1 **The antimicrobial peptide Defensin cooperates with Tumour Necrosis** 2 **Factor to drive tumour cell death in *Drosophila***

3

4 Jean-Philippe Parvy^{1*}, Yachuan Yu¹, Anna Dostalova², Shu Kondo³, Alina
5 Kurjan⁵, Philippe Bulet⁴, Bruno Lemaitre², Marcos Vidal¹ and Julia B.
6 Cordero^{1, 5*}

7

8 ¹ CRUK Beatson Institute, Gartcube Estate, Switchback Road, Glasgow, G61
9 1BD, UK

10 ² Global Health Institute, School of Life Sciences, Ecole Polytechnique
11 Federale de Lausanne, Station 19, 1015 Lausanne, Switzerland

12 ³ Invertebrate Genetics Laboratory, Genetic Strains Research Center,
13 National Institute of Genetics, Mishima 411-8540, Japan

14 ⁴ Institute for Advanced Biosciences, CR University Grenoble Alpes, Inserm
15 U1209, CNRS UMR5309, Immunologie Analytique des Pathologies
16 Chroniques, Grenoble, France

17 ⁵ Institute of Cancer Sciences, University of Glasgow, Gartcube Estate,
18 Switchback Road, Glasgow, G61 1QH, U.K

19

20 * Corresponding Authors: j.pary@beatson.gla.ac.uk,
21 Julia.Cordero@glasgow.ac.uk

22

23 **Abstract**

24 Antimicrobial peptides (AMPs) are small cationic molecules best known as
25 mediators of the innate defence against microbial infection. While *in vitro* and

ex vivo evidence suggest AMPs' capacity to kill cancer cells, *in vivo* demonstration of an anti-tumour role of endogenous AMPs is lacking. Using a *Drosophila* model of tumourigenesis, we demonstrate a role for the AMP Defensin in the control of tumour progression. Our results reveal that Tumour Necrosis Factor mediates exposure of phosphatidylserine (PS), which makes tumour cells selectively sensitive to the action of Defensin remotely secreted from tracheal and fat tissues. Defensin binds tumour cells in PS-enriched areas, provoking cell death and tumour regression. Altogether, our results provide the first *in vivo* demonstration for a role of an endogenous AMP as an anti-cancer agent, as well as a mechanism that explains tumour cell sensitivity to the action of AMPs.

Introduction

Vast amount of evidence demonstrates the key role of systemic immunity in tumour progression and patient outcome. Efforts to manipulate the immune response to tumours are at the core of basic and translational cancer research. In mammals and flies, tumourigenesis triggers inflammation and activation of the immune system, leading to tumour cell death (Cordero et al., 2010; Parameswaran and Patial, 2010; Parisi et al., 2014; Teng et al., 2008). Tumour Necrosis Factor (TNF) is an important player in these tumour/immune interactions and has pleiotropic effects on tumours, including induction of cell death (Ham et al., 2016; Parameswaran and Patial, 2010). This function is conserved in *Drosophila*, where the single TNF homolog Eiger (Egr), produced by tumour-associated macrophages (TAMs), has been shown

to drive cell death of neoplastic tumours, generated in larval imaginal discs by the loss of apico-basal complex components such as Disc large 1, Scribble or Lethal giant larvae (Cordero et al., 2010; Parisi et al., 2014; Parvy et al., 2018). Moreover, using *disc large 1 Drosophila* mutant larvae (*dlg*^{40.2} hereafter referred to as *dlg*), we recently reported that tumour-derived Egr, acts on haemocytes to activate Toll pathway in the *Drosophila* fat body, which was necessary to mediate TNF-dependent tumour cell death (Parisi et al., 2014). However, the mechanisms by which Toll signalling activation in immune tissues executes tumour cell death remain unknown (Figure 1A).

In *Drosophila* as in mammals, Toll pathway is well known to play a central role in the innate immune response to infection (Lemaitre et al., 1996). Downstream effectors of the Toll pathway include antimicrobial peptides (AMPs), which possess microbicidal activities against various pathogens. They display potent antimicrobial activity *in vitro* by disrupting negatively-charged microbial membranes. While intracellular activities have been reported, many AMPs kill pathogens by inserting into the lipid bilayer and disrupting the membrane integrity (Brogden, 2005). Host cells are instead protected from AMP as they are positively charged and contain cholesterol (Brender et al., 2012). *In vitro* studies have revealed AMPs capacity to kill cancer cells (Deslouches and Di, 2017). However, whether this cancer-killing activity is a natural function of AMPs is unknown, as there are no reports on an *in vivo* paradigm addressing such question. Since the Toll pathway is activated in *dlg* mutant tumour bearing larvae and is required for optimal TNF-induced tumour cell death (Parisi et al., 2014), we hypothesised that AMPs may be involved in this process.

Here we show that *Drosophila defensin* is induced in the fat body and tracheal system of *dlg* mutant tumour bearing larvae. We find Defensin consistently associated to dying tumour cells. Critically, systemic and tissue specific knockdown of Defensin demonstrates a non-redundant role of the AMP in controlling tumour growth through the induction of tumour cell death. Anti-tumoural Defensin production relies on TNF-dependent activation of both Toll and Imd pathway. Our results demonstrate that *dlg* mutant tumours expose PS in response to haemocyte-derived TNF and that Defensin is present in PS enriched area on the tumour surface. Finally, we find that lack of TNF prevents PS exposure in tumours and makes them insensitive to the action of Defensin. Collectively, our results reveal an anti-tumoural role for Defensin *in vivo* and provide insights into the molecular mechanisms, which make tumours sensitive to the killing action of an endogenous AMP.

Results

dlg tumour bearing larvae express the AMP Defensin

In order to assess expression of several AMPs we performed RT-qPCR analysis on fat bodies dissected from wild type controls (*w¹¹¹⁸*) or *dlg* mutant larvae (Figure 1B). Results showed consistent and statistically significant upregulation of *defensin* in fat bodies of *dlg* larvae compared to wild-type ones (Figure 1B). Other Toll-dependent AMPs display a trend to be increased (*drosomycin* and *attacin A*), even though data was highly variable amongst biological replicates, while other AMPs were not transcriptionally regulated (*drosocyn*, *cecropin A1*) (Figure 1B). Interestingly, human β -Defensin 1 displays anticancer activity *in vitro* (Bullard et al., 2008; Sun et al.,

2006), and deletion of human *def* appears prevalent in some cancer types (Ye et al., 2018). This prompted us to explore the role of *Drosophila* Defensin in *dlg* mutant tumours. Using *dlg* larvae reared on antibiotics, we confirmed that *defensin* upregulation was independent of the presence of microbes (Figure 1C). Moreover, larvae bearing *dlg* imaginal discs tumours induced by RNAi (*en>UAS-dcr2; UAS-dlg-IR*) also displayed increased *defensin* expression, confirming AMP gene induction as a consequence of *dlg*-driven epithelial transformation rather than whole body gene loss (Figure 1D). We conclude that Defensin, an AMP known for its activity against microbes, is induced, in a sterile environment, by the presence of tumours.

Defensin restrains *dlg* tumour growth and promotes tumour cell death.

We next hypothesized that Defensin may be an important mediator of anti-tumour immunity *in vivo*. To test this hypothesis, we generated null mutant alleles for the *defensin* (*def*) gene using the CRISPR/Cas9 system (*def^{sk3}* and *def^{sk4}*) (Figure 1E) (Hanson et al., 2018). Survival analysis of *def^{sk3}* flies confirmed that this AMP contribute to resist systemic infection to certain Gram-positive bacteria (Figure supplement 1) (Levashina et al., 1995). To evaluate the effect of Defensin on tumour development, we combined *def* and *dlg* loss of function alleles. Compared to *dlg* mutant animals, *dlg;def* double mutants displayed a significant increase in tumour size (Figure 2A). Tumour growth is limited by apoptotic tumour cell death as revealed by Dcp1 staining (Parisi et al., 2014). Interestingly, tumours from *dlg;def* double mutants display a very strong decrease in apoptosis (Figure 2B-E'), suggesting that increased tumour size in absence of Defensin is due to a decrease in tumour cell death.

This was further supported by the similar proliferation rates measured in *dlg* and *dlg,def* mutant tumours as per quantification of anti-phosphoHistone H3 staining (Figure supplement 2A-E'). Importantly, the effect of Def on tumour size and cell death were still observed when *dlg* and *dlg,def* larvae were reared in sterile conditions (Figure supplement 2F-J') and further confirmed upon ubiquitous knock down of *def* using RNA interference (IR) (Figure 2F-J'). Furthermore, fat body overexpression of *def* significantly rescued tumour volume and tumour cell death of *dlg;def^{sk3}* double mutant animals (Figure 2K and L). Additionally, larval injection of a synthetic Defensin peptide increased tumour cell death of *dlg* or *dlg;def^{sk3}* imaginal discs (Figure 2M), while it had no effect on tissues from wild-type larvae, indicating that Defensin can selectively promote cell death of tumour cells. Altogether, these results demonstrate that Defensin is required to control *dlg*-dependent tumorigenesis *in vivo* through induction of tumour cell death.

Defensin remotely produced from immune tissues bind to tumour cells

Having shown that Defensin restrict tumour growth, we sought to determine the tissues that produced endogenous Defensin in the context of tumour bearing. Previous studies have shown that Defensin is not produced by imaginal discs or tumours (Bunker et al., 2015; Kulshammer et al., 2015). In the context of infection, Defensin can be produced by the fat body as well the tracheal and gut epithelium (Tzou et al., 2000). We monitored *defensin* expression by RT-qPCR in various immune tissues of tumour bearing animals. We observed that the fat body, homologue to the mammalian liver and adipose tissue, and the trachea, a network of tubes transporting oxygen

to cells that resembles the mammalian vasculature, were the main sources of *defensin* in these animals (Figure 3A). This was also supported by Defensin immunostaining, (Figure 3B and C). Transcript assessment upon targeted IR knock down of *defensin* in the respective tissues further confirmed the domains of endogenous gene expression (Figure 3D and I). Importantly, knocking down *defensin* expression specifically in the fat body or the trachea of *dlg* animals, resulted in increased tumour size and decreased tumour cell death (Figure 3E-H' and J-M') confirming the non-redundant functional requirement of *defensin* in both tissues to efficiently promote tumour cell death.

To investigate the possibility that Defensin produced by the fat body and the trachea can specifically target tumour cells, we made use of an inducible haemagglutinin (HA) tagged form of Defensin (*UAS-def-HA*). We noticed leaky HA protein expression in the tracheal system (Figure 4A) but not in the fat body (Figure 4B) of animals carrying the *UAS-def-HA* construct only. Therefore, we overexpressed *UAS-def-HA* in the fat body using a Gal4 specific driver (*lpp>def-HA*), which resulted in significant upregulation of Defensin in the fat body (Figure 4D) when compared to control conditions (Figure 4B), but also maintained the leaky expression of the transgene in the trachea (Figure 4C). Strikingly, overexpression of *UAS-def-HA* in the fat body resulted in Defensin-HA immunostaining in transformed imaginal discs from *dlg* mutant animals (Figure 4E and E') but not in normal tissues from *dlg* heterozygous animals (Figure 4F and F').

Consistently, using anti-Defensin antibody, we observed endogenous Defensin staining on *dlg* mutant tumour (Figure 4G-H'') but not in the wild-type

discs (Figure 4I). Interestingly, we predominantly observed Defensin bound to tumour areas enriched with apoptotic cells (Figure 4H-H’). High-resolution imaging showed Defensin enrichment at the membrane of these dying cells (Figure 4J-J’).

Altogether, these results show that Defensin produced by immune tissues selectively binds tumour cells to target them for apoptosis.

Toll and Imd pathway contribute to Defensin expression in *dlg* mutant larvae

defensin expression upon systemic infection relies on both Toll and Imd pathways (Lemaitre et al., 1996; Leulier et al., 2000). While we previously showed that Toll pathway activation in *dlg* mutant larvae is required to achieve maximal tumour cell death (Parisi et al., 2014), the involvement of the Imd pathway in tumour bearing animals was still elusive. To assess the contribution of the Imd pathway to both *defensin* expression and tumour burden, we analysed these two phenotypes in larvae deficient for the Imd-pathway. We observed a 55-60% decrease in *defensin* expression in *dlg* mutants carrying a loss of function allele affecting *imd* (*dlg;imd¹*) (Figure 5A) or the gene encoding the downstream transcription factor Relish (*dlg;rel^{E20}*) (Figure 5F). Consistently, analysis of tumour phenotypes revealed increased tumour volume and decreased tumour cell death in *dlg;imd¹* and *dlg;rel^{E20}* animals when compared with *dlg* counterparts (Figure 5B-E’ and G-J’). Altogether, these data demonstrate that the Imd pathway is required for *defensin* upregulation, impairment of tumour growth and induction of tumour cell death in *dlg* mutant larvae.

Upon infection, AMP expression in the trachea exclusively relies on the Imd pathway (Tzou et al., 2000). We therefore looked at *defensin* expression and tumour phenotype in *dlg* mutant larvae where *imd* expression had been knocked down specifically within tracheal cells (*dlg;btI>imd-IR*). In this setting, *defensin* expression was significantly reduced in the whole larvae (Figure 6A). Consistently, tumour volume was increased while tumour cell death was decreased (Figure 6B-E') showing the requirement of Imd pathway in the tracheal system to control tumour burden.

In the fat body, both Imd and Toll pathways contribute to AMPs expression during infection (Tzou et al., 2002). To evaluate the contribution of Toll and Imd pathways in fat body in tumour bearing larvae, we monitored *defensin* expression and tumour phenotype in *dlg* animals in which Toll or Imd pathways have been selectively knocked down in the fat body. Knocking down *myd88*, which encodes a common adaptor to Toll receptors (Imler and Zheng, 2004), or *imd* in the fat body resulted in a significant decrease in *defensin* expression (Figure 6F) and, consistently, increased tumour volume and decreased tumour cell death (Figure 6G-K'). Moreover, concomitant overexpression of *defensin* and *Myd88-IR* or *Imd-IR* in the fat body of *dlg* larvae was sufficient to significantly rescue the tumour volume and tumour cell death phenotypes resulting from fat body knockdown of *Myd88* or *Imd* in *dlg* animals (Figure 6L-O).

We conclude that, in *dlg* mutant animals, Toll and Imd pathways have non-redundant roles in restricting tumour growth and promoting tumour cell death through the control of *defensin* expression. Importantly, forced Defensin

expression in an otherwise immune-compromised animal is sufficient to reduce tumour growth and to promote tumour cell death.

Defensin is enriched in tumour areas exposing phosphatidylserine

AMPs targeting of pathogens, involves the recognition of negatively charged molecules exposed on the cell surface (Yeaman and Yount, 2003). A key question raised by our study is how Defensin can selectively bind and kill tumour cells (Figure 4 and Figure 2M). Selective action of cationic AMP is attributed to their ability to interact with negative charged membrane such as those found in bacteria. We hypothesized that the membrane of tumour cells from *dlg* mutant larvae might change their electrostatic properties, making them sensitive to the action of Defensin. Phosphatidylserine (PS) is a negatively charged phospholipid, normally restricted to the inner leaflet of the cell membrane. However, PS can be exposed on the outer leaflet for example in apoptotic cells, which tags these cells for phagocytosis (Birge et al., 2016; Shklyar et al., 2013; Tung et al., 2013). Moreover, PS exposure has been shown to occur independently of apoptosis in many cancer cell types (Riedl et al., 2011). Therefore, we investigated whether PS externalisation could be a factor allowing specific targeting of tumour cells by Defensin. Our data revealed that *dlg* but not wild-type tissues, displayed high levels of Annexin V staining (Figure 7A and B), indicating increased exposure of PS by *dlg* tumours. We next looked at the ability of Defensin to specifically associate with tumour cells exposing PS and found that Defensin localized in PS-enriched areas on *dlg* tumours (Figure 7A-A''). Thus, Defensin produced by

immune responsive tissues binds specifically to tumour cells and this ability correlated with the exposure of PS.

The TNF homolog Eiger is required for PS exposure and Defensin anti-tumoural activity.

Previous studies have shown that circulating macrophage-like cells in *Drosophila*, called haemocytes, bind to *dlg* mutant tumours and contribute to cell death (Parisi et al., 2014). This process is mediated by the release of Eiger from haemocytes (Cordero et al., 2010; Parisi et al., 2014), which then activates the JNK pathway in target cells to promote apoptosis (Igaki et al., 2009). Moreover, Toll pathway-activation in tumour bearing animals also requires Eiger (Parisi et al., 2014). Accordingly, we found that *defensin* upregulation observed in *dlg* animals was lost in *dlg;egr³* double mutants (Figure 7C). We next tested whether PS exposure in tumours was dependent on Eiger, and observed an almost complete loss of cell-surface PS in *dlg;egr³* tumours (Figure 7A, D and E). Therefore, Eiger is required for PS exposure in these tumours. We then analysed PS exposure in *dlg* mutant tumours, upon specific *eiger* knockdown in haemocytes (*dlg;hml>egr-IR*), and observed a strong decrease in cell surface-exposed PS compared to control tumours (*dlg;hml>ctrl-IR*) (Figure 7F-H). This indicates that PS exposure in tumour cells is likely triggered by the release of Eiger from haemocytes.

Importantly, Egr-dependent signalling and PS exposure were intact in *dlg;def* and *dlg;imd* mutants (Figure 7I-L and Figure supplement 3). This indicates that Eiger signalling and PS exposure precedes the action of Defensin in *dlg* tumours. To further assess whether Eiger was required for Defensin-induced

tumour cell death, we injected synthetic Defensin peptide into control, *dlg* or *dlg;egr³* mutant larvae. As expected, Defensin injection was able to robustly promote tumour cell death in *dlg* mutant tumours (Figure 7M). However, Defensin was unable to affect tumours derived from Eiger -deficient animals, demonstrating the requirement of Eiger for tumour cell death induced by Defensin (Figure 7M).

Discussion

While the role of antimicrobial peptides in innate immune defense has been well-recognized for decades (Bahar and Ren, 2013), recent reports revealed potential additional physiological functions of AMPs, including aging and neurodegeneration (Cao et al., 2014; Kounatidis et al., 2017; Lezi et al., 2018), wound-healing (Chung et al., 2017; Tokumaru et al., 2005), resistance to oxidative stress (Mergaert et al., 2017; Zhao et al., 2011), immune signaling (Tjabringa et al., 2003; van Wetering et al., 2002) and anti-cancer activity (Deslouches and Di, 2017). However, due to the absence of AMP mutants, most of these studies have relied on the use of exogenous sources of AMPs or genetic modification of upstream regulators of AMP expression. Recently, the use of loss of function alleles of *Drosophila* Diptericin (DptB), allowed to establish a role for the AMP in long-term memory (Barajas-Azpeleta et al., 2018). Pioneer work reporting systematic deletion of multiple AMPs in *Drosophila*, opens the door for in-depth analysis of the endogenous functions of these molecules (Hanson et al., 2018). Using such tools and a genetically defined *in vivo* tumour model, our study demonstrates a role for Defensin in the control of tumours and deciphers the molecular mechanism allowing

specific tumour cell targeting by the AMP. Collectively, our results suggest a model of tumour control in two steps: Firstly, the cellular immune response to tumours involves the binding of haemocytes to tumour cells and activation of TNF pathway leading to PS exposure (Figure 7N). This is followed by a humoral response achieved by the action of antimicrobial peptides remotely produced by the fat body and the trachea (Figure 7N). Thus, the two branches of *Drosophila* innate immunity contribute to tumour elimination.

A role for a *Drosophila* AMP in tumour control

Using mutants as well as ubiquitous or targeted knockdowns, our study reveals that Defensin is non-redundantly required to drive tumour cell death and restrict tumour growth in neoplastic tumours generated by loss the apico-basal determinant Dlg. This was reinforced by detection of Defensin on dying tumour cells as well as genetic rescue and injection experiments showing that Defensin can actively drive tumour cell death. Several *in vitro* studies in mammals have pointed to AMPs anti-tumoural potential (Deslouches and Di, 2017). Amongst these suggested anticancer peptides, Human β -Defensin 1 (hBD1) appears downregulated in 82% of prostate cancer and 90% of renal clear cell carcinomas (Donald et al., 2003). Furthermore, expression of hBD1 induces cell death in prostate and renal cancer cells *in vitro* (Bullard et al., 2008; Sun et al., 2006). Moreover, a recent report shows prevalent deletion of Human *defensin* gene cluster in some tumour types including prostate, lung and colorectal cancers, as well as a decrease overall survival in patients carrying these deletions (Ye et al., 2018). However, since the classification of a molecule as AMP is based on structural protein features, it is important to

mention that Human Defensins are not homologous of, but rather have structural resemblances, to *Drosophila* AMPs due to convergent evolution (Shafee et al., 2017). Further studies are required to determine if anticancer activity displayed by Defensins from different species is linked to their structural properties.

As upon infection (Tzou et al., 2000), we show that the fat body and the trachea are the main sources of Defensin in tumours bearing larvae. Moreover, our results indicated that Defensin's maximal expression, and anti-tumour properties rely on Imd and Toll pathways activation. As expected from previous studies on anti-pathogenic immunity, Imd appears to play a critical role in the tracheal system, while both Imd and Toll drive Defensin expression in the fat body (Hoffmann and Reichhart, 2002; Tzou et al., 2002). Consistently, overexpressing Defensin partially rescued the effect of Imd or Toll knockdown on *dlg* tumours. However, Toll and Imd pathways are major regulators of multiple AMPs in the *Drosophila* fat body. Then, it is conceivable that AMPs other than Defensin may possess similar anti-tumoural activity. In fact, a recent study in *Drosophila* shows that ectopic expression of several antimicrobial peptides, including the Defensin, can increase cell death in a haematopoietic tumour model (Araki et al., 2018). Authors of that study also reported activation of Toll and Imd pathways together with increased expression of several AMPs in those tumour bearing animals. Together with our study, this suggests potentially general anti-tumoural properties of *Drosophila* AMPs.

Defensin specifically targets tumour cells

Importantly, we show that Defensin targets tumour cells for apoptosis while sparing normal cells. As their human counterparts, we show that *Drosophila* tumours can expose PS independently of cell death (Riedl et al., 2011). Interestingly, an *in vitro* study showed selective anticancer activity of some synthetic peptides derived from beetle Defensin, against cancer cells exposing PS (Iwasaki et al., 2009). A similar targeting mechanism has also been proposed to explain temporin-1CEa or L-K6 anticancer activities towards melanoma cells and breast cancer cells respectively (Wang et al., 2016; Wang et al., 2017). PS is a mark of apoptotic cells, which is used as an “eat me” signal by phagocytes (Shklyar et al., 2013; Tung et al., 2013). In *Drosophila*, the phagocytic receptor Simu together with Draper contribute to the elimination of apoptotic to the recognition of PS (Shklyar et al., 2013; Tung et al., 2013). It would be interesting to test the role of these receptors in the control of *dIlg* tumour progression.

Our results indicate that PS exposure precedes Defensin action and is not just an “eat-me” signal but likely contributes to changing the membrane of tumour cells making them sensitive to the action of AMPs. It is tempting to speculate that deleterious effect of AMPs observed upon aging or neurodegeneration may involve a similar targeting mechanism of ‘foreign-looking’ or unfit cells (Cao et al., 2013; Kounatidis et al., 2017; Lezi et al., 2018).

Noteworthy, other negatively charged molecules enriched on tumour surface such as heparan sulfates and O-glycosylated mucins may also contribute to the targeting by AMPs (Hollingsworth and Swanson, 2004; Knelson et al., 2014).

Defensin action requires TNF

TNF is a major player in the tumour microenvironment by exerting pleiotropic effects on both the tumour and its surroundings (Ham et al., 2016; Parameswaran and Patial, 2010). In our *dlg* mutant model, we observed that defensin induction requires TNF, supporting previous observations that induction of innate immune response in tumour bearing animal relied on Eiger produced by the tumour (Parisi et al., 2014). Indeed, Toll activation in the fat body has been proposed to be a consequence of tumour-derived TNF, which drives haemocyte proliferation leading to an increase in Toll ligand Spatzle (Parisi et al., 2014). Whether Imd pathway is also indirectly activated by the changes in immune cell activity in response to tumour or by alternative mechanisms remains an open question.

Our results show that haemocyte-derived TNF is required for PS exposure by the tumour, a key process for tumour targeting by Defensin. Consistently, haemocyte-derived TNF is shown to drive cell death in *dlg* mutant tumours (Parisi et al., 2014). The precise molecular mechanisms driving PS exposure downstream of TNF remain unknown. However, haemocyte-derived TNF, activates JNK pathway, which triggers many changes in tumour cells including apoptosis (Igaki et al., 2002; Moreno et al., 2002), ROS production (Fogarty et al., 2016; Santabarbara-Ruiz et al., 2015), loss of cell polarity (Zhu et al., 2010), modification of extracellular matrix (Uhlirova and Bohmann, 2006), proliferation and cell migration (Beaucher et al., 2007; Igaki et al., 2006; Pastor-Pareja et al., 2004; Srivastava et al., 2007). Thus, TNF might also sensitize cells through activation of the JNK

pathway (Cordero et al., 2010) providing, in addition to PS exposure, a secondary sensitization mechanism of tumour cells to the action of the AMP. Further studies are needed to explore a potential link between JNK activation and PS exposure since pleiotropic effects of JNK may drive PS exposure downstream of TNF.

Another consequence of TNF-dependent JNK activation in tumours is the increased expression of matrix metalloproteases (Mmps) by tumour cells (Uhlirva and Bohmann, 2006). Importantly, we show that Mmp-1 induction and then JNK pathway activation are still present in tumour from larvae devoided of Defensin. This demonstrates that TNF signalling acts upstream of Defensin. Mmps degrade the basal membrane facilitating metastasis of primary tumour cells (Beaucher et al., 2007; Pastor-Pareja et al., 2004; Srivastava et al., 2007). Interestingly, a study reported that human Mmp-7 can cleave immature Defensins, promoting their activation (Wilson et al., 1999; Wilson et al., 2009). Indeed, the pro-domain present in AMPs is thought to keep their *in vivo* activity silent, allowing local activation of AMP upon cleavage. While the mechanisms of *Drosophila* Defensin pro-domain cleavage remain unknown, it would be interesting to explore whether the changes in the tumour microenvironment could also affect Defensin activation.

In conclusion, our study provides the first direct *in vivo* demonstration of the role of an endogenous AMP as an anti-cancer agent in *Drosophila*. Our data points to a conserved mechanism of tumour control by AMPs, a potent arm of the innate immune system. Importantly, we identify cellular features within

tumours, which may be predictive of their sensitivity to be targeted by AMPs. This study provides a new paradigm to decipher the molecular mechanisms influencing anti-tumoural functions of an AMP, which may extend to other non-canonical roles of AMPs, such as in ageing, long-term memory and wound healing. Moreover, together with the new genetic tools allowing targeting of all *Drosophila* AMPs (Hanson et al., 2018), our study establishes new bases to explore *in vivo* a potential important natural mechanism of defence against tumours.

Material and Methods

Drosophila housekeeping and rearing

Flies were reared at 25°C under 12h/12h light/dark cycles on standard oatmeal and molasses medium (Fernandez-Ayala et al., 2009). Details on fly strains used in this study are presented in Table supplement 1 and detailed genotypes are included in corresponding figure legends.

For assessment of tumour volume and tumour cell death, flies were transferred to medium embryo collection cages and allowed to lay eggs for 8h at room temperature (RT) on agar/grape juice plates (2.1% agar, 25% grape juice, 1.25% sucrose, 0.2% methyl 4-hydroxybenzoate) supplemented with yeast paste. Plates were kept at 25°C until hatching. 200 larvae hatched within a 4h time window were collected and transferred to rearing bottles containing 45mL of fly food to avoid any developmental delay and/or starvation effects due to overcrowding conditions. The tumour phenotype was analysed close to the normal developmental time, i.e. 7 days after hatching. Tumours were dissected and stained for further analysis (see below).

449

450 **Generation of *defensin* mutants**

451 *def^{SK3}* and *def^{SK4}* mutants were generated using CRISPR/cas9
452 technology (Hanson et al., 2018). Mutants were isogenized through
453 backcrosses with a *w¹¹¹⁸* *iso* line as previously described in Ferreira-Teixeira
454 et al, 2014.

455

456 **Survival assays**

457 Survival of *def^{SK3}* animals was compared to wild type controls
458 (isogenised background, *iso*). Male flies (5-7 days old) were pricked in the
459 thorax with a needle dipped in a concentrated pellet of fresh overnight
460 bacterial culture. Infected flies were kept at 29°C. *Listeria innocua* was
461 cultured in Brain heart infusion medium at 37°C and used at optical density of
462 200nm (OD₆₀₀). *Erwinia carotovora carotovora* 15 was cultured in Luria-
463 Bertani broth at 29°C and used at OD₆₀₀ 200. Experiments were repeated
464 three times independently and one representative experiment is shown.

465

466

467 **Immunohistochemistry**

468 Tissues were dissected in Phosphate Buffer Saline (PBS) and fixed for
469 20min in 4% formaldehyde. Fat body samples were fixed for 30min using the
470 same protocol. Tissues were washed three times in PBS containing 1% of
471 Triton-X100 (PBT) and incubated overnight at 4°C with primary antibodies.
472 Tissues were washed 5 times in PBT and incubated for 2h at RT with
473 secondary antibodies and 4',6-Diamidine-2'-phenylindole dihydrochloride

(DAPI, Sigma) (1µg/mL). After three washes in PBT, tissues were mounted in Vectashield (Vector Laboratories, Inc.) using Secured-Seal spacers (Thermo Fisher Scientific).

The following primary antibodies and corresponding dilutions were used: rabbit anti-Dcp1 (1/100), rabbit anti-phosphoHistone H3 (ser10 and ser28 mixed, 1/100) and mouse anti-HA (1/1000) (Cell Signalling Technology); mouse anti-Mmp1 (1/10) (Developmental Studies Hybridoma Bank, 3B8D12); chicken anti-GFP (1/4000) (Abcam) and guinea pig anti-Defensin (1/100) (Kind gift from Dahua Chen, (Ji et al., 2014)). Secondary goat antibodies were Alexa Fluor® 488nm or 594nm (Invitrogen) conjugated and were used at 1/500 dilution.

Confocal images were captured using a Zeiss 710 or Zeiss 880 with Airyscan confocal microscope and processed with Fiji 2.0.0 or Adobe Photoshop C.S5.1.

Quantification of tumour volume and tumour cell death

For analysing tumour phenotype, images were acquired using optimal slice parameter and 12bits using a Zeiss 710 confocal microscope. Quantifications were made as previously described (Parisi et al., 2014). Briefly, we used Volocity 3D imaging analysis software (Perkin Elmer) to quantify the total volume of tumour identified by DAPI staining and cell death visualised with anti-Dcp1 staining. When haltere or leg discs tumours were still associated with the wing disc, they were not considered for quantification. Quantifications were done in at least three independent biological replicates for each genetic setting. Single representative experiments are presented

except for *dlg;def* double mutant rescue experiments (Figure 1L-M) for which replicates are pooled due to the few larvae available. In all cases the difference between control and mutant tumour were strongly reproducible between replicates.

Sterile rearing conditions

To rule out any effect of infection on the tumour phenotypes, *Drosophila* larvae were reared on standard food supplemented with penicillin and streptomycin (100 IU/ml and 100 mg/ml, respectively) (Figure 1A, Figure supplement 3A-E' and Figure supplement 4G).

qRT-PCR analysis

Total RNA from 7 to 10 whole larvae or tissues collected from 10 to 20 larvae (10 larvae for fat body, 20 larvae for trachea) was extracted using TRIzol (Thermo Fisher Scientific), according to the manufacturer's instructions. RNA was treated with Turbo DNA free Kit (Life Technologies LTD) and RNA concentration and quality were monitored using a Nanodrop (Thermo Fisher Scientific). The same amount of total RNA (1µg for whole larvae and fat body or 100ng for trachea) was used to perform the three independent reverse-transcriptions using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNAs were pooled and qPCR was performed using PerfeCTa SYBR green (Quanta Bio), following the manufacturer's instructions. cDNA amplification was monitored with Applied Biosystems 7500 fast instruments. Serial 10-fold dilutions of an external standard were used to produce a standard curve, and RNA samples were

included to control for the absence of DNA contamination. *rp/32* expression was used to normalize expression levels of target genes. Data was analysed using the Ct method ($2^{-\Delta\Delta Ct}$). All qPCR experiments were carried out on three to seven independent biological replicates (see figure legends for details) and, larvae were sampled from the same bottle used to analyse the tumour phenotype. GraphPad Prism7 software was used for graphical representation and statistical analysis. Primer targets and sequences are presented in Table supplement 2.

PCR amplification of genomic DNA

To verify the sequence of *def^{sk3}* and *def^{sk4}* mutants, genomic DNA was extracted from single flies by grinding whole animals with a 200µL pipette tip containing 50µL of squishing buffer (10mM Tris-HCl pH 8.2, 1mM EDTA, 25mM NaCl, 200µg proteinase K) followed by incubation at 37°C for 30min. Proteinase K was heat-inactivated at 95°C for 2min. 1µL of genomic DNA was used for PCR amplification using PfuUltra II Fusion DNA Polymerase (Agilent) (for primers see Table supplement 2) and Eppendorf Mastercycler Ep Gradient Thermal Cycler.

Annexin V staining and quantification

Tumours were dissected in PBS and incubated in Annexin V binding buffer (Fisher Scientific) containing 5% of Alexa Fluor (488nm or 568nm) conjugated Annexin V (Life Technologies LTD) for 10min. Tissues were washed quickly in Annexin V binding buffer and fixed in 4% formaldehyde for

20min. Immunostaining, mounting and imaging were then carried out as described above.

We used Fiji 2.0.0 software to quantify Annexin V staining at the tumour surface. Maximal Z-projection of whole tumours were generated and the Colour Threshold tool was then used to detect the red pixels that represented Annexin V staining at the surface of tumours. The hue slider was set to include only the red signal, and the brightness slider was adjusted to exclude any background signal. This area was then selected and measured in pixels.

Synthetic Defensin production and injections

The mature Defensin peptide (ATCDLLSKWNWNHTACAGHCAKGFKGGYCNDKAVCVCRN) was synthesised (over 90% purity) and the purity controlled by Genepep using Ultra Performance Liquid Chromatography/ Mass Spectrometry. We subsequently compared the integrity of synthetic Defensin with authentic Defensin purified from *Drosophila* hemolymph using two complementary approaches, namely Matrix-Assisted Laser Desorption/Ionization-Mass Spectrometry (MALDI-MS) for molecular mass determination and trypsin digestion followed by nanoLiquid Chromatography/Electrospray Ionization tandem Mass Spectroscopy (nanoLC/ESI MS/MS) for molecular integrity confirmation. Those analyses confirmed the similar mass between natural and synthetic Defensin (respectively provided by Bulet EIRL and Genepep) as well the presence of disulphide bonds within synthetic Def, demonstrating it is the mature peptide (Data not shown).

For injection experiments, larvae were collected, washed three times in cold sterile PBS and injected on a fly pad (Flystuff-SLS UK) upon CO₂ anaesthesia using Nanoject II and glass capillaries 3.5 inch (Drummond). Larvae were injected with 69nL of PBS containing 7.5nmol of synthetic Defensin or 69nL of PBS only as control. Larvae were then gently transferred into agar/grape juice plates and kept at 25°C. Tumours were dissected 4h after injection and stained for visualization of nuclei and tumour cell death as describe above.

Statistical analyses

All data are presented as mean $\pm$ SD and n values are indicated in the figure legends. Statistical analyses were carried out using GraphPad Prism7 software and only significant differences are indicated in dot-plots. Survival upon infection was analysed using Log-rank test. qPCR data were analysed using unpaired t-test with a two-tailed p value. Tumour volume and tumour cell death comparing 2 samples were analysed using Mann-Whitney test with a two-tailed p value and the one comparing 3 genotypes were analysed using One-way ANOVA followed by Turkey's multiple comparisons test. Tumour volume from injected larvae was analysed using Two-way ANOVA followed by Turkey's multiple comparisons test and only significant statistical differences between ctrl and Defensin injected larvae for each genotype tested are indicated.

Acknowledgments: We would like to thank Dr. Dahua Chen for the anti-Defensin antibody, the Vienna *Drosophila* Resource Centre and the

Bloomington *Drosophila* Stock Centre for fly stocks, and Dr. Sébastien Voisin for his critical contribution in mass spectrometry analyses. JPP and YY are supported by Cancer Research UK core funding through the CRUK Beatson Institute (A17196). JBC is a Sir Henry Dale Fellow jointly funded by the Wellcome Trust and the Royal Society (grant number 104103/Z/14/Z). *In memory of Marcos Vidal (1974-2016).*

Author contributions: MV initiated this project. JPP performed most of the experiments in this study; YY and AK provided technical assistance; AD assayed fly survival upon infection; SK and BL generated the *defensin* mutant flies; PB analysed the quality of synthetic Def; JPP and JBC designed experiments and analysed the data; BL provided important insights into AMPs and *Drosophila* immunity; JPP, PB, BL and JBC wrote the paper; JBC directed the study.

Competing interests: Authors declare no competing interests.

Data and material availability: All data and materials supporting the findings of this study are available from the corresponding authors upon request.

References

- Araki, M., Awane, R., Sato, T., Ohkawa, Y., and Inoue, Y. (2018). Anti-tumor effects of antimicrobial peptides, targets of the innate immune system, against hematopoietic tumors in *Drosophila mxc* mutants. *BioRxiv*.
- Bahar, A.A., and Ren, D. (2013). Antimicrobial peptides. *Pharmaceuticals (Basel)* 6, 1543-1575.
- Barajas-Azpeleta, R., Wu, J., Gill, J., Welte, R., Seidel, C., McKinney, S., Dissel, S., and Si, K. (2018). Antimicrobial peptides modulate long-term memory. *PLoS Genet* 14, e1007440.
- Beaucher, M., Hersperger, E., Page-McCaw, A., and Shearn, A. (2007). Metastatic ability of *Drosophila* tumors depends on MMP activity. *Dev Biol* 303, 625-634.
- Birge, R.B., Boeltz, S., Kumar, S., Carlson, J., Wanderley, J., Calianese, D., Barcinski, M., Brekken, R.A., Huang, X., Hutchins, J.T., *et al.* (2016). Phosphatidylserine is a global immunosuppressive signal in efferocytosis, infectious disease, and cancer. *Cell Death Differ* 23, 962-978.
- Brender, J.R., McHenry, A.J., and Ramamoorthy, A. (2012). Does cholesterol play a role in the bacterial selectivity of antimicrobial peptides? *Front Immunol* 3, 195.
- Brogden, K.A. (2005). Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat Rev Microbiol* 3, 238-250.
- Bullard, R.S., Gibson, W., Bose, S.K., Belgrave, J.K., Eaddy, A.C., Wright, C.J., Hazen-Martin, D.J., Lage, J.M., Keane, T.E., Ganz, T.A., *et al.* (2008). Functional analysis of the host defense peptide Human Beta Defensin-1: new insight into its potential role in cancer. *Mol Immunol* 45, 839-848.
- Bunker, B.D., Nellimoottil, T.T., Boileau, R.M., Classen, A.K., and Bilder, D. (2015). The transcriptional response to tumorigenic polarity loss in *Drosophila*. *Elife* 4.
- Cao, J., Ni, J., Ma, W., Shiu, V., Milla, L.A., Park, S., Spletter, M.L., Tang, S., Zhang, J., Wei, X., *et al.* (2014). Insight into insulin secretion from transcriptome and genetic analysis of insulin-producing cells of *Drosophila*. *Genetics* 197, 175-192.
- Cao, Y., Chtarbanova, S., Petersen, A.J., and Ganetzky, B. (2013). Dnr1 mutations cause neurodegeneration in *Drosophila* by activating the innate immune response in the brain. *Proc Natl Acad Sci U S A* 110, E1752-1760.
- Chung, E.M.C., Dean, S.N., Propst, C.N., Bishop, B.M., and van Hoek, M.L. (2017). Komodo dragon-inspired synthetic peptide DRGN-1 promotes wound-healing of a mixed-biofilm infected wound. *NPJ Biofilms Microbiomes* 3, 9.
- Cordero, J.B., Macagno, J.P., Stefanatos, R.K., Strathdee, K.E., Cagan, R.L., and Vidal, M. (2010). Oncogenic Ras diverts a host TNF tumor suppressor activity into tumor promoter. *Dev Cell* 18, 999-1011.
- Deslouches, B., and Di, Y.P. (2017). Antimicrobial peptides with selective antitumor mechanisms: prospect for anticancer applications. *Oncotarget*.
- Donald, C.D., Sun, C.Q., Lim, S.D., Macoska, J., Cohen, C., Amin, M.B., Young, A.N., Ganz, T.A., Marshall, F.F., and Petros, J.A. (2003). Cancer-specific loss of beta-defensin 1 in renal and prostatic carcinomas. *Lab Invest* 83, 501-505.
- Fernandez-Ayala, D.J., Sanz, A., Vartiainen, S., Kemppainen, K.K., Babusiak, M., Mustalahti, E., Costa, R., Tuomela, T., Zeviani, M., Chung, J., *et al.* (2009). Expression of the *Ciona intestinalis* alternative oxidase (AOX) in *Drosophila* complements defects in mitochondrial oxidative phosphorylation. *Cell Metab* 9, 449-460.

667 Fogarty, C.E., Diwanji, N., Lindblad, J.L., Tare, M., Amcheslavsky, A., Makhijani, K.,
668 Bruckner, K., Fan, Y., and Bergmann, A. (2016). Extracellular Reactive Oxygen
669 Species Drive Apoptosis-Induced Proliferation via *Drosophila* Macrophages. *Curr*
670 *Biol* 26, 575-584.

671 Ham, B., Fernandez, M.C., D'Costa, Z., and Brodt, P. (2016). The diverse roles of
672 the TNF axis in cancer progression and metastasis. *Trends Cancer Res* 11, 1-27.

673 Hanson, M.A., Dostalova, A., Ceroni, C., Poidevin, M., Kondo, S., and Lemaitre, B.
674 (2018). Synergy and remarkable specificity of antimicrobial peptides in vivo
675 using a systematic knockout approach. *BioRxiv*.

676 Hoffmann, J.A., and Reichhart, J.M. (2002). *Drosophila* innate immunity: an
677 evolutionary perspective. *Nat Immunol* 3, 121-126.

678 Hollingsworth, M.A., and Swanson, B.J. (2004). Mucins in cancer: protection and
679 control of the cell surface. *Nat Rev Cancer* 4, 45-60.

680 Igaki, T., Kanda, H., Yamamoto-Goto, Y., Kanuka, H., Kuranaga, E., Aigaki, T., and
681 Miura, M. (2002). Eiger, a TNF superfamily ligand that triggers the *Drosophila*
682 JNK pathway. *EMBO J* 21, 3009-3018.

683 Igaki, T., Pagliarini, R.A., and Xu, T. (2006). Loss of cell polarity drives tumor
684 growth and invasion through JNK activation in *Drosophila*. *Curr Biol* 16, 1139-
685 1146.

686 Igaki, T., Pastor-Pareja, J.C., Aonuma, H., Miura, M., and Xu, T. (2009). Intrinsic
687 tumor suppression and epithelial maintenance by endocytic activation of
688 Eiger/TNF signaling in *Drosophila*. *Dev Cell* 16, 458-465.

689 Imler, J.L., and Zheng, L. (2004). Biology of Toll receptors: lessons from insects
690 and mammals. *J Leukoc Biol* 75, 18-26.

691 Iwasaki, T., Ishibashi, J., Tanaka, H., Sato, M., Asaoka, A., Taylor, D., and
692 Yamakawa, M. (2009). Selective cancer cell cytotoxicity of enantiomeric 9-mer
693 peptides derived from beetle defensins depends on negatively charged
694 phosphatidylserine on the cell surface. *Peptides* 30, 660-668.

695 Ji, S., Sun, M., Zheng, X., Li, L., Sun, L., Chen, D., and Sun, Q. (2014). Cell-surface
696 localization of Pellino antagonizes Toll-mediated innate immune signalling by
697 controlling MyD88 turnover in *Drosophila*. *Nat Commun* 5, 3458.

698 Knelson, E.H., Nee, J.C., and Blobe, G.C. (2014). Heparan sulfate signaling in
699 cancer. *Trends Biochem Sci* 39, 277-288.

700 Kounatidis, I., Chtarbanova, S., Cao, Y., Hayne, M., Jayanth, D., Ganetzky, B., and
701 Ligoxygakis, P. (2017). NF-kappaB Immunity in the Brain Determines Fly
702 Lifespan in Healthy Aging and Age-Related Neurodegeneration. *Cell Rep* 19, 836-
703 848.

704 Kulshammer, E., Mundorf, J., Kilinc, M., Frommolt, P., Wagle, P., and Uhlirova, M.
705 (2015). Interplay among *Drosophila* transcription factors Ets21c, Fos and Ftz-F1
706 drives JNK-mediated tumor malignancy. *Dis Model Mech* 8, 1279-1293.

707 Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., and Hoffmann, J.A. (1996).
708 The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the
709 potent antifungal response in *Drosophila* adults. *Cell* 86, 973-983.

710 Leulier, F., Rodriguez, A., Khush, R.S., Abrams, J.M., and Lemaitre, B. (2000). The
711 *Drosophila* caspase Dredd is required to resist gram-negative bacterial infection.
712 *EMBO Rep* 1, 353-358.

713 Levashina, E.A., Ohresser, S., Bulet, P., Reichhart, J.M., Hetru, C., and Hoffmann, J.A.
714 (1995). Metchnikowin, a novel immune-inducible proline-rich peptide from

715 *Drosophila* with antibacterial and antifungal properties. *Eur J Biochem* 233, 694-
716 700.

717 Lezi, E., Zhou, T., Koh, S., Chuang, M., Sharma, R., Pujol, N., Chisholm, A.D., Eroglu,
718 C., Matsunami, H., and Yan, D. (2018). An Antimicrobial Peptide and Its Neuronal
719 Receptor Regulate Dendrite Degeneration in Aging and Infection. *Neuron* 97,
720 125-138 e125.

721 Mergaert, P., Kikuchi, Y., Shigenobu, S., and Nowack, E.C.M. (2017). Metabolic
722 Integration of Bacterial Endosymbionts through Antimicrobial Peptides. *Trends*
723 *Microbiol* 25, 703-712.

724 Moreno, E., Yan, M., and Basler, K. (2002). Evolution of TNF signaling
725 mechanisms: JNK-dependent apoptosis triggered by Eiger, the *Drosophila*
726 homolog of the TNF superfamily. *Curr Biol* 12, 1263-1268.

727 Parameswaran, N., and Patial, S. (2010). Tumor Necrosis Factor- α Signaling in
728 Macrophages. *Crit Rev Eukaryot Gene Expr* 20, 87-103.

729 Parisi, F., Stefanatos, R.K., Strathdee, K., Yu, Y., and Vidal, M. (2014). Transformed
730 epithelia trigger non-tissue-autonomous tumor suppressor response by
731 adipocytes via activation of Toll and Eiger/TNF signaling. *Cell Rep* 6, 855-867.

732 Parvy, J.P., Hodgson, J.A., and Cordero, J.B. (2018). *Drosophila* as a Model System
733 to Study Nonautonomous Mechanisms Affecting Tumour Growth and Cell Death.
734 *Biomed Res Int* 2018, 7152962.

735 Pastor-Pareja, J.C., Grawe, F., Martin-Blanco, E., and Garcia-Bellido, A. (2004).
736 Invasive cell behavior during *Drosophila* imaginal disc eversion is mediated by
737 the JNK signaling cascade. *Dev Cell* 7, 387-399.

738 Riedl, S., Rinner, B., Asslaber, M., Schaidler, H., Walzer, S., Novak, A., Lohner, K.,
739 and Zweytick, D. (2011). In search of a novel target - phosphatidylserine exposed
740 by non-apoptotic tumor cells and metastases of malignancies with poor
741 treatment efficacy. *Biochim Biophys Acta* 1808, 2638-2645.

742 Santabarbara-Ruiz, P., Lopez-Santillan, M., Martinez-Rodriguez, I., Binagui-Casas,
743 A., Perez, L., Milan, M., Corominas, M., and Serras, F. (2015). ROS-Induced JNK
744 and p38 Signaling Is Required for Unpaired Cytokine Activation during
745 *Drosophila* Regeneration. *PLoS Genet* 11, e1005595.

746 Shafee, T.M., Lay, F.T., Phan, T.K., Anderson, M.A., and Hulett, M.D. (2017).
747 Convergent evolution of defensin sequence, structure and function. *Cell Mol Life*
748 *Sci* 74, 663-682.

749 Shklyar, B., Levy-Adam, F., Mishnaevski, K., and Kurant, E. (2013). Caspase
750 activity is required for engulfment of apoptotic cells. *Mol Cell Biol* 33, 3191-3201.

751 Srivastava, A., Pastor-Pareja, J.C., Igaki, T., Pagliarini, R., and Xu, T. (2007).
752 Basement membrane remodeling is essential for *Drosophila* disc eversion and
753 tumor invasion. *Proc Natl Acad Sci U S A* 104, 2721-2726.

754 Sun, C.Q., Arnold, R., Fernandez-Golarz, C., Parrish, A.B., Almekinder, T., He, J., Ho,
755 S.M., Svoboda, P., Pohl, J., Marshall, F.F., *et al.* (2006). Human beta-defensin-1, a
756 potential chromosome 8p tumor suppressor: control of transcription and
757 induction of apoptosis in renal cell carcinoma. *Cancer Res* 66, 8542-8549.

758 Teng, M.W., Swann, J.B., Koebel, C.M., Schreiber, R.D., and Smyth, M.J. (2008).
759 Immune-mediated dormancy: an equilibrium with cancer. *J Leukoc Biol* 84, 988-
760 993.

761 Tjabringa, G.S., Aarbiou, J., Ninaber, D.K., Drijfhout, J.W., Sorensen, O.E.,
762 Borregaard, N., Rabe, K.F., and Hiemstra, P.S. (2003). The antimicrobial peptide
763 LL-37 activates innate immunity at the airway epithelial surface by

transactivation of the epidermal growth factor receptor. *J Immunol* **171**, 6690-6696.

Tokumaru, S., Sayama, K., Shirakata, Y., Komatsuzawa, H., Ouhara, K., Hanakawa, Y., Yahata, Y., Dai, X., Tohyama, M., Nagai, H., *et al.* (2005). Induction of keratinocyte migration via transactivation of the epidermal growth factor receptor by the antimicrobial peptide LL-37. *J Immunol* **175**, 4662-4668.

Tung, T.T., Nagaosa, K., Fujita, Y., Kita, A., Mori, H., Okada, R., Nonaka, S., and Nakanishi, Y. (2013). Phosphatidylserine recognition and induction of apoptotic cell clearance by *Drosophila* engulfment receptor Draper. *J Biochem* **153**, 483-491.

Tzou, P., De Gregorio, E., and Lemaitre, B. (2002). How *Drosophila* combats microbial infection: a model to study innate immunity and host-pathogen interactions. *Curr Opin Microbiol* **5**, 102-110.

Tzou, P., Ohresser, S., Ferrandon, D., Capovilla, M., Reichhart, J.M., Lemaitre, B., Hoffmann, J.A., and Imler, J.L. (2000). Tissue-specific inducible expression of antimicrobial peptide genes in *Drosophila* surface epithelia. *Immunity* **13**, 737-748.

Uhlirova, M., and Bohmann, D. (2006). JNK- and Fos-regulated Mmp1 expression cooperates with Ras to induce invasive tumors in *Drosophila*. *EMBO J* **25**, 5294-5304.

van Wetering, S., Mannesse-Lazeroms, S.P., van Sterkenburg, M.A., and Hiemstra, P.S. (2002). Neutrophil defensins stimulate the release of cytokines by airway epithelial cells: modulation by dexamethasone. *Inflamm Res* **51**, 8-15.

Wang, C., Chen, Y.W., Zhang, L., Gong, X.G., Zhou, Y., and Shang, D.J. (2016). Melanoma cell surface-expressed phosphatidylserine as a therapeutic target for cationic anticancer peptide, temporin-1CEa. *J Drug Target* **24**, 548-556.

Wang, C., Dong, S., Zhang, L., Zhao, Y., Huang, L., Gong, X., Wang, H., and Shang, D. (2017). Cell surface binding, uptaking and anticancer activity of L-K6, a lysine/leucine-rich peptide, on human breast cancer MCF-7 cells. *Sci Rep* **7**, 8293.

Wilson, C.L., Ouellette, A.J., Satchell, D.P., Ayabe, T., Lopez-Boado, Y.S., Stratman, J.L., Hultgren, S.J., Matrisian, L.M., and Parks, W.C. (1999). Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. *Science* **286**, 113-117.

Wilson, C.L., Schmidt, A.P., Pirila, E., Valore, E.V., Ferri, N., Sorsa, T., Ganz, T., and Parks, W.C. (2009). Differential Processing of {alpha}- and {beta}-Defensin Precursors by Matrix Metalloproteinase-7 (MMP-7). *J Biol Chem* **284**, 8301-8311.

Ye, Z., Dong, H., Li, Y., Ma, T., Huang, H., Leong, H.S., Eckel-Passow, J., Kocher, J.A., Liang, H., and Wang, L. (2018). Prevalent Homozygous Deletions of Type I Interferon and Defensin Genes in Human Cancers Associate with Immunotherapy Resistance. *Clin Cancer Res* **24**, 3299-3308.

Yeaman, M.R., and Yount, N.Y. (2003). Mechanisms of antimicrobial peptide action and resistance. *Pharmacol Rev* **55**, 27-55.

Zhao, H.W., Zhou, D., and Haddad, G.G. (2011). Antimicrobial peptides increase tolerance to oxidant stress in *Drosophila melanogaster*. *J Biol Chem* **286**, 6211-6218.

Zhu, M., Xin, T., Weng, S., Gao, Y., Zhang, Y., Li, Q., and Li, M. (2010). Activation of JNK signaling links lgl mutations to disruption of the cell polarity and epithelial organization in *Drosophila* imaginal discs. *Cell Res* **20**, 242-245.

Figure 1

Figure Legends

Figure 1 | *def* is induced in *dlg* mutant tumour bearing animals. A, Working model showing the cooperation between haemocyte-derived TNF and the immune response in the fat body in tumour cell death (Parisi et al, 2014). B, RT-qPCR analyses showing expression of several AMPs in the fat body of *dlg*^{40.2} mutant tumour bearing larvae compared to wild-type (*w*¹¹¹⁸) larvae (n=4). C, RT-qPCR analysis of *def* expression in *w*¹¹¹⁸ and *dlg*^{40.2} whole larvae reared on antibiotics (n=7). D, RT-qPCR analysis showing *def* expression in larvae expressing a *ctrl-IR* or a *dlg-IR* in the posterior part of the wing disc (*en>;UAS-dcr2*) (n=3). E, Schematic representation of the *def* gene locus showing mutant alleles generated (UTR: Untranslated Regions, SP: Signal Peptide, PrD: Pro-Domain, Def: Mature Defensin). Statistical analysis: B-D, Student t-test, B, ***p=0.0003, C, **p=0.0074, D, *p=0.042.

Figure 2

Figure 2 | Def restricts tumour growth and promotes tumour cell death.

A-E', Quantification of tumour volume (TV) (A) and tumour cell death (TCD) (B) in wing imaginal discs from *dlg*^{40.2} (n=17), *dlg*^{40.2};*def*^{sk3} (n=19) and *dlg*^{40.2};*def*^{sk4} (n=20) mutant larvae and representative immunofluorescence images of tissues quantified (C-E'). F, RT-qPCR analysis showing *def* expression upon ubiquitous knockdown (*dlg*^{40.2};*tub*>) (n=3). G-J', Quantification of TV (G) and TCD (H) in wing imaginal discs from larvae ubiquitously expressing a *control-IR* (*ctrl-IR*: n=10) or a *def-IR* (n=15) and representative immunofluorescence images of tissues quantified (I-J'). K-L, Quantification of TV (G) and TCD (H) in wing imaginal discs from *dlg* mutant controls (*dlg*^{40.2};*lpp*>: n=28), *dlg*;*def*^{sk3} mutants controls (*dlg*^{40.2};*def*^{sk3};*lpp*>: n=31) or *dlg*;*def*^{sk3} mutants expressing *def* in the fat body (*dlg*^{40.2};*def*^{sk3},*UAS-def*;*lpp*>: n=27). M, Effect of PBS (ctrl) or synthetic Def injection on TCD from wild-type larvae (*w*¹¹¹⁸, ctrl: n=10, Def: n=9), *dlg*^{40.2} (ctrl: n=10, Def: n=10) and *dlg*^{40.2};*def*^{sk3} (ctrl: n=11, Def: n=18) mutant larvae. Tissues were stained with 4',6-diamidino-2-phenylindole (DAPI, blue) for nuclei visualization and with anti-cleaved Decapping protein 1 (Dcp1) antibody (red) to detect apoptotic cell death. Scale bars=50 µm. Statistical analysis: A, B, K, N, One way ANOVA, *p<0.05, ****p<0.0001; F, Student t-test, ****p<0.0001, G-H, Mann-Whitney test, G, **p=0.0054, H, ****p<0.0001; M, Two way ANOVA (only relevant significant statistics are indicated), *p<0.05, ****p<0.0001.

Figure 3

Figure 3 | Def from the trachea and the fat body mediates tumour cell death. A, RT-qPCR analysis of *def* expression in gut, fat body and trachea dissected from *w¹¹¹⁸* or *dlg^{40.2}* mutant larvae (n=3). B-C, Fat body (B) and trachea (C) from *dlg^{40.2}* mutant larvae stained with DAPI (blue) and anti-Def antibody (red). D, RT-qPCR analysis of *def* expression from *dlg^{40.2}* mutant larvae (*dlg^{40.2};lpp>*) expressing a *ctrl-IR* or *def-IR* in the fat body (n=4). E-H', Quantification of TV (E) and TCD (F) in wing imaginal discs from *dlg^{40.2}* mutant larvae (*dlg^{40.2};lpp>*) expressing a *ctrl-IR* (n=15) or *def-IR* (n=17) in the fat body and representative immunofluorescence images of tissues quantified (G-H'). I, RT-qPCR analysis of *def* expression from *dlg^{40.2}* mutant larvae (*dlg^{40.2};btl>*) expressing a *ctrl-IR* or *def-IR* in the trachea (n=3). J-M', Quantification of TV (J) and TCD (K) in wing imaginal discs from *dlg^{40.2}* mutant larvae (*dlg^{40.2};btl>*) expressing a *ctrl-IR* (n=14) or *def-IR* (n=16) in the trachea and representative immunofluorescence images of tissues quantified (L-M'). Tumours were stained with DAPI (blue) and anti-Dcp1 antibody (red). Scale bars=50 μ m. Statistical analysis: A, D, I, Student t-test, D, **p=0.0054, I, ****p<0.0001; E, F, J, K, Mann-Whitney test, E, F, ****p<0.0001, J, ***p=0.0009, K, ***p=0.0004.

Figure 4

Figure 4 | Def produced by immune tissues specifically targets tumour cells. A-B, DAPI (blue) and anti-HA antibody (red) staining showing expression of *def*-HA construct in trachea (A) but not in fat body (B) of *UAS-def*-HA larvae. C-D, DAPI (blue) and anti-HA antibody (red) staining showing expression of a *UAS-def*-HA transgene in the fat body and the trachea when driven by a fat body specific driver (*lpp*>*UAS-def*-HA). E-E', DAPI (white), anti-Dcp1 (green) and anti-HA antibody (red) staining of *dlg*^{40.2} mutant tumour from larvae expressing the *def*-HA construct in the fat body and the trachea (*dlg*^{40.2}; *lpp*>*UAS-def*-HA). F-F', DAPI (white), anti-Dcp1 (green) and anti-HA antibody (red) staining of *dlg*^{40.2} heterozygous wing disc from larvae expressing the *def*-HA construct in the fat body (*dlg*^{40.2}/FM7; *lpp*>*UAS-def*-HA). G-H'', *dlg*^{40.2} mutant tumour stained with DAPI (blue), anti-Def (red) and anti-Dcp1 (green) antibodies. I, wild type (*w*¹¹¹⁸) wing imaginal disc stained with DAPI (blue), anti-Def (red) and anti-Dcp1 (green) antibodies. J-J'', High-resolution imaging of a single dying tumour cell stained with DAPI (blue), anti-Def (red) and anti-Dcp1 (green) antibodies. A-I, Scale bars=50 µm, J-J'', Scale bar=2.5 µm.

Figure 5

Figure 5 | Imd pathway activation is required for *def* expression and Def-mediated tumour cell death. A, RT-qPCR analysis of *def* expression from *dlg*^{40.2} and *dlg*^{40.2};*imd*¹ mutant larvae (n=4). B-E', Quantification of TV (B) and TCD (C) in wing imaginal discs from *dlg*^{40.2} (n=15) and *dlg*^{40.2};*imd*¹ (n=14) mutants larvae and representative immunofluorescence images of tissues quantified stained with DAPI (blue) and anti-Dcp1 antibody (red) (D-E'). F, RT-qPCR analysis showing *def* expression in *dlg*^{40.2} and *dlg*^{40.2};*rel*^{E20} mutant animals (n=5). G-J', Quantification of TV (G) and TCD (H) from *dlg*^{40.2} (n=12) and *dlg*^{40.2};*rel*^{E20} (n=10) mutant larvae and representative pictures of the corresponding tumours (I-J'). Scale bars=50 µm. Statistical analysis: A, F, Student t-test, A, ***p<0.0003, F, ****p<0.0001; B, C, G, H, Mann-Whitney test, B, C, H, ****p<0.0001, G, **p=0.009.

Figure 6

Figure 6 | Imd and Toll pathway activation are required in the trachea and the fat body to promote Defensin-dependent tumour cell death. A, RT-qPCR analysis of *def* expression from *dlg*^{40.2} mutant larvae (*dlg*^{40.2}; *btl*>) expressing a *ctrl-IR* or *imd-IR* in the trachea (n=3). B-E', Quantification of TV (B) and TCD (C) from *dlg*^{40.2} mutant larvae (*dlg*^{40.2}; *btl*>) expressing a *ctrl-IR* (n=18) or *imd-IR* (n=15) in the trachea and representative immunofluorescence images of tissues quantified stained with DAPI (blue) and anti-Dcp1 antibody (red) (D-E'). F, RT-qPCR analysis of *def* expression from *dlg*^{40.2} mutant larvae (*dlg*^{40.2}; *lpp*>) expressing a *ctrl-IR*, an *imd-IR* or a *myd88-IR* in the fat body. G-K', Quantification of TV (G) and TCD (H) from *dlg*^{40.2} mutant larvae (*dlg*^{40.2}; *lpp*>) expressing a *ctrl-IR*, an *imd-IR* or a *myd88-IR* in the fat body and representative immunofluorescence images of tissues quantified (I-K'). L-O, Quantification of TV (L, N) and TCD (M, O) from *dlg*^{40.2} mutant larvae (*dlg*^{40.2}; *lpp*>) expressing a *ctrl-IR*, an *imd-IR* alone or in combination with a *UAS-def* in the fat body (L, M) and from *dlg*^{40.2} mutant larvae (*dlg*^{40.2}; *lpp*>) expressing a *ctrl-IR*, a *myd88-IR* alone or in combination with a *UAS-def* in the fat body (N, O). Scale bars=50 µm. Statistical analysis: A, Student t-test, A, ***p=0.0001; B, C, Mann-Whitney test, B, ***p=0.0003, C, ***p=0.0002; F, G, H, L-O, One way ANOVA, *p<0.05, **p<0.01, ****p<0.0001.

Figure 7

Figure 7 | TNF is required for PS exposure and Defensin-driven tumour cell death. A-A'', DAPI (blue), Annexin V (green) and anti-Def (red) staining of *dlg*^{40.2} mutant tumours. B, D, E, Annexin V (green) and DAPI (blue) staining of wing imaginal discs from larvae of the indicated genotypes. C, RT-qPCR analysis showing *def* expression in wild-type, *dlg*^{40.2} or *dlg*^{40.2};*egr*³ mutants. F, G, Annexin V (green) and DAPI (blue) staining of wing imaginal discs from *dlg* mutant larvae (*dlg*^{40.2},*hml*>) expressing a *ctrl-IR* (n=5) or an *egr-IR* (n=9) in the haemocytes. H, Quantification of Annexin V signal on tumours from the corresponding genotypes. I-L, Annexin V (green) and anti-Dcp1 (red) antibody staining of tumours from *dlg*^{40.2}, *dlg*^{40.2};*def*^{sk3}, *dlg*^{40.2};*def*^{sk4} and *dlg*^{40.2};*imd*¹ mutant larvae. M, Quantification of TCD upon PBS (ctrl) or Def injection in wild-type (*w*¹¹¹⁸, ctrl: n=10, Def: n=14), *dlg*^{40.2} (ctrl/Def: n=18) or *dlg*^{40.2};*egr*³ (ctrl: n=20, Def: n=13) mutant larvae. N, A model for Def antitumoural activity. Scale bars=50 µm. Statistical analysis: C, One way ANOVA, *p<0.05; H, Mann-Whitney test, ***p=0.001; M, Two way ANOVA (only relevant significant statistics are indicated), **p<0.01.