.*, 2006). For these reasons we decided to analyze *totA* activation as a readout of the Mekk1 activation. Our results showed that in *mth* mutant flies totA is highly expressed upon the challenge with Gram-negative bacteria when compared to wild-type flies. Since expression of AMPs in *mth* mutants is comparable to that in wild-type flies, the observed resistances is not due to the

action of AMPs. It can be proposed that the high activation of the signalling pathway involved in *totA* induction seen in mth^1 and mth^2 mutant flies might indeed explain their resistance to *E. cloacae*.

B. Imd-independent activation of the immune response

We observed that *totA* and AMPs gene expression was induced in Imd^{Sha}; mth^2 double mutant, while they were not upregulated in Imd^{Sha} single mutant. The fact that mutation in the *methuselah* gene is able to restore the wild-type phenotype in the imd^{Sha} mutant suggests that *methuselah* is involved in the Imd pathway regulation.

The MAPKKK Mekk1 is required for *totA* gene expression. The *totA* gene transcription is also induced after the clean injury and more strongly after the septic one with *E. coli* (Ekengren and Hultmark 2001; Brun *et al.*, 2006). In opposition to *mth* mutants, *Mekk1* mutants show hyper-sensitivity to higher temperatures and oxidative stress (Inoue *et al.*, 2001; Zhuang *et al.*, 2002b; Brun *et al.*, 2006). However, the absence of *totA* expression in *Mekk1* mutant does not lead to the reduced survival of flies upon bacterial infection (Brun *et al.*, 2006). We hypothesize that the Methuselah receptor inhibits somehow Mekk1. The overexpression of Mekk1 due to the absence of Methuselah in the mutant, might be able to induce the activation of IKK complex (Kenny+Ird5), resulting in Imd-independent Relish phosphorylation and AMP genes transcription (Fig.11).

The *methuselah* mutation restores a wild-type phenotype of AMPs expression in Imd^{Sha} mutant flies but not in Imd^1 mutants. This result could be interpreted as followed. Upon direct binding with bacterial elicitors (monomeric or polymeric DAP-type PGN), PGRP-LC recruits the adaptor Imd (Kaneko *et al.*, 2006 ; Choe *et al.*, 2005). Imd contains a death domain and can interact with another death-domain protein, FADD (Naitza *et al.*, 2002), which itself

binds the apical caspase Dredd (Hu *et al*., 2000). This caspase has been proposed to associate with Relish, which it might cleave directly once Relish is phosphorylated (Stöven *et al*., 2000 ; Stöven *et al*., 2003). In the absence of the Death Domain in the Imd ^{Sha} protein, AMP genes transcription could still take place in *mth* mutant flies. Then after bacterial challenge, *Imd* ^{Sha}, mutated in its Death Domain would be unable to recruit the caspase DREDD (Zhou *et al*., 2005 ; Paquette *et al*., 2010). Thus, DREDD would be free to cleave Relish (Stöven *et al*., 2003 ; Erturk-Hasdemir *et al*., 2009) previously activated by the Mekk1 pathway and the IKK complex (independently of TAK1). *Imd*^{1} and *Imd* ^{Sha} mutant expected to behave in the same way but they did not. In the light of what was proposed above, the most probable hypothesis is that *Imd*^{1} can not be cleaved nor ubiquitinated (Paquette *et al*., 2010), would sequester DREDD into the Imd/FADD complex which therefore cannot cleave Relish. This would consequently lead to a lack of AMP expression.

Here we suggest that bacterial challenge generates stress activating Mekk1 signalling pathway. This leads to *totA* expression and the activation of the IKK complex in order to induce the NF-kB factor translocation and the *attacin* and *cecropin* gene transcription. This is emphazise by the observation that, *in vivo*, in a *TAK1 m*utant flies, Relish cleavage still takes place (Kleino *et al*., 2005 ; Delanay *et al*., 2006), meaning that Relish can be activated independently of TAK1 and Imd.

C. The *methuselah* mutation affects the transcriptional response of AMP genes upon *E. coli* challenge.

The transcription of AMPs genes upon *E. cloacae* challenge was not affected in *methuselah* mutant flies. On the contrary, upon *E. coli* challenge, the transcription of AMP genes is clearly affected in *methuselah* mutant flies. Transcripts of *drosocin* and *diptericin* were almost not induced in *mth*^{2} mutant flies. The gene for cecropin is overexpressed while attacin

transcription was delayed in the *mth*^{2} mutant. In the light of our experiments Methuselah receptor might be required for full activation of the immune response upon septic injury with *E. coli.*

The JNK pathway activation is delayed in *methuselah* mutant flies upon *E. coli* challenge. The *puckered gene* induction is concomitant with the absence of the Diptericin and Drosocin genes transcription. This result is consistent with a previous study, which showed that the knockdown of *Drosophila* JNK or activator protein 1 (AP1) enhanced the expression of IKK-NF-kB dependent genes. (Kim *et al.*, 2005). In addition, we found that after challenge, *imd* and *tak1* genes are not transcribed, whereas *dreed* and *relish* genes are transcribed. These demonstrate further observations that upon *E. coli* challenge, of the *mth* mutant flies, *attacin* and *cecropin* expression is independent of TAK1 and Imd activation. We assume that upon septic injury with *E. coli*, the Methuselah receptor would be required to inhibit the JNK activation and thus shifting the immune activation towards Imd pathway. We believe that this might be the reason why after septic injury with *E. coli* the Methuselah receptor is required to induce a complete antimicrobial response.

D. Competition between the JNK pathway and the Imd pathway upon *E. coli* challenge

Assuming that the Methuselah receptor functions as an inhibitor of Mekk1, a mutation leading to loss of its function would lead to the activation of Mekk1. Mekk1 activation could lead to the activation of the IKK complex and the further activation of NF-kB as it has been previuosly describted in mammals (Lee *et al.*, 1997). Mekk1 is also able to activate the JNK pathway (Ryabinina *et al.*, 2006). Kim *et al.*, showed that the activation of the JNK pathway induces the nuclear translocation of AP-1 and his fixation to promoters activated by the transcription factor NF-kB (Kim *et al.*, 2005; Kim *et al.*, 2007). Considering our results, it

seems that the activation of the JNK pathway enters conteract with the Imd pathway upon *E. coli* challenge. Indeed, we observed that the activation of the JNK pathway coincides with the absence of the transcriptional induction of the *diptericin* and *drosocin* genes.

E. Model for regulation of the activation of immune responses to Gram-negative bacteria by the Methuselah receptor

Upon septic injury with *E. cloacae*, Mekk1 (MAPKKK) would promote the synthesis of initial AMPs (Cecropin at 3 h and Attacin at 6 h after septic injury) and the *turandot A* gene through the activation of the IKK complex, by bypassing the upstream Imd pathway. Mekk1 would also activate the JNK pathway through the phosphorylation of Hempiterous (MAPKK) thus protecting flies against oxidative stress and helping to repair damaged tissues. In addition, the JNK Mekk1-dependent pathway also induces AP-1 nuclear translocation. Once in the nucleus, AP-1 would enter into competition with Relish at the level of the AMPs promoters, leading to downregulation of the early transcription of genes, *cecropin* and *attacin*. Later, PGRP-LC would be activated by an increasing concentration of PGN released by proliferating bacteria. This would lead to the activation of IMD pathway and Relish nuclear translocation. Relish would also enter in a competition with AP-1 in order to induce the transcription of *diptericin* at 12 h and *drosocin* at 48 h.

Upon *E. coli* challenge, Mekk1 would participate in the synthesis of the first AMPs (*cecropin* at 3h and *attacin* at 6h) and the *totA* gene by direct activation of the IKK complex. Mekk1 would again activate the JNK pathway through Hempiterous (MAPKK) phosphorylation thus protecting flies against oxidative stress and helping to repair damaged tissues. The JNK-Mekk1-dependent pathway also induces AP-1 nuclear translocation. Once in the nucleus, AP1 would enter into competition with Relish at the level of the AMP promoters, leading to downregulation of the early genes *cecropin* and *attacin* transcription. However, in the case of

E. coli challenge the Methuselah receptor would be activated directly or indirectly. This would lead to Methuselah-dependent Mekk1 inhibition and the resulting JNK inhibition, would allow flies to transcribe *diptericin* and *drosocin* genes, later, between 12 h and 48 h following the challenge.

Our data suggest that transcription of genes encoding early AMPs (*cecropin* and *attacin)* could be induced through Mekk1 by activation of the IKK complex and that in the case of *E. coli* challenge, Methuselah is activated in order to reduce the JNK activation.

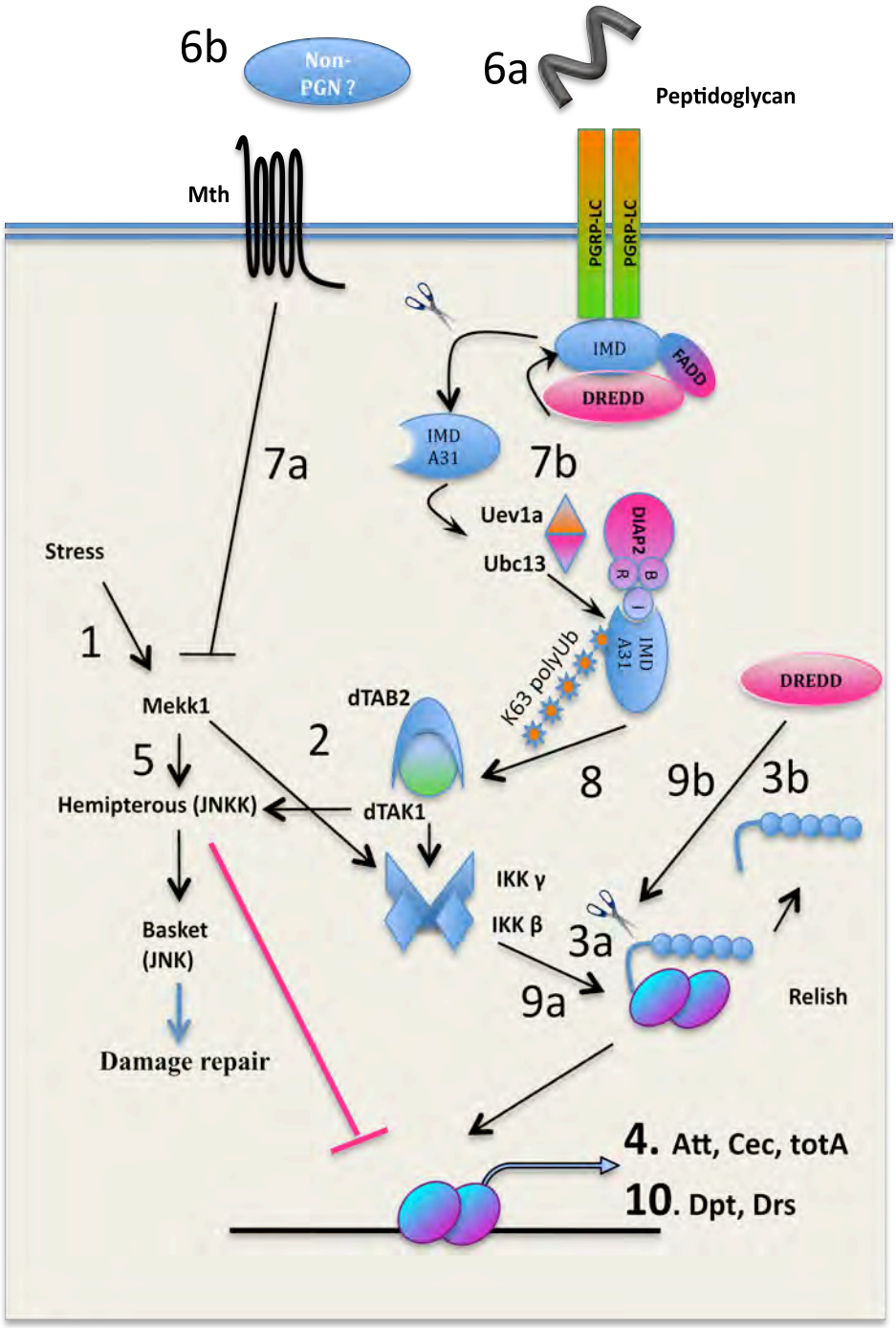


Figure 11. Antimicrobial response upon septic injury with *E. coli*: (1) Stress induced by bacterial challenge activates, Mekk1. (2) Mekk1would phosphorylate IKK complex. (3a) IKK complex activation induces Relish phosphorylation and (3b) its subsequent cleavage by DREDD. (4) and then the transcription of *Att*, *Cec*, and TotA. (5) Mekk1 activates JNK pathway and AP-1 nuclear translocation. Bacteria that are still dividing activate (6a) the PGRP-LC receptor via the PGN, (6b) Methuselah receptor via Stunted or a non PGN molecule to inactivate Mekk1 and JNK pathway activation. (7b) PGRP-LC dimerization induces Imd protein recruitment and the formation of the Imd-FADD-DREDD complex. Imd protein is cleaved and liberates an extremity A31 where DIAP2 proteins is fixed and recruit Uev1a and Ubc13 to ubiquitinate Imd on a K63 position. (8) K63-polyubiquitin chains will recruit both the TAB2/TAK1 complex and the IKK complex, induce Relish phosphorylation (9a) and cleavage (9b) to transcribe (10) *diptericin* and *drosocin*.

V. Perspectives

The results obtained during this study are preliminary data that have to be confirmed and validated. First, an epistatic analysis must be conducted to demonstrate that the *methuselah* mutation is able to compensate the absence of TAK1, without compensating the absence of DREDD, FADD and IKK (Kenny+Ird5) mutations. Second, in order to establish Mekk1 as a kinase able to phosphorylate IKK, we should study the phosphorylation of IKK and Relish in a double mutant *imd;mth* and *mth;Mekk1,* as well as in the double mutant *TAK1*;*mth*. Finally, I would analyse early AMP genes (*cecropin* and *attacin*) and puckered transcription after challenge of the *Mekk1* mutant flies.

Recently, Wang *et al.*, indentified the JNK signalling pathway as a significant genetic determinant of longevity in *Drosophila*. Activation of JNK in response to oxidative challenge and to other environmental stressors has been well described in a number of model systems and was proposed to trigger the expression of genes that could mediate protective functions in the organism at least in certain cell types (Wang *et al.*, 2003). A missregulation of the JNK pathway is able to reduce lifespan. On the contrary, an overexpression of the JNK pathway increases *Drosophila* lifespan and resistance towards bacterial challenge (Libert *et al.*, 2008).

Here, we observed that the *methuselah* mutation, which increases the lifespan of *Drosophila* up to 35%, confers resistance to Gram-negative bacterial challenge. The Methuselah receptor might be involved in negative régulation of the JNK pathway upon *E. coli* challenge and therefore in the positive régulation of Imd pathway. The *mth* mutation represents an excellent opportunity to study the interaction between the JNK and the Imd pathways.

Conclusion

Nous avons montré que la mutation du récepteur Methuselah augmente la résistance des drosophiles à l'infection par la bactérie à Gram-négatif, *Enterobacter cloacae (E. cloacae)*. Le patron d'expression des peptides antimicrobiens induit suite à l'infection par cette bactérie s'est révélé similaire entre des mouches mutantes (*mth*) et des mouches sauvages. Cependant une différence très importante a été observée concernant l'activation de la MAP3 kinase, Mekk1, impliquée dans la résistance au stress oxydatif. Dans le mutant *mth*, la voie Mekk1 semble être suractivée après infection par des bactéries à Gram-négatif. Nous en déduisons que le phénotype de résistance observé dans le mutant *mth* est probablement lié à la suractivation de la voie Mekk1. Cependant, il faudrait vérifier si la surexpression de Mekk1 augmente la résistance des mouches l'infection bactérienne.

La voie Imd participe à la réponse anti-microbienne spécifiquement dirigée vers les bactéries à Gram-négatif. Il est admis que cette voie est activée par la reconnaissance du peptidoglycane au niveau du récepteur transmembranaire PGRP-LC qui active successivement la protéine Imd puis la MAP3 kinase TAK1, le complexe IKK pour mener à l'activation du facteur NF-κB. Cependant, l'ensemble de nos résultats suggèrent qu'en cas d'infection par des bactéries à Gram négatif, l'activation du facteur NF-κB passe tout d'abord par l'activation de la MAP3 kinase Mekk1 puis par l'activation de la MAP3 kinase TAK-1 entrainant respectivement la transcription des gènes précoce codant pour de la Cécropine et l'Attacine puis des gènes tardifs codant pour la Diptéricine et la Drosocine. Partant de cette observation, nous avons l'intention de vérifier l'hypothèse selon laquelle chez la drosophile, Mekk1 est capable de mener à l'activation de la voie NF-κB.

Nous avons également mis en évidence une nuance importante dans la réponse contre les bactéries à Gram négatif. Nos recherches montrent que les bactéries *E. coli* et *E. cloacae* n'activent pas la réponse humorale de la même façon. Ainsi comme nous l'avons déjà mentionné, l'infection microbienne activerait non seulement la transcription des gènes codant pour des peptides antimicrobiens précoces via l'activation de IKK (Mekk1-dépendante), mais il serait également capable d'activer la voie JNK. Dans la littérature, il est décrit que l'activation de la voie JNK entrainer la translocation nucléaire du facteur AP-1 qui rentre en compétition avec le facteur Relish au niveau des sites promoteur activés par le facteur de NF-κB. Il semblerait que l'infection par *E. cloacae* entraine une activation de la voie PGRP-LC suffisamment important pour contourner la régulation négative de la voie JNK sans avoir

besoin de réprimer son activation. Par contre, concernant la bactérie *E. coli*, il semblerait qu'elle ne soit pas capable d'activer la voie Imd sans avoir au préalable inhibée la voie JNK par l'activation du récepteur Methuselah. Son activation mènerait à l'inhibition de Mekk1 et de la voie JNK pour permettre l'activation de la transcription des gènes codant pour des peptides antimicrobiens tardifs.

Ici nous suggérons que Mekk1 puisse activer le facteur NF-κB en cas d'infection, mais également que le récepteur Methuselah participe à la reconnaissance directe ou indirecte de la bactérie *E. coli.*

Involvement of Argonaute-2 in gut integrity upon *Beauveria bassiana* infection

Introduction

L'activation des facteurs NF-kB est le principal signe de la réponse immunitaire innée dans tout le règne animal. À ce jour, la plupart des voies de signalisation activées lors d'infections ont été identifiées. Cependant, les mécanismes de contrôle sont très peu connus. Le troisième projet que j'ai développé au cours de ma thèse consiste en l'analyse du rôle potentiel des petits ARN dans la régulation de la réponse immunitaire de la drosophile.

Différents types de petits ARN non codants impliqués dans la régulation génique ont été identifiés au cours des dernières années. Dans les lignées somatiques ils se répartissent en deux catégories. D'une part les microARN (miARN), synthétisés par le génome, se lient par complémentarité à un ARNm cible afin de bloquer sa traduction voire induire sa dégradation. Ils constituent un mécanisme très important de régulation de l'expression génique. D'autre part les small interfering ARN (siARN) sont produits lorsqu'une molécule d'ARN double brin est produite ou introduite dans la cellule comme par exemple lors d'infections virales. Ils induisent la dégradation de cette molécule et de toute molécule possédant la même séquence. Ils sont un instrument de lutte anti-virale et servent maintenant d'outil d'inactivation génique.

Afin de tester l'implication des petits ARN non codant au cours de la réponse immunitaire, j'ai testé divers mutants affectant la production des miARN (mutants *dcr1*, *loqs* et *Ago1*) ou des siARN (mutants *dcr2, R2D2* et *ago2*). Les mutations *dcr-1* et *Ago1* ne sont pas viables à l'état homozygote. En revanche, l'analyse des mutants *loqs*, présentant un phénotype hypomorphe viable, ainsi que des mutants *dcr2* et *ago2* a montré un phénotype de forte sensibilité à l'infection par voie naturelle par le champignon entomopathogène *Beauveria bassiana* ainsi que la bactérie *Serratia marcescens*. Cependant, ces mêmes mutants ne présentent aucune sensibilité aux infections bactériennes lorsque les bactéries sont injectées par piqûre dans la cavité corporelle. Par ailleurs, les mouches mutantes pour *R2D2* ne sont sensibles à aucune infection. Ces phénotypes se comprennent à la lumière de la récente mise en évidence d'une troisième classe de petits ARN interférents, codés dans le génome, les endo-siARNs. Tout comme les siARNs, la synthèse des endo-siARNs est dépendante de Dcr-2 et Ago2. Mais la protéine associée à Dcr2 n'est pas ici R2D2 mais Loqs. Ces ARN dérivent de la transcription de transposons, de paires de transcrits sens et anti-sens et de structures en tiges boucles, formant naturellement dans tous les cas des ARNs double brin. A ce jour, le rôle de ces endo-siARNs n'est pas connu mais leur profil transcriptionnel spécifique suggère qu'ils jouent un rôle régulateur de l'expression du génome.

C’est donc la première fois que l’on attribue une fonction aux endo-siARNs. J’ai pu montrer que leur absence affecte la survie des mouches suite à une infection par voie naturelle, principalement au niveau de l’intestin. Cependant, la réponse humorale (synthèse des peptides antimicrobiens suite à l’activation des voies Toll ou Imd) des mutants *ago2*, *dcr-2* et *loqs* reste comparable à celle de mouches sauvages. Afin d’identifier les gènes affectés par l’absence d’endo-siARNs et expliquer le phénotype observé, j’ai entrepris une analyse transcriptomique des mutants *ago2* après infection par *Beauveria bassiana*.

J’ai entrepris par ailleurs une analyse phénotypique de ces mutants et pu montrer que le gène ago2 est requis dans les cellules souches intestinales pour résister aux infections. L’intestin de la mouche est très fortement affecté par une infection et doit être rapidement reconstruit grâce à la multiplication des cellules souches. Notre hypothèse de travail actuelle est donc que les endo-siARNs sont nécessaires au maintien ou au fonctionnement normal des cellules souches et qu’un dysfonctionnement au niveau de ces cellules souches lorsqu’ une infection affecte la capacité de la mouche à récupérer un intestin fonctionnel après une infection.

I. Introduction

The term RNA interference (RNAi) describes the concept of small RNA mediated gene silencing. The first endogenous small RNA was discovered 17 years ago in the nematode *Caenorhabditis elegans* (Lee *et al.*, 1993). The number of known small RNAs has since substantially expanded. What distinguishes and defines eukaryotic small RNAs in the RNA silencing pathways is their limited size and their association with Argonaute (Ago)-family proteins. Ago family proteins can be divided into two groups: the Ago subfamily and the Piwi subfamily (for review see Farazi *et al.*, 2008).

So far, three main small RNA classess have been identified in flies and mammals: 21-22 nt microRNAs (miRNAs) are associated with Argonaute-1 proteins and repress partially complementary mRNAs in both germline and somatic tissues. 21–22 nt small interfering RNAs (siRNAs) are generated from exogenous double-stranded RNA (dsRNA) such as viral genomes and loaded into complexes containing Argonaute-2. The last class, 24–30 nt Piwi-interacting RNAs (piRNAs) loaded into complexes containing Piwi-class proteins, are mostly restricted to the germline, where they repress transposons and can activate transcription in heterochromatin (Yin *et al.*, 2007; Brennecke *et al.*, 2007).

Mutants in core components of the *Drosophila* miRNA pathway are lethal, reflecting the fundamental roles of miRNAs in host-gene regulation in particular in development. Mutants in the core piRNA pathway are sterile and exhibit massive deregulation of transposon activity in the germline (Aravin *et al.*, 2007 a and b). Mutants in the *Drosophila* siRNA pathway, such as the main siRNA-generating enzyme Dicer-2 and the siRNA effector Argonaute-2, are hypersensitive to viruses, indicating that RNAi restricts viral replication (Ding *et al.*, 2007). Yet, these mutants are viable and fertile (Okamura *et al.*, 2004).

miRNAs are endogenous small RNAs found in the genomes of plants, animals and viruses. miRNAs are most often transcribed by polymerase II as long pri-miRNA precursors containing ≈70 nt stem-loop structures. Once excised from the pri-miRNA by the nuclear enzyme Drosha (Lee *et al.*, 2003), the stem-loop becomes a pre-miRNA. These pre-miRNAs are exported from the nucleus to the cytoplasm via the Exportin-5 system, where they are further processed by the ribonuclease Dicer-1 to yield ≈22 nt long double stranded RNAs (Lee *et al.*, 2004). Dicer-1 in complex with the double stranded RNA binding protein R3D1 (Loquacious) (Jiang *et al.*, 2005) facilitates dissociation and loading of the resulting single stranded mature miRNA into the RNA induced silencing complex (RISC). The core part of the complex is the RNaseH-like enzyme Argonaute-1 (Okamura *et al.*, 2004). How miRNAs regulate mRNA targets is unclear. Most animal miRNAs bind to imperfectly complementary sequences in the 3′-untranslated region of target mRNAs, and either repress translation or promote mRNA deadenylation and decay (Eulalio *et al.*, 2008; Eulalio *et al.*, 2007; Franks *et al.*, 2008).

RNAi guided by siRNAs is a conserved eukaryotic response to foreign nucleic acids and an important anti-viral response for plants and animals. The second Dicer enzyme encoded by the *Drosophila* genome, Dicer-2, senses dsRNA produced by the viral RNA-dependent polymerase during replication of the virus. Following recognition, Dicer-2 is found in a complex with the dsRNA binding protein R2D2 and processes dsRNA into siRNAs. These siRNAs are then incorporated into the RISC complex, mediating a sequence specific slicing of viral single stranded RNA by its core component, Argonaute-2 (Marques and Carthew, 2007).

Now, recent studies reveal interplay between the siRNA and miRNA canonical pathways leading to generation of the fourth class of small RNAs, the endogenous small interfering RNAs (endo-siRNAs) (Forstemann *et al.*, 2007; Tomari *et al.*, 2007). Recent work demonstrates different sources for endo-siRNAs in *Drosophila*. They derive from

transposons, convergent transcription and transcripts which fold to form long regions of dsRNAs. Their biogenesis and function depend on the canonical siRNA pathway components Dicer-2 and Argonaute-2. These proteins act with Loquacious (Loqs) that normally functions together with Dicer-1 in miRNA biogenesis (Forstemann *et al.*, 2005; Jiang *et al.*, 2005; Saito *et al.*, 2005; Park *et al.*, 2007a), whereas the usual partner for Dicer-2 is R2D2 (Liu *et al.*, 2003). The dependence of endo-siRNAs on Dicer-2 in combination with Loqs, but not R2D2, was unexpected, and it clearly distinguishes the small RNA-mediated response against transposons from the response to infection with an RNA virus (Wang *et al.*, 2006b).

Recent papers show deregulation of retrotransposon transcripts, pseudogene transcripts and *cis*-natural antisense transcript (*cis*-NAT) pairs in *Dicer-2* and/or *Ago2* mutants, suggesting that they are regulated by endo-siRNAs. So far, direct targets of *Drosophila* endo-siRNAs have been proposed only for some hairpin RNAs (hpRNAs) (Okamura *et al.*, 2008; Kawamura *et al.*, 2008; Czech *et al.*, 2008). These were DNA-binding proteins (Okamura *et al.*, 2008; Kawamura *et al.*, 2008). In *D. melanogaster cis*-NAT-siRNA loci are significantly enriched for DNA and RNA-binding proteins (Okamura *et al.*, 2008). Furthermore, it has been reported that the endo-siRNA pathway is involved in the formation of heterochromatin in somatic tissues in *Drosophila* (Fagegaltier *et al.*, 2009). Of note, *D. melanogaster* Dicer-2 mutants exhibit abnormal nucleolar morphology (Peng *et al.*, 2007), whereas Argonaute-2 mutants were reported to have chromosome segregation defects (Deshpande *et al.*, 2005). This suggests that the endo-siRNA pathway might be involved in cell division.

Since the biological function of endo-siRNAs is not well known, we aimed to explore their role in the innate immune system of *D. melanogaster*. Knowing that Argonaute-2 (*Ago2)* and Dicer-2 *(Dcr-2*) mutants are sensitive to viral infection (Rij *et al.*, 2006; Galiana-Arnoux *et al.*, 2006), we examined their response to other pathogens such as bacteria and fungi. Although the humoral response after septic injury in *Ago2*, *Dcr-2* and *Loqs* mutants is comparable to that of wild-type flies after bacterial and fungal infection, we observed that the

mutants are sensitive to *Beauveria bassiana* natural infection. Moreover, we found that flies mutant for *Ago2* are also more susceptible to natural infection with *Serratia marcescens*. We observed that the *Drosophila* gut is strongly affected by fungal infection by feeding and must be quickly rebuilt. Argonaute-2 is likely required in intestinal stem cells to resist natural infections. To identify genes affected by the absence of endo-siRNAs and explain the observed phenotype, we undertook a transcriptomic analysis of *Ago2* ^{414} mutants after infection by *B. bassiana*. It is the first time that small RNAs are shown to play a role in *Drosophila* response to fungal and bacterial infections.

II. Experimental procedures

Drosophila melanogaster strains and maintenance

Fly stocks were raised on standard cornmeal-agar medium at 25°C. The *Spätzle* mutant spz[20] and Yw or Ago2/+ were used as Toll mutant and wild-type control respectively. *Dcr-2* ^{A500V} is a point mutation in the helicase domain (Lee *et al.*, 2004; Deddouche *et al.*, 2008) (Fig1). The Loquacious mutant (*Loqs*) is a piggyBac transposon insertion f00791 laying 57 bp upstream of the reported transcription start site for *Loqs* (Förstemann *et al.*, 2005). *Ago2* ^{414} is an imprecise excision of the EP element generating a 2.3-kb deletion of genomic DNA, which included exons 1 and 2 of the *Ago2* gene (Okamura *et al.*, 2004). R2D2 mutation is a substitution of two conserved alanines by lysine (Quinghua *et al.*, 2003). *Dcr-2* ^{A500V}, *Loqs*, *Ago2* ^{414}, and R2D2 mutants are in a background yellow white. *Escargot Gal4-Gal80 (esg);Ago2* ^{414} flies were crossed with *UAS-GFP;Ago2*^{414} or UAS-Ago2;*Ago2*^{414} flies and the progeny was kept at 29°C.

UAS Ago2 construction

Ago2 full-length cDNA sequence was subcloned as an NotI-KpnI fragment into the pUAS-T vector.

Overexpression of transgenes

Transgenes were overexpressed using the UAS-GAL4 system (Brand and Perrimon, 1993). In this system, a transgenic “driver” line expresses the yeast transcriptional activator GAL4 under the control of a chosen *Drosophila* promoter. When this line is crossed with another that carries the transgene of interest under an Upstream Activating Sequences (UAS) recognized by GAL4, the progeny that contains both transgenes will express the transgene of interest under the control of the promoter that drives GAL4 expression. Heat-shocks were

performed as follows : 1 hour at 37°C, and then incubation at 29°C. Escargot-Gal4 driver flies also express a transgenic thermosensitive GAL4 repressor, $GAL80^{ts}$. Under permissive conditions (18°C), the repressor is active and inhibe his targets, GAL4. However, when the flies are placed at 29°C, a restrictive temperature, the repressor is inactive and the GAL4-UAS system is fully functional.

Natural infection and survival experiments

Infection experiments were performed on 4–6 days old adult flies (10 males and 10 females), repeated two or three times (n=2 or n=3). For natural fungal infections, flies were shaken on a lawn of sporulating *B. bassiana* (80.2 strain). They were then put back into vials.

For the natural bacterial infection, *Serratia marcescens* (*Db11* strain spontaneously streptomycin-resistant) was grown in LB (Luria Bertani medium) at 37 °C. Batches of 20–25 adult wild-type and mutant flies were used in these experiments. The food solution containing bacteria was prepared from a culture grown exponentially at 37 °C to OD_{600} = 1. This culture was diluted with a sterile 50 mM sucrose solution to a final OD_{600} = 0.1. Two absorbent filters (37 mm; Millipore) were placed into fly culture tubes and soaked with 2 ml of the *S. marcescens* sucrose solution. The flies were then transferred to these vials and fed continuously on this solution.

Surviving flies were counted once a day. Experiments were performed at 29 °C. Results are expressed as a percentage of infected flies at different time points after infection.

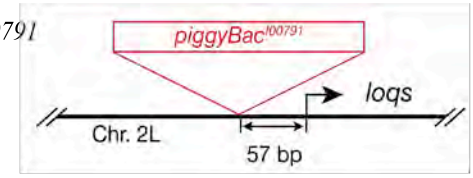
Gut preparation / microscopy

Intestines were dissected in PBS and fixed 30 min in 4% paraformaldehyde (PFA) and stained overnight by 10 µM Texas Red-labeled phalloidin in PBS + 0.1% Triton X100. The next day they were incubated 10 min with 0,1 % calcofluor-white (Uvitex 2B, Polysciences, Inc. Cat. # 19517) and then mounted in Vectashield HardSet ^{TM} with or without DAPI (CliniSciences).

Microarray analysis

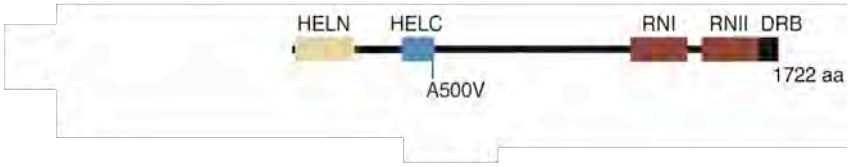
Total RNA from 30 flies of 4 days old males was isolated with RNeasy kit (Qiagen) and treated with DNase (Qiagen). RNA was quantified by NanoDrop ND-1000 and its quality was assed using Bioanalyzer chips. For each sample, 1 μg of total RNA was amplified and labeled using a Labeling Kit according to the protocol provided by the supplier. Transcriptome data were generated using Affymetrix Drosophila Genome 2.0 arrays. Were labeled cRNA, washed, stained, and scanned according to the protocol described in Affymetrix Manual. The experiment was performed in duplicate.

A. *Loqs* ^{f00791}

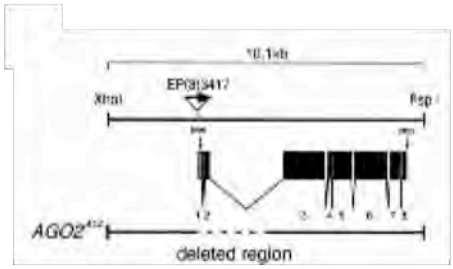


Förstemann et al., 2005

B. *Dcr-2* ^{A500V}



C. *Ago2* ^{414}



Okamura et al., 2004

D. *R2D2*

GxGxSKKxAKxxAAxxALxxL
KK

Quinghua et al., 2003

Figure 1. Molecular identity of *Loqs, Dcr-2, Ago2* and *R2D2* mutations. A. *Loqs* ^{f00791} PiggyBac transposon insertion f00791 is laying 57 bp upstream of the reported transcription start site for *Loqs.* B. *Dcr-2* ^{A500V} point mutation is in the helicase domain. HELN: DExH box; HELC: C-helicase domain; RN: is a tandem repeats of RNase III domains; DRB: C-terminal dsRNA binding domain. C. *Ago2*^{414} Imprecise excision of the EP element generating a 2.3-kb deletion of genomic DNA, which included exons 1 and 2 of the *argonaute-2* gene. D. *R2D2* The blue shaded boxes refer to dsRNA-binding (dsrb) domains. The consensus dsrb motif sequence is shown. Two conserved alanines (A) were substituted by lysines (K) in both dsrb motifs in the mutant R2D2 protein. G, glycine; S, serine; L, leucine; and x, any amino acid.

III. Results

A. Endo-siRNA pathway is involved in the defense against *B. bassiana*.

In *Drosophila*, injection of bacteria into the body cavity induces the secretion of antimicrobial peptides (AMPs) into the hemolymph. The expression of AMPs after infection with fungi and Gram-positive bacteria is activated through the Toll pathway, and Gram-negative bacteria infection through the IMD pathway. *Dcr-2* ^{A500V}, *Ago2* ^{414} and *Loqs* ^{f00791} mutants were challenged with different pathogens. We used a Gram-positive bacterium (*Enterococcus faecalis*), an entomopathogenic fungus (*Beauveria bassiana*) and a Gram-negative bacterium (*Agrobacterium tumefaciens*). After natural infection with *B. bassiana*, we observed that *Dcr-2* ^{A500V}, *Loqs* ^{f00791} and *Ago2* ^{414} mutants are as sensitive as *Spätzle* mutant (Fig.2), the ligand of the Toll transmembrane receptor protein. When challenging the flies with *E. faecalis* and *A. tumefaciens*, *Dcr-2*, *Ago2* and *Loqs* mutants behaved as wild-type flies. *R2D2* mutant flies were also examined, but did not show increased sensitivity to any of the pathogens.

To confirm the role of the endo-siRNA pathway upon *B. bassiana* infection, we overexpressed the Flock House virus B2 protein, known to suppress antiviral RNAi in insects (review Ding and Voinnet 2006). This protein is also an inhibitor of the endo-siRNA pathway (Fagegaltier *et al.*, 2009). B2 binds to one face of an A-form RNA duplex, independent of its length. As a consequence, B2 prevents the processing of long dsRNAs into siRNAs by *Drosophila* Dicer-2 (Chao *et al.*, 2005). We found that flies overexpressing B2 protein are sensitive to *B. bassiana* (Fig.3). These results show that the endo-siRNA pathway is involved in the defense of *Drosophila* against *B. bassiana*.

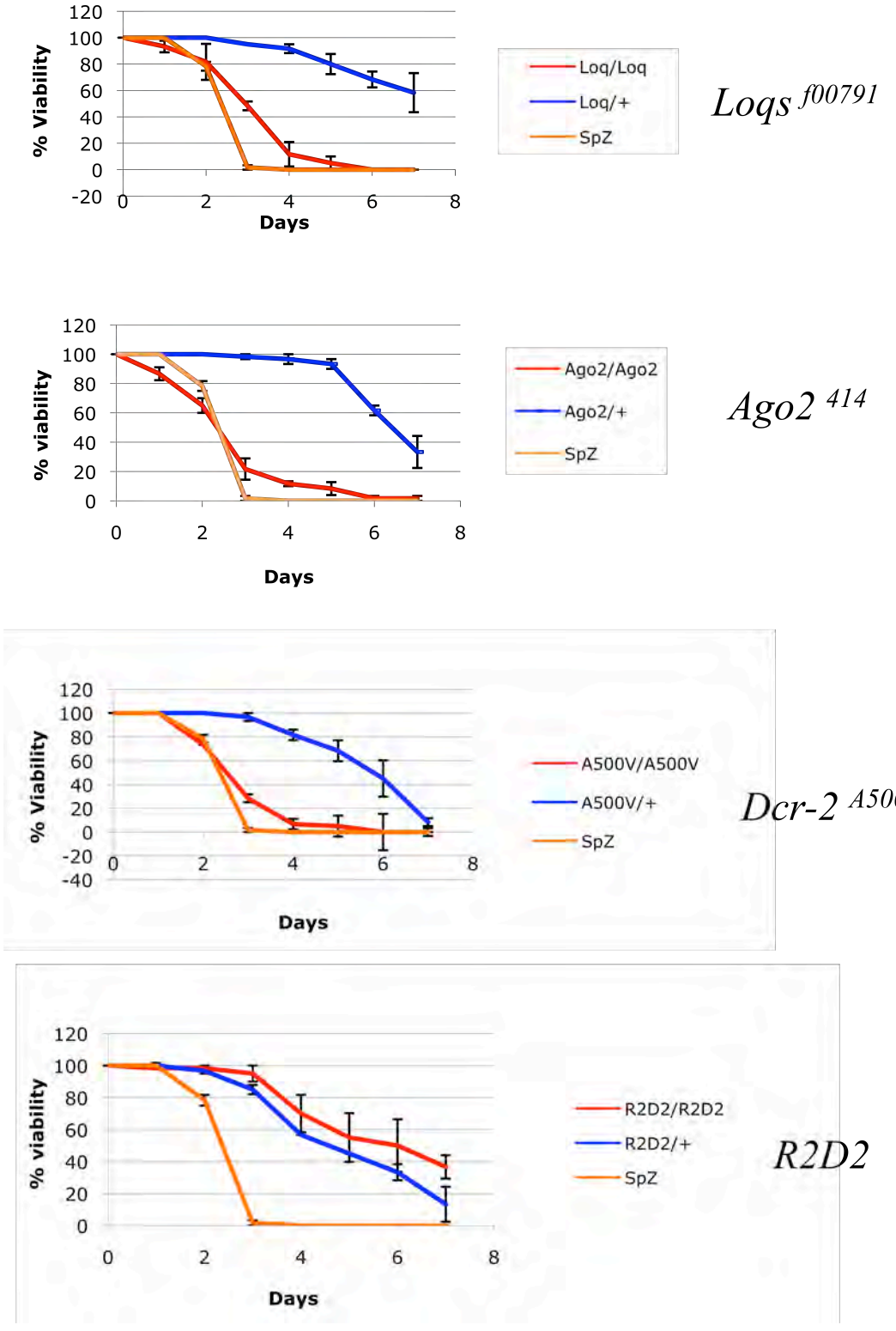


Figure 2. *Beauveria bassiana* natural infection. Mutants in the endo-siRNA pathway (*Loqs*, *Ago2* and *Dcr-2*, but not *R2D2*) are sensitive to *B. bassiana* infection (n=3). Flies deficient in the Toll pathway are susceptible to fungi and Gram-positive infections. Spätzle mutant flies (Spz) were used as control.

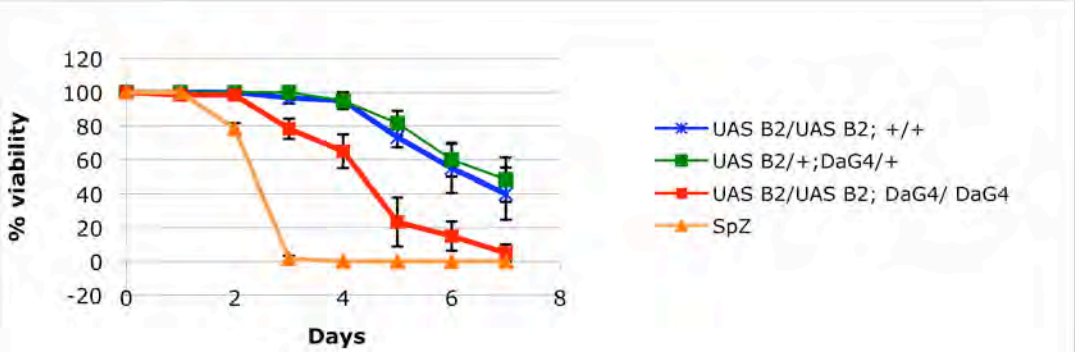


Figure 3. *Beauveria bassiana* natural infection. Flies overexpressing B2 protein are sensitive to *B. bassiana* infection (n=3). Flies deficient in the Toll pathway are susceptible to fungi and Gram-positive infections. Spätzle flies (Spz) are mutant for the Toll pathway and were used as control.

In order to explain why the endo-siRNA pathway mutant flies are sensitive to *B. bassiana* natural infection, we examined expression of the main anti-fungal peptide *drosomycin*, induced through the Toll pathway, in the *Ago2* ^{414} and *Loqs* ^{f00791} mutants by Northern blot. Surprisingly, *drosomycin* expression was not affected by the mutations (data not shown), indicating that the humoral repsonse is not sufficient to protect the flies against *B. bassiana* infection. This together with the fact that the mutant flies are not sensitive to septic injury, led us to investigate if the mutant sensitivity is specific for natural/oral infections. In particular, we were interested whether it is associated with the gut.

B. *Ago2* ^{414} mutant flies are sensitive to *Serratia marcescens* natural infection.

Three years ago, infection of *Drosophila* with the Gram-negative bacteria *Serratia marcescens* was described as an intestinal infection model. *S. marcescens* (Db11) escapes from the digestive tract into the hemocoel through the intestinal epithelium (Nehme *et al.*, 2007).

As demonstarted above, flies mutant for genes involved in the endo-siRNA pathway are sensitive to *B. bassiana* natural infection and we wondered if they were also sensitive to natural infection by *S. marcescens*. *Ago2* ^{414} is a homozygous viable stock, easier to maintain than *Dcr-2* ^{A500V} and *Loqs* ^{f00791} stocks. Therefore, we decided to use it for this and the rest of our experiments.

Ago2 ^{414} mutant flies naturally infected with *S. marcescens* are as sensitive as *Kenny (key)* flies, mutant for the IMD pathway (Fig.4).

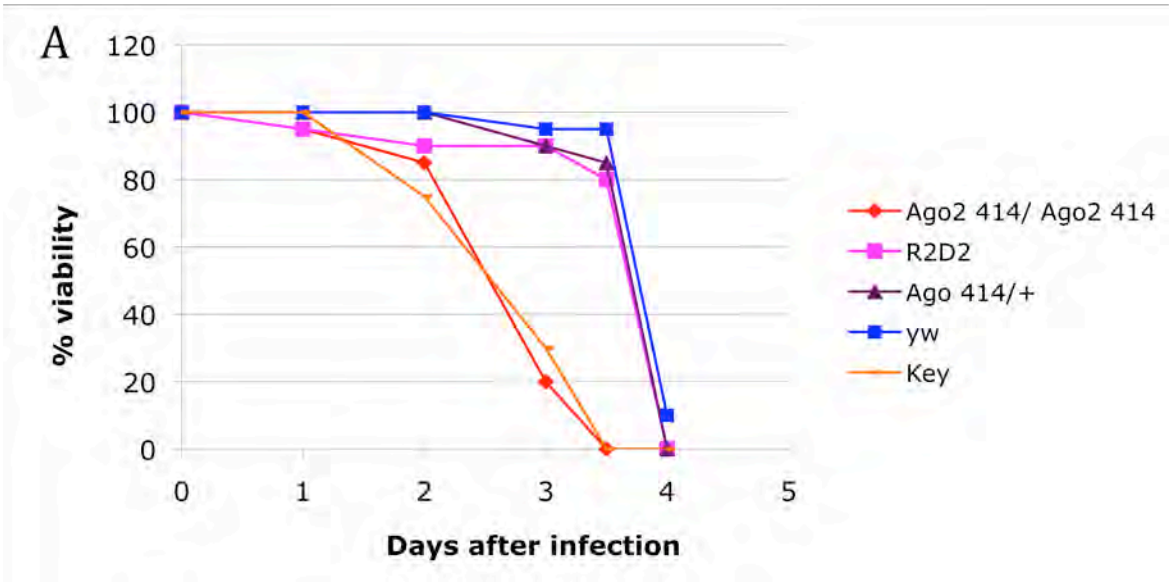


Figure 4. ***Ago2*** ^{414} **mutant flies are sensitive to *Serratia marcescens* infection**. Survival of *Ago2* ^{414} mutant upon *S. marcescens* natural infection. yw and *Ago2* ^{414}/+ flies are considered wild-type (n=2)

C. *Beauveria bassiana* spores are found in *Drosophila* intestine.

Natural infection of *Drosophila* by *B. bassiana* is initiated by applying fungal spores on the cuticle. Until now, it was believed that *B. bassiana* infects many insect species by penetrating this cuticle. After application of the spores, they are visible on their cuticle and we can easily observe that in approximately 12 hours the flies clean their bodies. The only part where spores

can be found is the dorsal part of the thorax, inaccessible for the fly. Conidia of *B. bassiana* germinate on the host cuticle and differentiate to form an appressorium. An infecting hypha penetrates through the host cuticle and eventually reaches the hemocoel. It has been shown that Termites exposed to *Beauveria bassiana* or *Metarhizium anisoplae* contain spores in their hindguts within 8 hours of exposure (Kramm and West, 1982).

In order to examine if *B. bassiana* is present inside the adult gut of *Drosophila*, we performed a calcofluor-white staining on wild-type intestine 48h after *B. bassiana* infection.

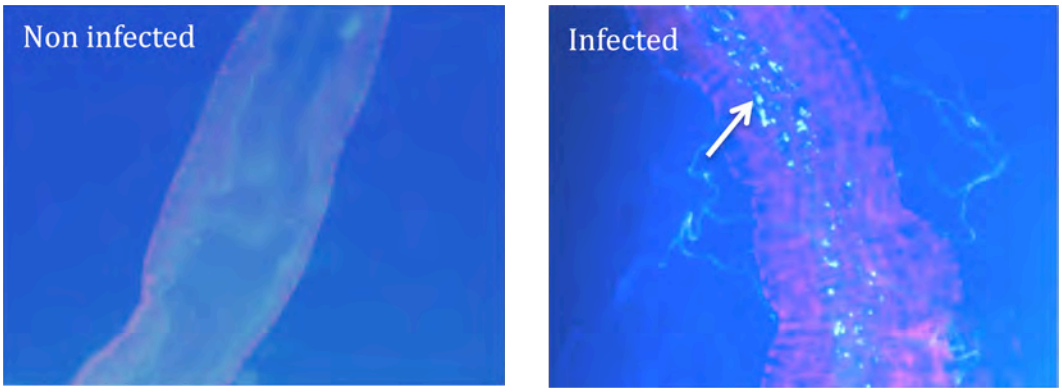


Figure 5. *Beauveria bassiana* spores in wild-type *Drosophila*'s intestine was stained with calcofluor-white before (left) and 48h after infection (right). Spores are stained with calcofluor and epithelial cells are labeled with the phalloïdin-Texas red.

Calcofluor-white is a dye that is used for identification of fungi. It is nonspecific, and binds to the cellulose and chitin in the fungal cell walls. Before infection, flies have been fed 24 hours on 50 mM sucrose, in order to empty their gut from yeast that could be also stained by calcofluor-white.

Fig.5 clearly shows that *B. bassiana* spores are present in the gut of *Drosophila* wild-type (see the white arrow). The same was observed in the mutant (data not shown). We conclude that *B. bassiana* is able to infect *D. melanogaster* through cuticle but is also present in the digestive tract.

D. *Ago2*^{414} mutant flies phenotype is rescued when Argonaute-2 is expressed in stem cells.

In order to understand the function of Argonaute-2 in the defense against natural infection with *B. bassiana*, we investigated in which tissue Argonaute-2 is required. We therefore set up rescue experiments of the *Ago2* ^{414} phenotype. The wild-type *argonaute-2* cDNA was introduced to various tissues, using different drivers. In particular, we expressed *argonaute-2* in hemocytes (Hemolectin driver), trachea (Brist Less driver), gut (NP1 and Caudal drivers), stem cells (Escargot driver) and we also used an inducible ubiquitous driver (Heat Shock). None of them rescued *Ago2* ^{414} phenotype except for the driver specific for stem cells, Escargot Gal4 (esg) (Fig.6).

Subsequently, using the Gal80 thermo-sensitive system, we expressed *argonaute-2* only at the adult stage. At 18°C the Gal 80 protein to inhibits fixation of the Gal4 protein to the UAS promoter. At 25°C and 29°C the thermo-sensitive Gal80 protein is denatured, allowing the binding of Gal4 to its promoter and transcription of the target gene Argonaute-2. Flies were left at 18°C from early embryogenesis to pupal emergence. Adults were then incubated at 29°C for 3 days before infection. We observed that in these condition the expression of *argonaute-2* in adult stem cells is not sufficient to rescue the *Ago2* ^{414} phenotype (data not shown). Also, the heat-shock Gal4 driver was activated only during adult stage and this was not sufficient to rescue *Ago2* ^{414} phenotype. This result clearly shows that the Argonaute-2 protein is required during development in order to protect against *B. bassiana* infection at adult stage.

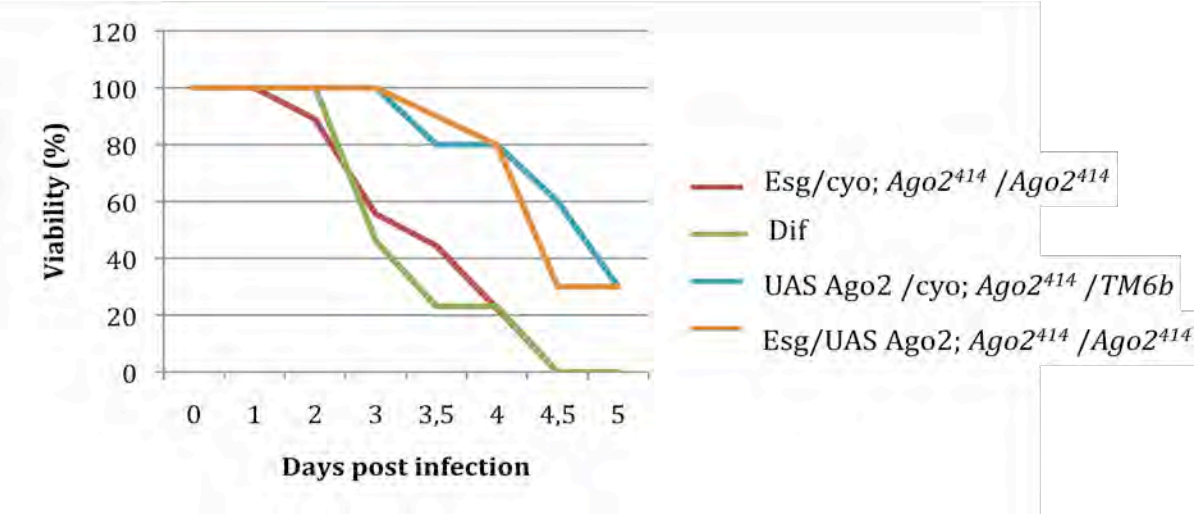


Figure 6. Survival after *B. bassiana* infection. Overexpression of Argonaute-2 in stem cells with the Escargot Gal 4 driver (esg) is able to rescue the *Ago2* ^{414} mutant phenotype. Dif is a transcription factor of the Toll pathway. Flies deficient in the Toll pathway are susceptible to fungi and Gram-positive infections. *Dif* mutant was used as a control (n=2).

E. Abnormal structure of the gut in *Ago2* ^{414} mutant flies.

To maintain homeostasis, the gut epithelium is constantly renewed through division and differentiation of intestinal stem cells. In order to have a general view of the gut epithelium structure in mutant and wild-type flies, we used red phalloïdin to stain actin filaments in epithelial cells.

It appears that the intestine of *Ago2* ^{414} mutant flies exhibits a quite different structure than that of wild-type. In mutants, the epithelial cells are differently organized than in wild-type guts (Fig.7 A and C). 48h after infection with *B. bassiana*, the gut epithelium in *Ago2* ^{414} mutant flies is totally disorganized in comparison to wild-type (Fig.7 B and D).

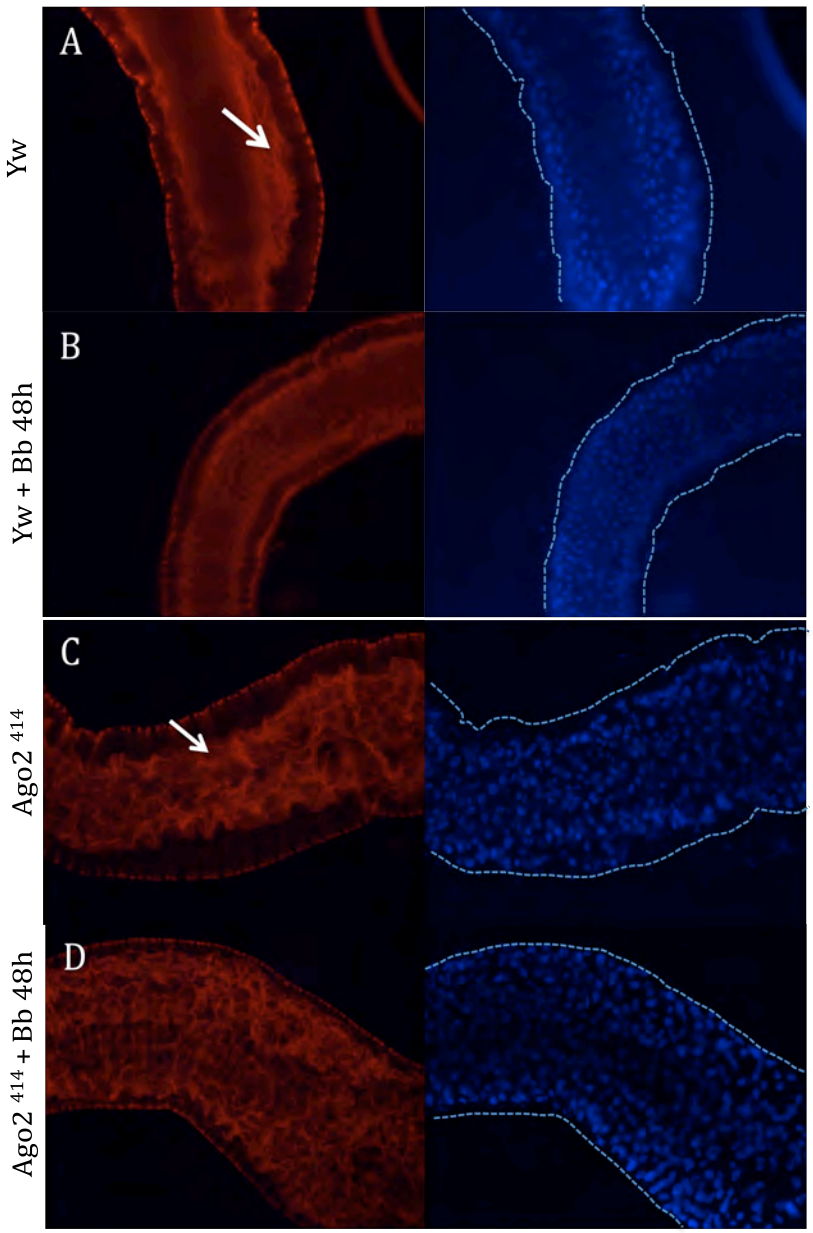


Figure 7. Comparison of the gut structure in wild-type and mutant flies, before and after infection. Epifluorescence microscopy: Phalloïdin Texas-Red labels actin filaments and DAPI (blue) labels the nuclei in the gut of control (yw) and *Ago2* ^{414} mutant flies 2 days after infection with *B. bassiana* at 29°C compared to the non-pathogenic conditions. The difference between intestines of *Ago2* ^{414} mutant and wild-type flies is indicated by the arrows.

To corroborate this observation, we stained the peritrophic membrane with calcofluor-white and the actin filaments with phalloïdin. The peritrophic membrane is a cylindrical membranous sheet, which encloses food in the midgut of most insects and protects the epithelium from the abrasive action of solid food. The peritrophic membrane is secreted by cardia and/or the midgut epithelium and is composed of chitin and several other proteins (Elvin *et al.*, 1996).

Flies were infected with *B. bassiana* and after 48h incubation guts have been dissected and observed using microscopy. We confirmed that the layer of epithelial gut cells in the mutant is thick and totally disorganized after infection (Fig.9 E) in comparison to non infected mutant (Fig.9 D) or infected wild-type (Fig.8 B), whereas we did not observe any difference in the wild-type and the mutant before infection (Fig.8 A and 9 D).

As we could not observe the peritrophic membrane because the gut was full of yeast (Fig. 8 and 9 D) we decided to feed flies with 50 mM sucrose solution for 24 hours before infection, in order to empty guts. Then, *Ago2*^{414} mutant flies were infected with *B. bassiana* and the guts were dissected 48h later. The peritrophic membrane is large in the mutant before infection, while intestinal cells form a single layer (Fig.9 A, B and C). After infection, the peritrophic membrane is shrunken in the mutant flies (Fig.9 F). These results indicate that the peritrophic membrane is present in *Ago2*^{414} mutant flies and that the intestinal lumen is reduced due to the thickening of the gut epithelium.

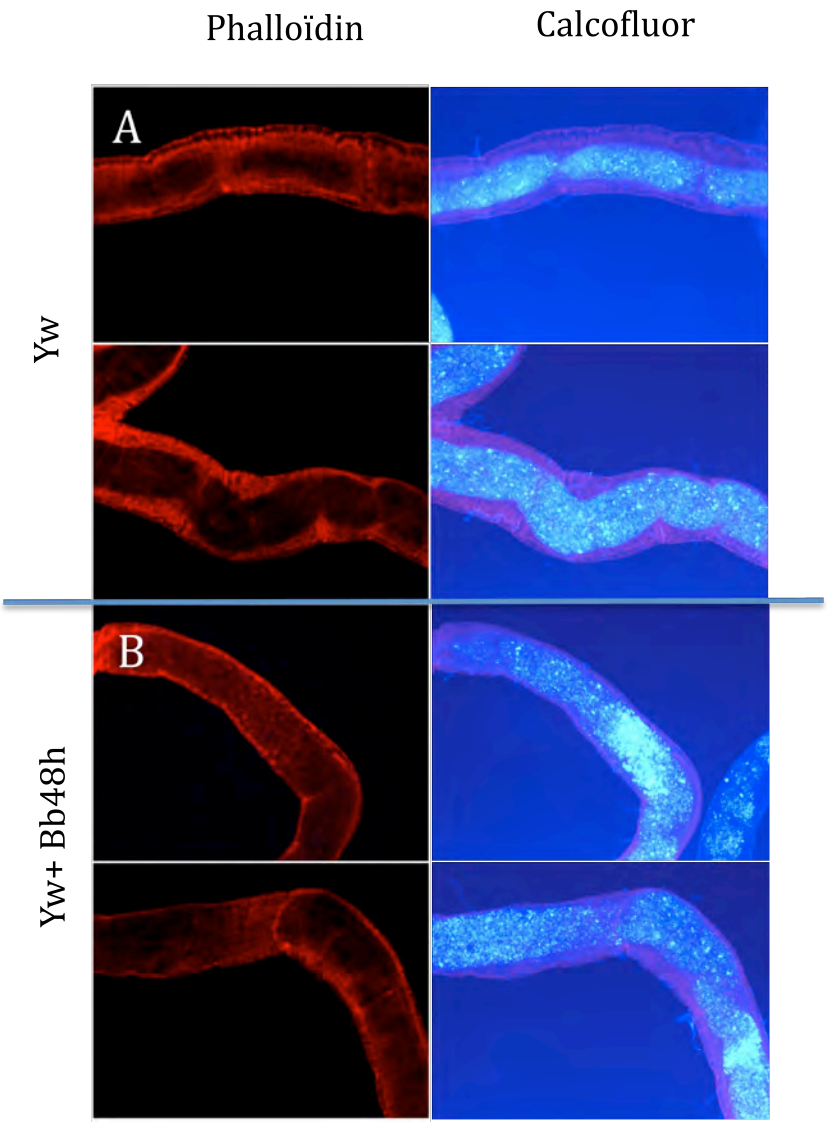


Figure 8. Epithelial gut cells in wild-type flies. Epifluorescence microscopy: Phalloïdin-Texas Red labels filaments of actin and the calcofluor labels the peritrophic membrane in the gut of control flies (yw) on day 2 after infection with *B. bassiana* at 29°C compared to the non-pathogenic conditions.

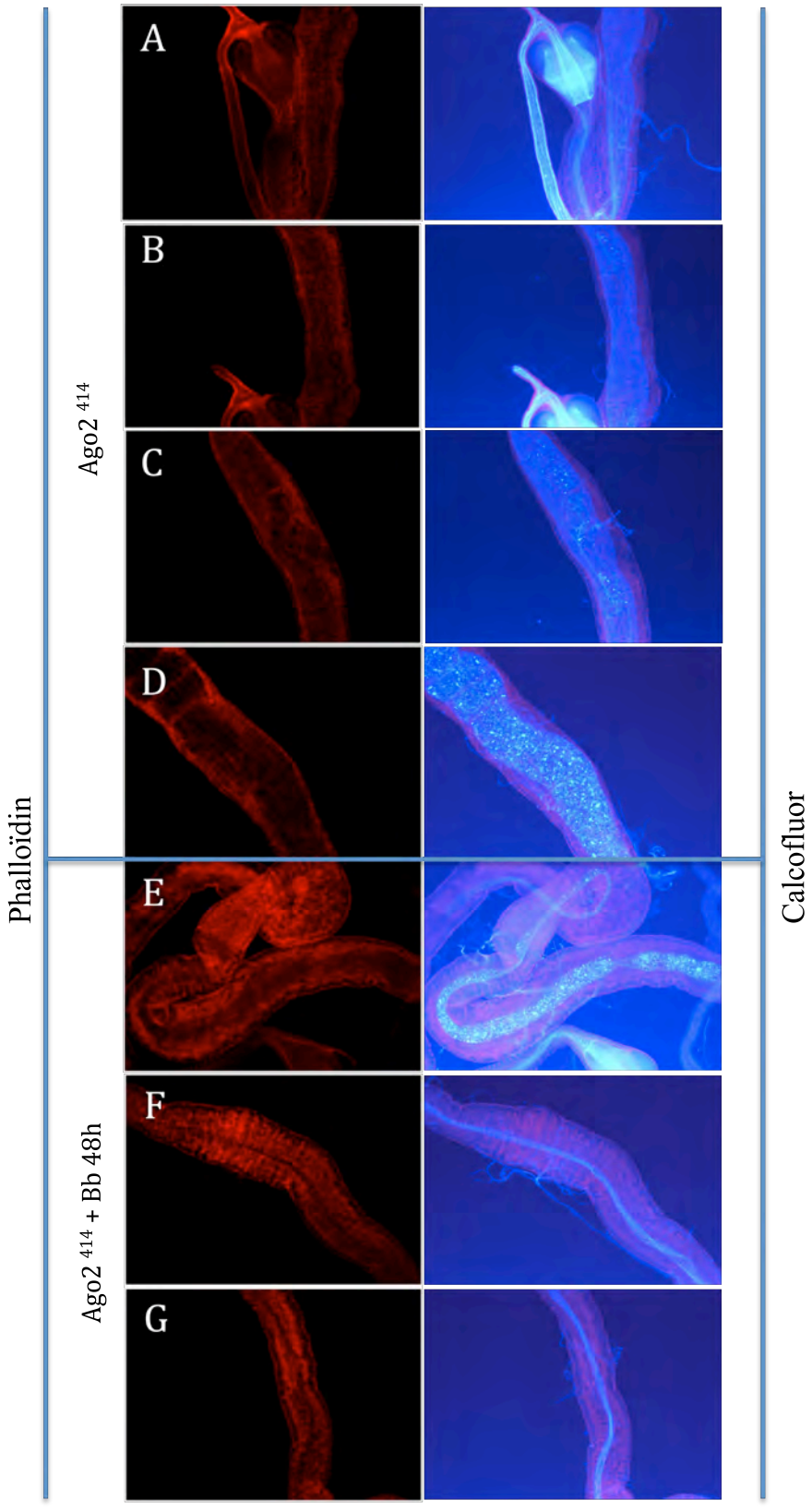


Figure 9. Epithelial gut cells in *Ago2* ^{414} mutant flies. Epifluorescence microscopy: Phalloïdin-Texas Red labels filaments of actin and the calcofluor labels the peritrophic membrane in the gut of *Ago2* ^{414} mutant flies (on day 2 after infection with *B. bassiana* at 29°C) compared to the non-pathogenic conditions.

F. Argonaute-2 is required for stem cell proliferation.

The adult *Drosophila* midgut is composed of absorptive enterocytes (ECs) interspersed with secretory enteroendocrine cells (EEs). Although this tissue has long been considered quiescent, ground-breaking work published in 2006 revealed that the *Drosophila* midgut is constantly renewed by a pool of intestinal stem cells (ISCs). ISCs divide asymmetrically to produce both ISCs and enteroblasts (EBs) that differentiate into ECs or EEs (Fig.10) (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006).

It is known that oxidative stress caused by bacteria or chemicals accelerates stem cell division as well as differentiation resulting in replenishment of enterocytes and epithelial repair after damage (Chatterjee and Ip, 2009). *B. bassiana* likely inflicts damage to the gut epithelium. Stem cell proliferation and differentiation repair and may be required to protect the fly against *B. bassiana.*

We saw previously that the $Ago2^{414}$ mutant phenotype is rescued by the overexpression of the full length cDNA *argonaute-2* in the stem cells, using the Escargot Gal4 driver (esgG4). This means that Argonaute-2 protein is required in the stem cells and that the absence of Argonaute-2 protein reduces *Drosophila* resistance after *B. bassiana* infection.

We compared stem cells in the wild-type and the mutant flies after infection with *B. bassiana.* In order to observe stem cells in the gut upon infection we generated flies expressing the GFP protein using the esgG4 driver.

Upon infection, the number of stem cells is increased in the mutant in comparison with the wild-type (Fig.11). It suggests a high ISC proliferation and/or a failure of EBs to correctly differentiate.

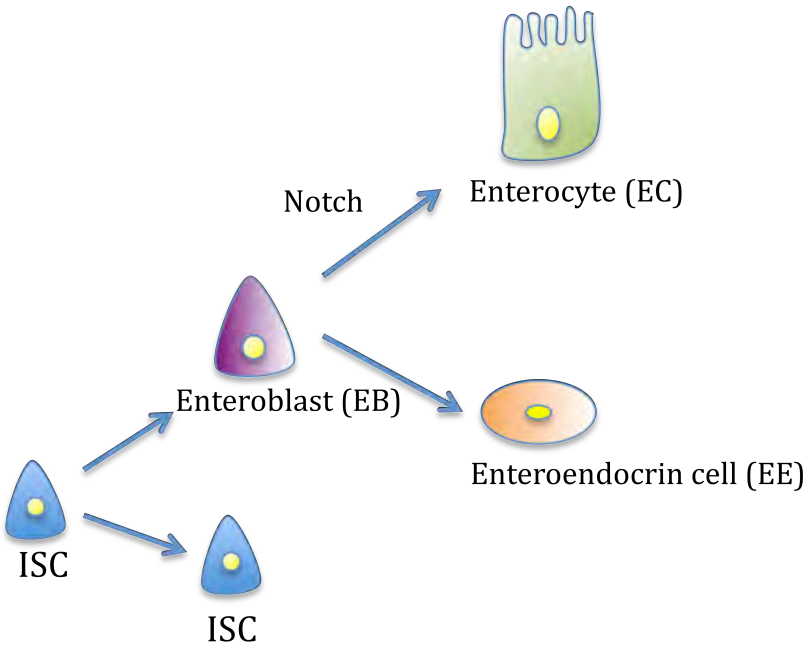


Figure 10. Epithelium renewal in drosophila intestine: Intestinal stem cells (ISCs) divide asymmetrically to produce both ISCs and EBs that differentiate into ECs or EEs. Delta protein is expressed on the surface of ISC, and interacts with Notch receptor on the adjacent EBs. This results in EB differentiation into an EC and prevents differentiation into an EE. Lower expression of Delta by ISCs facilitates EB differentiation into EEs. ISCs are characterized by expression of the transcription factor Escargot, and by expression of the Notch ligand Delta. EBs express Escargot and have a high level of Notch signalling. The gene *prospero* is a marker of EEs.

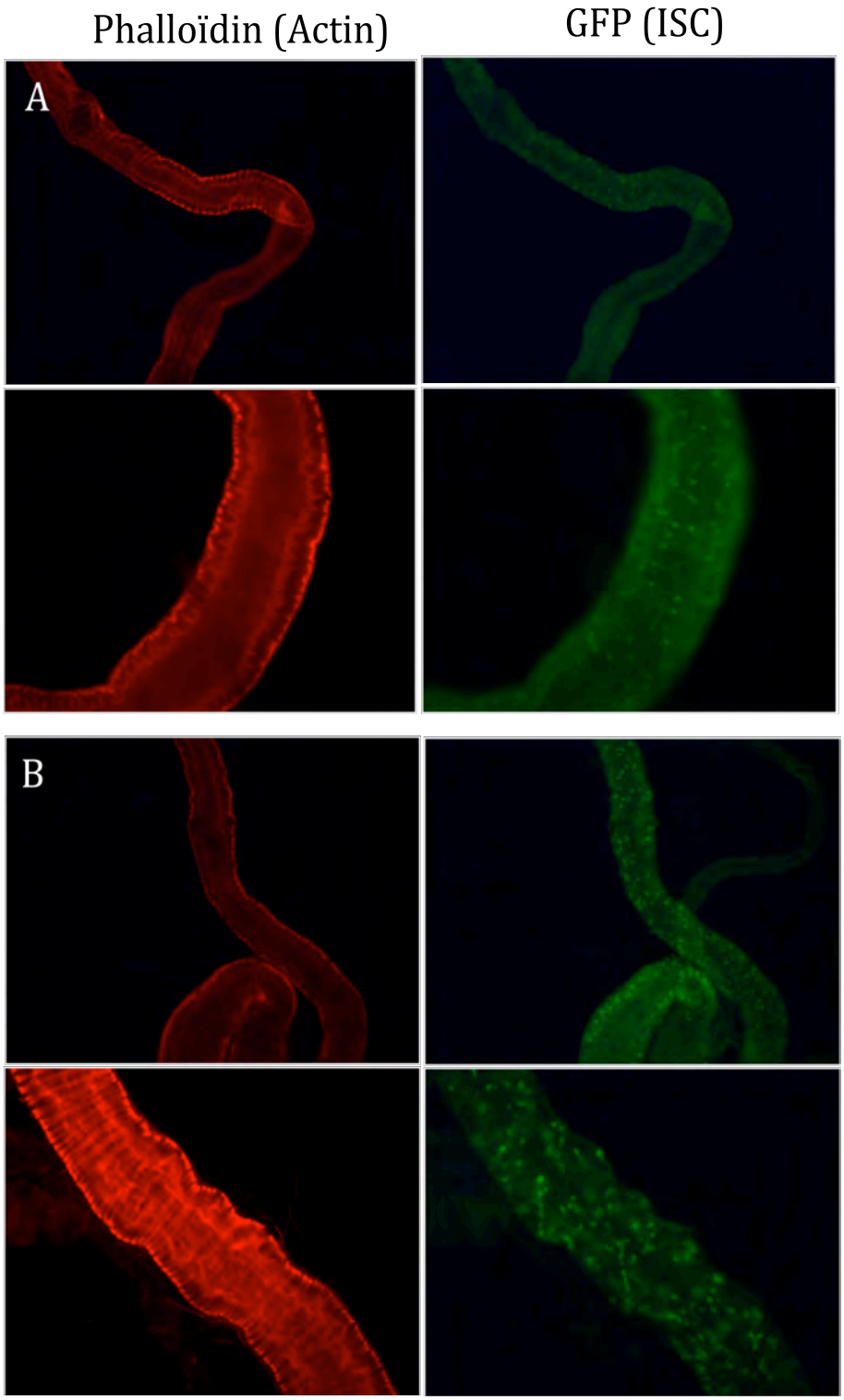


Figure 11. **Stem cells in the intestine after B. bassiana infection.** Intestinal stem cells (ISC) in A. the wild-type (esgG4/UAS-GFP; *Ago2*^{414}/TM6b) and B. *Ago2* mutant (esgG4/UAS-GFP; *Ago2* ^{414}/*Ago2* ^{414}) were labeled by expression of Green Fluorescent Protein (GFP) 48h after infection with *B. bassiana*. Phalloïdin-Texas Red labels filaments of actin.

G. Transcriptome analysis of *Ago2* ^{414} mutant flies.

To determine genes specifically induced or repressed in the *Ago2* ^{414} mutant we investigated transcriptome variations in *Ago2* ^{414} whole mutant flies (Fig.13). Following infection, with *B. bassiana* transcriptomic data were generated using Affymetrix Gene Chip, *Drosophila* genome 2.0 Array. RNA from control (*Ago2* ^{414} /+) and mutant flies (*Ago2* ^{414} / *Ago2* ^{414}) non infected or infected with *B. bassiana* for 24h was examined in duplicate. Bioinformatics analysis confirmed that our microarray data were consistent between the duplicates.

1. Transposons are overexpressed.

Genetic and molecular evidence suggests that in addition to suppressing viral infection, the RNAi pathway silences selfish genetic elements in the fly soma. Depletion or mutation of either *dicer-2* or *argonaute-2* elevates transcript levels of transposable elements (Ghildiyal *et al.*, 2008 ; Kawamura *et al.*, 2008 ; Czech *et al.*, 2008 ; Chung *et al.*, 2008). In our microarray results we observed overexpression of several transposons in our *Ago2* ^{414} mutants, thus validating our microarray experiment.

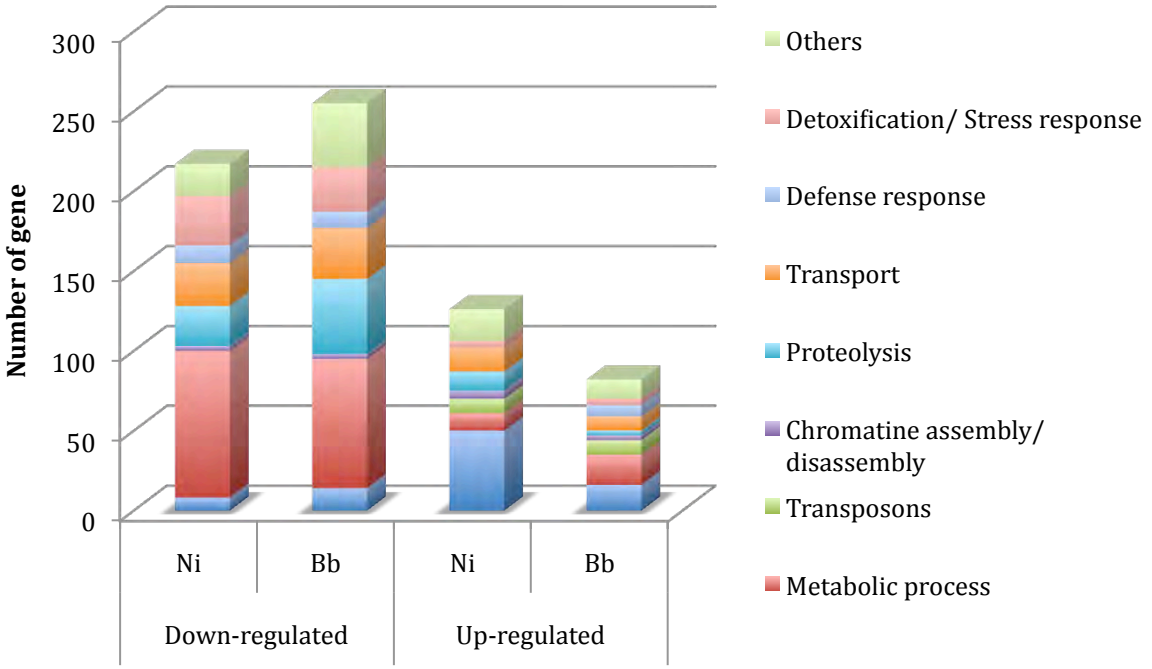


Figure 12. Microarray data. Wild-type flies (*Ago2* ^{414} /+) and mutant flies (*Ago2* ^{414} / *Ago2* ^{414}) were infected (Bb) or not infected (Ni) with *B. bassiana* for 24h. Changes (≥2 fold) in mRNA accumulation are shown for genes grouped into various categories.

2. Genes involved in DNA replication, transcription and translation are up-regulated when Ago2 is missing.

It has been reported that *Drosophila Ago2* ^{414} mutant embryos exhibit asynchronous nuclear division cycles, due to an incomplete separation of nuclei. The mitotic spindle apparatus is disrupted and the centrosome is not correctly assembled. These embryos also have defects in the actomyosin cytoskeleton (Deshpande *et al.*, 2005). Another report shows that the endo-siRNA pathway is involved in cell cycle regulation during *Drosophila* embryonic development (Lucchetta *et al.*, 2009). This effect has never been described in adult flies. Here according to our microarray results, it seems that in adult flies the mutation of *argonaute-2* affects cell division, gene transcription and translation.

Among 50 genes overexpressed in *Ago2* ^{414} mutant flies and involved in replication, transcription and translation processes, 24 genes are involved in mitosis and cytoskeleton, 19 genes in transcription, and only 7 in translation process. However, after infection only 16 genes out of 50 are overexpressed.

3. Genes related to metabolic processes, proteolysis, detoxification and stress response are down-regulated in *Ago2* ^{414} mutant flies.

A number of genes involved in detoxification and stress response are repressed. Moreover, we observed in the *Ago2* ^{414} mutants, the expression of genes involved in metabolic processes and proteolysis is also reduced. Despite all these transcriptional perturbation, flies mutant for *argonaute-2* are viable and fertile.

4. Toll pathway activation is not affected in *Ago2* ^{414} mutant flies.

Natural infection of *Drosophila* by *B. bassiana* leads to the expression of Toll-dependent immune genes, including the antifungal peptide genes Drosomycin and Metchnikowin (De Gregorio *et al.*, 2001 Lemaitre *et al.*, 1997). In our microarray experiment, *drosomycin* and *metchnikowin* are not affected by the mutation of *argonaute-2*. This confirmed the results of our Northern blot analysis of *drosomycin* in both *Ago2* ^{414} and *Loqs* ^{f00791} mutants. This result suggests that a different mechanism of defense against *B. bassiana* is affected by the mutation.

H. CG10102 is a nuclear protein involved in protection against *B. bassiana*.

In our microarray data, we identified the gene *CG10102* which was expressed 2.4 times more in wild-type flies after *B. bassiana* infection than in non-infected flies. In the *Ago2* ^{414} mutant, the level of *CG10102* transcription was similar before and after infection. The gene *CG10102* is one of three *Drosophila* homologs of mammalian activity-regulated cytoskeleton-associated protein (ARC) (Mattaliano *et al.*, 2007).

During *Drosophila* oogenesis, germline stem cell (GSC) identity is maintained largely by preventing the expression of factors that promote differentiation. The DNA-associated protein Stonewall (Stwl) promotes GSC maintenance by repressing expression of many genes involved in GSC differentiation. To identify Stwl targets Maines *et al.* carried out a microarray analysis of undifferentiated germ cells that lack Stwl. *CG10102* is among the genes upregulated in the absence of Stwl. *CG10102* encodes a nucleic acid binding protein involved in transcription and is supposed to induce GSCs differentiation (Maines *et al.*, 2007).

We wondered if *CG10102* was involved in the protection against *B. bassiana* infection perhaps by regulation of intestinal stem cells differentiation. In order to verify this hypothesis we decided to study two different P-element insertion lines in *CG10102*. The *BL20918* insertion is located exactly between the first exon and the intron.

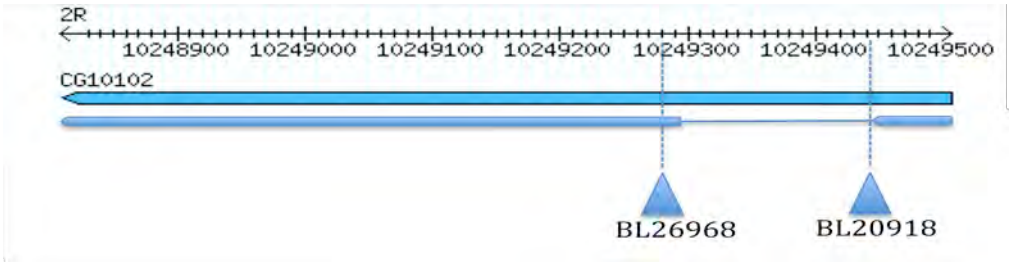


Figure 13. P-element insertion in CG10102 gene. (modified from http://flybase.org/)

The *BL26968* insertion is located downstream the beginning of the second exon (Fig.13). The two lines have been challenged with *B. bassiana*. *BL20918* is more sensitive than *BL26968* and is as sensitive as *Ago2* ^{414} flies to *B. bassiana* infection (Fig.14). To confirm this result, and to be sure that the result observed in the survival is really due to the inactivation of *CG10102*, we crossed a strain with inserted P-element (BL20918) with a deficiency strain (Df26552), which covers the *CG10102* genomic region and challenged the hemizygous flies with *B. bassiana* (Fig.14). As expected, the *BL20918/Df26552* flies are as sensitive as *BL20918* and *Ago2* ^{414} strains. This result confirms the specificity of the *CG10102* mutation phenotype.

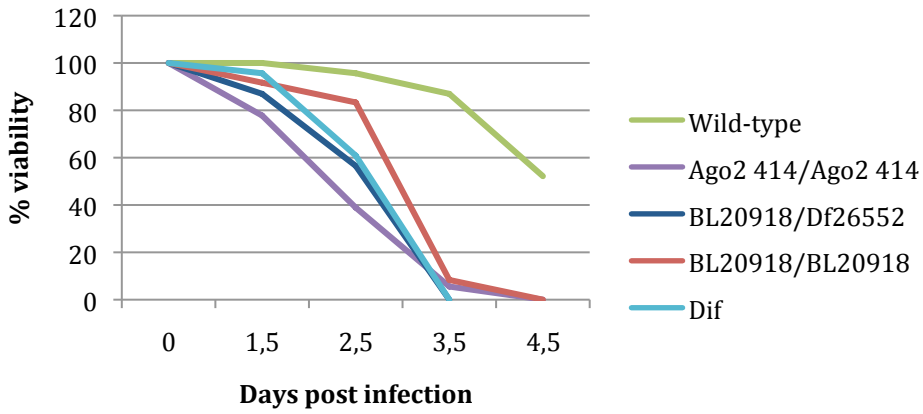


Figure 14. Survival after *B. bassiana* infection. Hemizygous flies (BL20918/Df26552), the strain containing inserted P-element (BL20918), *Ago2* $^{414/414}$ mutant and wild-type flies were challenged with *B. bassiana* and incubated at 29°C. Dif was used as a control.

We then dissected the gut of *BL20918* flies (Fig.16). The gut phenotype observed in the *BL20918* strain is the same as the one of *Ago2* ^{414} mutant flies. Fungal infection induces a réduction of the gut lumen of the intestine wall in *Ago2* ^{414} and *BL20918* mutant flies intestinal lumen.

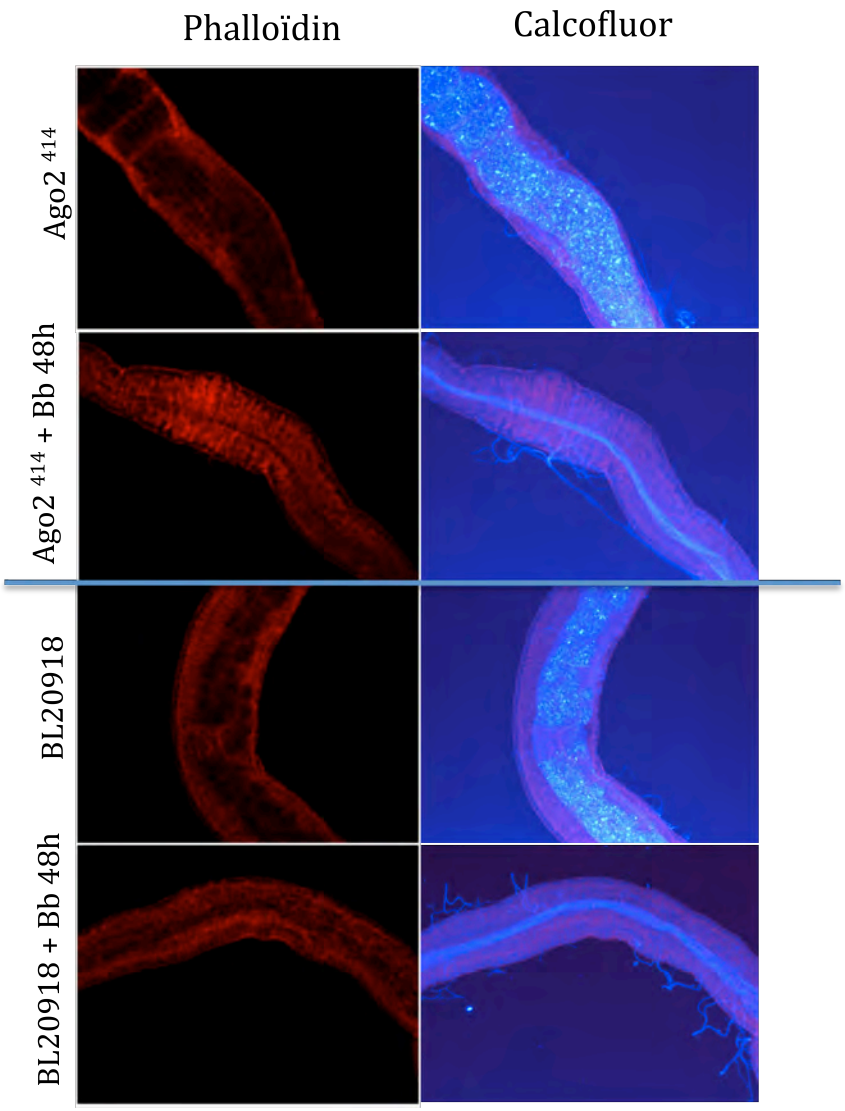


Figure 15. Comparison of the gut structure in *Ago2* ^{414} mutant flies and the BL20918 strain. Epifluorescence microscopy: Phalloïdin-Texas Red labels filaments of actin and the calcofluor labels the peritrophic membrane in the gut of mutant flies, *Ago2* ^{414} and *BL20918* 48h after infection with *B. bassiana* at 29°C compared to the non-pathogenic conditions.

IV. Discussion and perspectives

A. *Beauveria bassiana* infects Drosophila intestinal tract.

The first microorganism described in silkworms (*Bombyx mori*) as a disease agent was the fungus *B. bassiana* (Agostino Bassi, 1835). It is occasionally found in the lungs of wild rodents and nasal passages of horses, giant tortoises and also humans. Among the imperfect fungi, it has one of the largest host lists and occurs in soil as a ubiquitous saprophyte. The fungus also invades the larval alimentary canal of *Helicoverpa zea* to cause starvation and nutrient depletion that may lead to larval death (Cheung and Grula, 1982). In adult *Drosophila* it has never been shown that *B. bassiana* could invade the digestive tract. Here we show that after *B. bassiana* natural infection, spores are found inside the gut of the flies.

B. The absence of Argonaute-2 protein renders flies vulnerable to gut infection.

Our results show that *Dcr-2* ^{A500V}, *Ago2* ^{414} and *Loqs* ^{f00791} mutants are sensitive to *B. bassiana* natural infection. In *Ago2* ^{414} flies, intestines have roughly the same shape as in wild-type flies. However, the gut structure totally changes after infection. Intestinal wall becomes thicker and the intestinal light is much reduced. The mutant is not only sensitive to *B. bassiana* infection, but also to *S. marcescens* natural infection. Our observations, although they need to be confirmed for all mutants in the endo-siRNA pathway, suggest that the endo-siRNA pathway is involved in defense against *B. bassiana* infection, more likely by regulation of epithelial cell renewal in the gut.

In this chapter we aimed to examine the role of endo-siRNA pathway in *Drosphila* response to *B. bassiana*. As mentioned before, an overlap between the miRNA and endo-siRNA pathways exists. Indeed, it has been shown that some miRNAs are loaded into complexes

containing Argonaute-2 protein (Forstemann *et al.*, 2007; Tomari *et al.*, 2007). Moreover, the mutation in the *loquacious* gene, involved in the miRNA pathway, makes flies more susceptible to *B. bassiana* natural infection. That suggests that miRNA might also be responsible for the phenotype observed in *Ago2*^{414} mutant flies. The nature of small RNAs involved in protection of *Drosophila* from B. bassiana in cooperating with Argonaute-2 remains to be established.

C. Abnormal gut structure in Ago2^{414} mutant.

Previous reports have shown that bacteria and stress-inducing agents (*e.g.* oxidative agents like paraquat) can cause pathological changes in adult *Drosophila* midgut (Liehl *et al.*, 2006; Nehme *et al.*, 2007; Biteau *et al.*, 2008; Choi *et al.*, 2008). Epithelial damage has been observed after feeding with the two pathogenic bacteria *P. entom ophila* and *S. marecescens* (Liehl *et al.*, 2006; Nehme *et al.*, 2007). These bacteria can elicit complex reactions in the midgut, in particular increase ISC prolifération, by an unknown mechanism. It has been proposed that bacterial feeding is a cause of the oxidative stress in the gut. To confirm this idea, Chatterjee and Ip show that different oxidizing agents such as paraquat and hydrogen peroxide also have a prominent and similar effect on stem cells in the fly gut (Chatterjee and Ip, 2009).

The Argonaute proteins constitute a highly conserved family whose members have been implicated in RNAi in several organisms. They influence development and are involved in stem cell fate determination. The capacity of stem cells to self-renew is crucial for the development and maintenance of a wide range of tissues, from germ cells to hematopoietic progenitor cells and meristem cells (Kuramochi-Miyagawa *et al.* 2001; Deng and Lin 2002 ; Qiao *et al.* 2002 ; Sharma *et al.* 2001). The best-characterized *Drosophila* Argonaute family

member, Piwi, is required for the self-renewing and asymmetric division of both male and female germline stem cells (Cox *et al.* 1998). There are reasons to believe that the connection between Argonaute family members and stem cell character might also exist in mammals. Human Hiwi is expressed in primitive hematopoietic progenitors (CD34+ bone marrow stem cells) but not in more differentiated hematopoietic lineages (Sharma *et al.* 2001).

Here, we show that there is an increased number of cells expressing Escargot (specific for ISCs and EBs) in *Ago2* ^{414} mutant flies infected with *B. bassiana* in comparison with wild-type flies. In addition, the gut epithelium looks very thick and gives the impression that cells proliferate abnormally. Until now Argonaute proteins were described to play a role in GSCs maintenance. It is the first time that a link between the ISC function and the Argonaute-2 protein is demonstrated.

Cell fate determination after ISC division requires Delta and Notch (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2007). *Drosophila* midgut ISC division is morphologically symmetrical, giving rise to two daughter cells that are initially similar. However, soon after division one cell retains high level of Delta and remains as an ISC, while the other cell quickly loses Delta and becomes an enteroblast (EB) (Ohlstein and Spradling, 2007). EBs function as precursor cells that can no longer divide but can differentiate into either enterocytes (ECs), the absorptive cells, or enteroendocrine cells (EEs), the hormone producing cells. Ninety percent of EBs differentiate into ECs and ten percent into EEs (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2007). The decision to differentiate into the two different cell types depends on the strength of Notch signalling in EB, which is determined by the level of Delta in the original ISC (Ohlstein and Spradling, 2007). Complete loss of Delta/Notch signalling leads to ISC tumours within 1–3 weeks in the gut, indicating that Notch signalling, in addition to promoting daughter cell differentiation, also restricts ISC proliferation (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Ohlstein and

Spradling, 2007). Moreover, inhibition of the Notch processing by the γ-secretase inhibitor DAPT causes ISC and EE tumours in the *Drosophila* intestine as well as formation of large clusters of prospero-positive cells in the midgut (Biteau *et al.*, 2008; Micchelli *et al.*, 2003 ; Ohlstein and Spradling, 2006). This indicates that EEs are overrepresented and Notch acts early in the intestinal cell lineage to limit the production of EEs (Ohlstein and Spradling, 2006).

Our microarray data show that after infection the Notch receptor is only slightly induced in *Ago2* ^{414} mutant flies (130%) in contrast to the wild-type flies (480%). This suggests that in *Ago2* ^{414} mutant flies *B. bassiana* infection is unable to fully activate the Notch signalling pathway. We suppose that ISC differentiation into EBs might be impaired, causing increased proliferation of ISC/EB-like cells and EE accumulation. In order to test this, it would be interesting to label the gut for prospero, which is specific for EEs (Ohlstein and Spradling, 2006).

D. CG10102 is transcriptional factor involved in defense against *B. bassiana* natural infection.

In our microarray data, *CG10102* is expressed at a very high level after *B. bassiana* natural infection. However, in the *Ago2* ^{414} mutant flies, *CG10102* is no longer induced by the infection. The insertion of a P-element in CG10102 results in high sensitivity to *B. bassiana* infection. According to Maines *et al.*, *CG10102* is involved in GSCs differentiation (Maines *et al.*, 2007). It is tempting to speculate that CG10102 is required also for *Drosophila* ISC proliferation and/or differentiation. Perhaps CG10102 is regulated, directly or not, by small RNAs in complex with Argonaute-2. To test this hypothesis, it would be essential to establish whether CG10102 can rescue the *Ago2* ^{414} mutant phenotype.

Conclusion

Nous avons montré que la mutation du récepteur Methuselah augmente la résistance des drosophiles à l'infection par la bactérie à Gram-négatif, *Enterobacter cloacae (E. cloacae)*. Le patron d'expression des peptides antimicrobiens induit suite à l'infection par cette bactérie s'est révélé similaire entre des mouches mutantes (*mth*) et des mouches sauvages. Cependant une différence très importante a été observée concernant l'activation de la MAP3 kinase, Mekk1, impliquée dans la résistance au stress oxydatif. Dans le mutant *mth*, la voie Mekk1 semble être suractivée après infection par des bactéries à Gram-négatif. Nous en déduisons que le phénotype de résistance observé dans le mutant *mth* est probablement lié à la suractivation de la voie Mekk1. Cependant, il faudrait vérifier si la surexpression de Mekk1 augmente la résistance des mouches l'infection bactérienne.

La voie Imd participe à la réponse anti-microbienne spécifiquement dirigée vers les bactéries à Gram-négatif. Il est admis que cette voie est activée par la reconnaissance du peptidoglycane au niveau du récepteur transmembranaire PGRP-LC qui active successivement la protéine Imd puis la MAP3 kinase TAK1, le complexe IKK pour mener à l'activation du facteur NF-κB. Cependant, l'ensemble de nos résultats suggèrent qu'en cas d'infection par des bactéries à Gram négatif, l'activation du facteur NF-κB passe tout d'abord par l'activation de la MAP3 kinase Mekk1 puis par l'activation de la MAP3 kinase TAK-1 entrainant respectivement la transcription des gènes précoce codant pour de la Cécropine et l'Attacine puis des gènes tardifs codant pour la Diptéricine et la Drosocine. Partant de cette observation, nous avons l'intention de vérifier l'hypothèse selon laquelle chez la drosophile, Mekk1 est capable de mener à l'activation de la voie NF-κB.

Nous avons également mis en évidence une nuance importante dans la réponse contre les bactéries à Gram négatif. Nos recherches montrent que les bactéries *E. coli* et *E. cloacae* n'activent pas la réponse humorale de la même façon. Ainsi comme nous l'avons déjà mentionné, l'infection microbienne activerait non seulement la transcription des gènes codant pour des peptides antimicrobiens précoces via l'activation de IKK (Mekk1-dépendante), mais il serait également capable d'activer la voie JNK. Dans la littérature, il est décrit que l'activation de la voie JNK entrainer la translocation nucléaire du facteur AP-1 qui rentre en compétition avec le facteur Relish au niveau des sites promoteur activés par le facteur de NF-κB. Il semblerait que l'infection par *E. cloacae* entraine une activation de la voie PGRP-LC suffisamment important pour contourner la régulation négative de la voie JNK sans avoir

besoin de réprimer son activation. Par contre, concernant la bactérie *E. coli*, il semblerait qu'elle ne soit pas capable d'activer la voie Imd sans avoir au préalable inhibée la voie JNK par l'activation du récepteur Methuselah. Son activation mènerait à l'inhibition de Mekk1 et de la voie JNK pour permettre l'activation de la transcription des gènes codant pour des peptides antimicrobiens tardifs.

Ici nous suggérons que Mekk1 puisse activer le facteur NF-κB en cas d'infection, mais également que le récepteur Methuselah participe à la reconnaissance directe ou indirecte de la bactérie *E. coli.*

Discussion générale

Ces dernières années, la réponse immunitaire de la *Drosophile,* et plus particulièrement l'expression des PAM a fait l'objet de nombreuses études. Ces recherches ont menées à une description détaillée mais encore incomplète des mécanismes moléculaires impliqués dans la reconnaissance et la transduction du signal de la réponse immunitaire chez les insectes. L'ensemble des résultats présentés ici mettent en évidence plusieurs mécanismes de régulation de la réponse immunitaire de *Drosophila melanogaster* face à l'infection par différents microorganismes.

La *Drosophile* est dépourvue de système immunitaire adaptatif, sa réponse immunitaire repose seule sur le système immunitaire innée. Elle partage de multiples réactions de défenses avec les organismes plus évolués. L'épithélium formant le tractus digestif ou encore les trachées représente la première ligne de défense contre les microorganismes mais est également capable de produire des peptides anti-microbiens ainsi que des réactifs oxygénés. A cela s'ajoute la participation des hémocytes au phénomène de phagocytose et d'encapsulation des organismes pathogènes. Et pour finir, le corps gras, analogue au foie chez les mammifères est le principal site de la réponse humorale ou systémique. La production de peptides antimicrobiens est régulée par les voies Toll et Imd qui partagent de nombreux points communs avec les voies des TLR et du TNFα chez les mammifères. La voie Toll est activée par les bactéries à Gram positif et les champignons. Au contraire, la voie Imd est activée en réponse à l'infection par des bactéries à Gram négatif.

Les protéines de mammifères RIP1, FADD, cIAP1/2, TAK1 et la caspase-8 sont impliquées dans des cascades intracellulaires menant à l'activation du facteur NF-kB. Chez la *Drosophile*, les homologues de ces protéines font partie de la voie Imd. L'étude de la voie Imd chez la *Drosophile* est une excellente opportunité afin de mieux comprendre les mécanismes qui participent à l'activation de ces mêmes molécules chez les mammifères. C'est ainsi que nous avons montré que la protéine Imd est rapidement ubiquitinée après activation de la voie Imd par le PGN puis clivée par la caspase-8 DREDD au niveau du motif de reconnaissance ^{27}LEKD/A^{31}. Pour induire *in fine* la phosphorylation et l'activation du facteur Relish et la transcription des gènes cibles.

Les récepteurs couplés aux protéines G sont des protéines à sept hélices transmembranaires capables d'intégrer un signal extra-cellulaire en réponse physiologique. Ces récepteurs sont remarquablement bien conservés au cours de l'évolution. Le génome de la *Drosophile* contient un nombre important de gènes codant pour des récepteurs à protéine G. Lors d'une étude réalisée sur le mutant *methuselah* nous avons mis en évidence une relation entre le récepteur à protéine G Methuselah et l'activation de la voie Imd. Il semblerait que la mutation de récepteur Methuselah ai pour effet d'activer la transcription des peptides anti-microbiens Imd indépendante après infection par la bactérie *Enterobacter cloacae* et soit capable de restaurer le phénotype sauvage d'un mutant *Imd*. Ces résultats permettent d'établir un lien entre l'activation de la voie NFκB connue en temps normal pour être sous l'influence de la protéine Imd et le récepteur Methuselah.

Chez la *Drosophile*, la lumière intestinale est considérée comme un environnement hostile aux microorganismes, due aux propriétés physiques (acide) et physiologiques (péristaltisme) de l'intestin ainsi qu'à la présence de lysozymes (Hultmark, 1996). Deux autres mécanismes participent au contrôle de l'infection bactérienne dans l'intestin de *Drosophile* : la production de ROS et la production de peptides anti-microbiens. Tout comme dans le cas de la réponse

systémique, la production de peptides anti-microbiens est induite par les cellules épithéliales de l'intestin via l'activation de la voie Imd après reconnaissance des bactéries à Gram négatif. La voie Toll ne semble pas être activée dans l'intestin. Elle apparaît être restreinte au corps gras et aux hémocytes. Alors que l'impact des bactéries sur le système immunitaire mucosal est de mieux en mieux compris, l'effet des bactéries sur le l'épithélium intestinal est peu connu. Il a été montré récemment que les bactéries indigènes ou infectieuses modulent l'activité des cellules souches intestinales de la *Drosophile.* L'infection par certaines bactéries entraine un stress oxydatif menant à une augmentation du renouvellement de l'épithélium intestinal. De plus, nous savons que les voie JNK et JAK-STAT participent à la prolifération des cellules souche intestinale (Buchon *et al*., 2009). Après avoir étudier le rôle des petits ARNs sur la réponse immunitaire innée de *Drosophila melanogaster*, nous avons tout d'abord montré que le champignon entomopathogène *Beauveria bassiana* (*B. bassiana*) est capable d'infecter le tractus digestif de la *Drosophile* de manière naturelle. Puis enfin, nous avons mis en évidence la participation de la voie des petits ARNs endogènes dans le maintient de l'intégrité de l'épithélium intestinal dans le cas d'une infection naturelle par *B. bassiana*.

Alors que les mécanismes de reconnaissance et de signalisation de la réponse immunitaire chez la *Drosophile* ont été décrits dans les grandes lignes, aujourd'hui, une étude détaillée des différents phénomènes impliqués dans la régulation de la réponse immunité innée apparaît être une priorité. C'est ainsi qu'au cours de ma thèse j'ai eu l'occasion de m'intéresser à différents aspects de la régulation de la réponse immunitaire chez la *Drosophile*. J'ai tout d'abord analysé les mécanismes moléculaires mis à l'œuvre dans la voie de transduction du signal de la voie Imd, similaire à celle en aval du récepteur du TNFα. Puis j'ai mis en évidence l'implication du récepteur Methuselah dans la régulation de la voie Imd. Et pour

finir, j’ai étudié le rôle de la voie des endo-siARNs dans la lutte contre l’infection par le champignon *B. bassiana.*

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