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RESEARCH ARTICLE

The Nimrod transmembrane receptor Eater is required for hemocyte attachment to the sessile compartment in Drosophila melanogaster

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ABSTRACT

Eater is an EGF-like repeat transmembrane receptor of the Nimrod family and is expressed in Drosophila hemocytes. Eater was initially identified for its role in phagocytosis of both Gram-positive and Gram-negative bacteria. We have deleted eater and show that it appears to be required for efficient phagocytosis of Gram-positive but not Gram-negative bacteria. However, the most striking phenotype of eater deficient larvae is the near absence of sessile hemocytes, both plasmatocyte and crystal cell types. The eater deletion is the first loss of function mutation identified that causes absence of the sessile hemocyte state. Our study shows that Eater is required cell-autonomously in plasmatocytes for sessility. However, the presence of crystal cells in the sessile compartment requires Eater in plasmatocytes. We also show that eater deficient hemocytes exhibit a cell adhesion defect. Collectively, our data uncovers a new requirement of Eater in enabling hemocyte attachment at the sessile compartment and points to a possible role of Nimrod family members in hemocyte adhesion.

KEY WORDS: EGF-like, Eater, Hemocyte, Receptor, Sessile

INTRODUCTION

Circulating immune cells express many cell surface receptors, following their specialized role in host defense. These roles include cell adhesion, cell-cell recognition, phagocytosis, chemokine-binding and others (Alberts et al., 2002). In Drosophila and other insects, hemocytes are circulating immune cells, which participate in the humoral and cellular immune defense reactions against microbes and parasites (Lemaître and Hoffmann, 2007; Honti et al., 2014). Drosophila hemocytes express many surface receptors, some of whose functions are not well understood or have not been studied (Kurucz et al., 2003; Kurucz et al., 2007; Somogyi et al., 2008; Ulvila et al., 2011). Here, we re-visit the function of Eater, an EGF-like repeat Nimrod receptor that is specifically expressed in Drosophila hemocytes (Kocks et al., 2005; Kurucz et al., 2007).

Drosophila larvae have two types of hemocytes in the unchallenged state: plasmatocytes, which are macrophage-like, and crystal cells, rounded hemocytes which contain crystals of pro-phenoloxidases, the enzyme zymogen of phenoloxidase that catalyses the melanization reaction against parasites or septic injury (Rizki et al., 1980; Rizki and Rizki, 1992; Lanot et al., 2001). Larval hemocytes are found in three compartments: (i) the lymph glands that function as a reservoir releasing hemocytes after parasitic infection, (ii) in the circulation and, (iii) in the sessile patches (Lanot et al., 2001; Evans and Banerjee, 2003; Jung et al., 2005; Crozatier and Meister, 2007; Honti et al., 2010; Makhi jani et al., 2011; Makhi jani and Brückner, 2012). Sessile hemocytes are attached to the internal surface of the larval body wall, forming patches, some of which are closely associated with secretory cells called oenocytes, as well as the endings of peripheral neurons (Makhi jani et al., 2011; Makki et al., 2014). Hemocytes continuously exchange between sessile patches and the circulation (Babcock et al., 2008; Welman et al., 2010). Interestingly, hemocytes leave the sessile patches and enter the circulation upon wasp infestation or mechanical stimulation of the cuticle by brushing (Márkus et al., 2009; Makhi jani et al., 2011). The formation and function of sessile hemocyte patches is not yet established but it has been proposed that they form a diffuse hematopoietic organ (Márkus et al., 2009; Makhi jani et al., 2011).

Eater is an EGF-like repeat single pass transmembrane receptor of the Nimrod family (Kocks et al., 2005; Kurucz et al., 2007). Eater has 32 EGF-like or Nim repeats in the extracellular domain, of the Nimrod family (Kocks et al., 2005; Kurucz et al., 2007). Additionally, it has been identified as a plasmatocyte receptor encoding gene required for efficient phagocytosis of S. aureus and E. coli in Drosophila (Kocks et al., 2005). The contribution of eater to phagocytosis of different bacterial types was measured in S2 cells expressing an RNAi knock down of eater and in ex vivo hemocytes of larvae carrying overlapping deficiencies ablating eater and seven flanking genes (Kocks et al., 2005). Additionally, it has been shown that a recombinant fragment of the Eater extracellular domain can bind to bacteria or bacterial products and that Eater is cell-surface expressed (Chung and Kocks, 2011).

Here we have generated a knockout of eater by homologous recombination and showed its requirement for efficient phagocytosis of Gram-positive and but not Gram-negative bacteria. Larvae lacking eater have more than two times the...
number of circulating hemocytes compared to wild type controls. Imaging the sessile compartment reveals that eater deficient larvae lack nearly all sessile hemocytes, both plasmatocyte and crystal cell types. We show that Eater is required cell-autonomously in individual plasmatocytes for their presence at the sessile compartment. Allowing hemocytes to adhere to a glass slide reveals that eater deficient hemocytes exhibit a cell adhesion defect. Collectively, our data uncovers a new requirement for the transmembrane receptor Eater in the formation of the hemocyte sessile compartment.

**MATERIALS AND METHODS**

**Drosophila stocks and methodology**

Wild type Oregon R flies and w^1118^ (BL5905) were used as controls, unless otherwise indicated. Fly larvae were reared at a density of 30 female flies with 15 males per large vial laying for 24 hrs. We generated and used stocks w^1118^; eater^1^, w^1118^; Hml^4^; UAS-GFP and w^1118^; Hml^4^; UASGFP; eater^1^, w^1118^; Df(3R)6206/TM6c (derived from BL7685) and w^1118^; Df(3R)791/TM6c (derived from BL27363), yw, lzGal4, UAS-GFP and yw, lzGal4, UAS-GFP; eater^1^, Bc(I) and Bc(II); eater^1^, w^1118^; Hml^4^; UAS-GFP, UAS-eater::RNAi/TM3, actGFP, Ser and w^1118^; UAS-eater::RNAi/TM3, actGFP, Ser, yw, lzGal4, UAS-GFP; Hml^4^:Red,nls, Eater::Gal4, UAS-2xGFP:: BcF6-CFP (P1’); msn9-mCherry and w, Eater::Gal4, UAS-2xGFP:: BcF6-CFP (P1’); msn9-mCherry, eater^1^. The Hml^4^-transgene drives expression in plasmatocytes only (Sinenko et al., 2004; Makhijani et al., 2011). The UAS-eater::RNAi flies were derived from stock 6124R-2 of the National Institute of Genetics (NIG), Japan. Experiments were repeated at least twice in 2 separate days. Unless otherwise indicated, data was analysed in Excel 2011 (Microsoft) and Prism v5.0a (Graphpad) and significance tests performed using Students t test. For Fig. 2E, statistical analyses were performed using the R program (R Development Core Team, 2008) with the R commander graphical interface (Fox, 2005).

**Gene targeting of eater**

Deletion limits of the eater^1^ allele: 5’-GGTTGATACCTTAAGAC-ACC...[insert]...GGGATGATCGAGAACCT-3’. The 5’ and 3’ homology arms, 5.0 kb and 3.8 kb, respectively, were PCR amplified from BAC2R1010 clone (CHORI) using Hot-start PFusion Polymerase (New England Biolabs). The 5’ arm was inserted between NotI and Nhel sites, and the 3’ arm was inserted between SpeI and AscI sites of the gene targeting vector plHR (Baena-Lopez et al., 2013). A donor transgenic stock, w^1118^; eater::piHR (II), was generated by transformation (Fly Facility, France) of starting stock w^1118^ (BL5905) and used for hsFLP and hs-I-Sce mediated gene targeting (Baena-Lopez et al., 2013). Using this method, we recorded a knockout efficiency of ~1/5000 of the F2 progeny were bonafide eater knockouts.

**Ex vivo larval hemocyte phagocytosis assay**

We combined several existing protocols (Kocks et al., 2005; Watson et al., 2005; Kurucz et al., 2007) to measure phagocytosis by larval hemocytes. For full details, see Neyen and colleagues (Neyen et al., 2014). Briefly, phagocytosis of fluorescent heat-killed bacteria was quantified using a flow cytometer (BD Accuri, USA) to measure both the fraction of cells phagocytosing and the intensity of phagocytosis. Wandering third instar larvae were bled in cold Schneider’s medium (Gibco) containing 1 nM phenylthiourea (PTU, Sigma). Hemocytes were incubated in 100 μl volumes in ultra low attachment 96-well plates (Costar no. 3474, Corning) at room temperature (RT) for 10 min. Then, 10 μl of a homogeneous suspension of Alexa-Fluor AF488 heat-killed bacteria (Molecular Probes) of titre 2 × 10^6, 2 × 10^7 or 2 × 10^8 in Schneider’s/PTU was added and the plate incubated at RT for 20 min. After incubation, the fluorescence of extracellular bacterial particles was quenched by adding trypan blue (Sigma). The fluorescence intensity of single hemocytes, not part of multicellular hemocyte clusters, was measured on red and green fluorescence channels with a 488 nm laser and BP530/30 and BP585/40 band-pass filters, respectively. The mean fluorescence intensity of a hemocyte population without bacteria added was used to define the gate for the phagocytosing hemocyte population. The phagocytic index was calculated as follows:

\[
\text{Phagocytic index, P.I. = } \frac{\text{mean fluorescence intensity of hemocytes in fluorescence positive gate}}{f} \times f,
\]

where \(f\) is equal to the fraction of hemocytes phagocytosing:

\[
\frac{\text{number of hemocytes in fluorescence positive gate}}{\text{total number of hemocytes}}.
\]

Note, the number of circulating hemocytes per larva can vary dramatically between genotypes. On average, 20 Oregon R larval bleeds yield ~5000 hemocytes, 20 w^1118^ (BL5905) larval bleeds yield ~7,500 hemocytes and 20 w^1118^; eater^1^ larval bleeds yield ~16,000 hemocytes (Fig. 3A). Therefore to achieve cell-matched assays across genotypes, we adjusted the number of larval bleeds between genotypes. Therefore, we used 13 w^1118^ (BL5905) larval bleeds and 6 w^1118^; eater^1^ larval bleeds per assay to achieve 20 Oregon R larval bleed-equivalents across all genotypes.

**Live imaging of third instar larvae**

For whole larval imaging, cleaned third instar larvae were mounted in cold PBS between two glass slides. Images were captured on a Leica MZ-16F fluorescence microscope with Leica Application Suite version 2.8.1. For live imaging of sessile patches, larvae were mounted dorsal side up on a 2% agarose pad on a glass slide atop a 9 cm petri plate filled with ice. Dermabond glue (Ethicon, US) was applied along the length of the
APO objective. Images were captured on an EMCCD ImagEM B/W following standard protocols (Evans et al., 2014). Lymph glands were dissected from synchronized early third instar larvae. Lymph gland and hemocyte immunostaining

shock induction (38˚C, 1 h) of the MARCM GFP+ hemocyte clones were generated by embryonic heat induction (38˚C, 1 h) of the MARCM GFP+ hemocyte clones were generated by embryonic heat

Generation of MARCM clones

MARCM GFP+ hemocyte clones were generated by embryonic heat shock induction (38˚C, 1 h) of the hsFLP gene as described in previously (Minakhina et al., 2007). To generate MARCM clones, we used y, hsFLP, ptubGAL4, UAS-GFP; FRT82B, ptubGAL80/TM6B, yw++; FRT82B and yw; FRT82B, eater’.

Lymph gland and hemocyte immunostaining

Lymph glands from synchronized early third instar larvae were dissected following standard protocols (Evans et al., 2014). Lymph glands were stained with mouse anti-Hemese primary antibody and horseradish peroxidase HRP-conjugated anti-mouse Ig and AEC as a chromogen. Hemocyte immunostaining was performed as previously described (Märkus et al., 2009), except that hemocytes were allowed to adhere in Schneiders medium supplemented with 5% fetal bovine serum (FBS) to HCl-cleaned glass slides at 25 ˚C for 3 h. Glass slides were washed in detergent and water, washed extensively with running tap water, incubated in 1 M HCl overnight, re-rinsed extensively with running tap water, rinsed in distilled water, rinsed in 70% ethanol and dried at 37˚C.

Hemocyte cell area measurement

Spread hemocytes were prepared as for immunostaining, except that cells were stained with AF488-phalloidin (Molecular Probes) and mounted in Vectashield-DAPI (Vector labs). Mosaic 2×2 images of hemocytes were captured with a ×20 objective on GFP and DAPI channels using Zeiss Axiosimaging Z.1 and Axiovision software (Carl Zeiss).

For confocal imaging, live samples were inverted and mounted on an inverted Olympus IX 81 microscope with confocal scanner unit (CSU-W1, Yokogawa, Japan) and imaged with a ×60/NA 1.42 oil U PLAN S APO objective. Images were captured on an EMCCD ImageEM B/W camera (detector size 6.45 μm) mounted on a Zeiss AxioImager Z.1 and Axiovision software (Carl Zeiss).

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Crystal cell counting methods

For crystal cell visualization by heating, ten third instar larvae were heated in 0.5 ml PBS in eppendorf tubes for 30 min at 67°C. Larvae were recovered, mounted between two glass slides over a white background and imaged. For quantification, black puncta were counted circumferentially in the posteriormost segments A6, A7 and A8.

To count live crystal cells, wandering third instar larvae, five at a time, carrying the BcF6-CFP label were selected, washed and vortexed for 1 min at max speed to release sessile crystal cells. Larval hemocytes were dissected to 5.5 ml of Schneiders medium containing 1 nM phenylthiourea (PTU, dissolved in DMSO) in 10 mm diameter wells of 8-well glass slides (silane surface, Teflon mask, Tekdon Inc., Florida). This volume is sufficient to fill the well when a coverslip (12 mm diameter, Menzel Glaüser) is placed over the well. Five larvae were dissected per well. Circular mosaic images (13 columns x 17 rows) of the entire well under CFP illumination were immediately captured and the number of CFP-expressing crystal cells counted directly by eye from the mosaic image. To count black cells, Bc-carrying larvae were treated similarly but 20 larvae were vortexed and hemocytes dissected to 120 ml of Schneiders medium, 1 nM PTU. The resulting cell suspension was mounted over a 1 mm² grid hemocytometer (Preciss, France), the number of black cells counted and the number of black cells per larva derived.

Live imaging of crystal cell rupture

Two third instar larvae were dissected in 6 ml PBS-0.1% BSA on glass slides (Menzel-Glaser Superfrost). Immediately a 12 mm diameter cover glass (Menzel-Glaser) was placed and sample mounted. Crystal cells were located under CFP illumination and imaged with a 6x100 oil objective under DIC III on a Zeiss AxioImager Z.1. Time between dissection and imaging was typically less than 2 minutes.

RESULTS

Deletion of the eater gene by homologous recombination

To investigate Eater function, we deleted eater using an optimized method of gene-targeting (Baena-Lopez et al., 2013). Gene targeting in the w¹¹¹⁸ genetic background yielded eater¹, a 745 bp deletion removing the ATG translation start site, the first and second exons, 18 bp of the third exon together with insertion of a 7.9 kb cassette carrying the white+ gene (Fig. 1A; Materials and Methods). We confirmed functional deletion of eater and integrity of flanking genes by RT-PCR from total RNA (Fig. 1B). Flies carrying the eater¹ lesion appear developmentally wild type, consistent with previous findings that plasmatocyte-deficient flies are mostly viable (Charroux and Royet, 2009; Defaye et al., 2009).
Eater appears to be required for efficient phagocytosis of Gram-positive but not Gram-negative bacteria

We first used the eater<sup>1</sup> deletion to ask whether eater is required for phagocytosis of heat-killed, labeled bacteria, as previously reported (Kocks et al., 2005). We employed an ex vivo phagocytosis assay in which larval hemocytes were incubated with 2 × 10<sup>6</sup>, 2 × 10<sup>7</sup> or 2 × 10<sup>8</sup> Alexa Fluor 488-labeled heat-killed bacteria and run on a flow cytometer to measure the fluorescence of hemocytes. To quantify phagocytosis, we used a phagocytic index (P.I.) equal to the fraction of cells phagocytosing multiplied by the mean fluorescence intensity of the phagocytosing cell population. As controls, we used hemocytes from w<sup>1118</sup> larvae, carrying the same genetic background as eater<sup>1</sup> mutant larvae, and hemocytes from wild type Oregon<sup>R</sup> larvae. We observed that phagocytosis of Gram-negative E. coli by eater<sup>1</sup> deficient hemocytes was similar or even more than that of control Oregon<sup>R</sup> wild type hemocytes (Fig. 2A,B). Contrastingly, phagocytosis of Gram-positive Staphylococcus aureus was defective in eater<sup>1</sup> null hemocytes compared to wild-type controls at all titres tested (Fig. 2C,D), consistent with previous analyses (Kocks et al., 2005). We extended our analysis to other Gram-positive and Gram-negative bacteria. eater<sup>1</sup> null hemocytes phagocytosed the Gram-negative Serratia marcescens to wild type levels, but were deficient in phagocytosis of the Gram-positive Staphylococcus epidermidis and Micrococcus luteus (Fig. 2E). Together these data indicate that phagocytosis of the Gram-positive bacteria S. aureus, S. epidermidis and M. luteus, but not the Gram-negative bacteria E. coli and S. marcescens by plasmatocytes, the major macrophage-like cell type in Drosophila, is defective in eater<sup>1</sup> null larvae.

Sessile plasmatocytes are absent or almost absent in eater<sup>1</sup> null larvae

While examining phagocytosis, we noticed that dissected eater<sup>1</sup> null larvae release more than three times the number of hemocytes that wild type Oregon<sup>R</sup> larvae release and more than two times more than w<sup>1118</sup> larvae (Fig. 3A). The high number of circulating hemocytes prompted us to investigate the anatomy of the hemocyte compartments in third instar eater<sup>1</sup> larvae. In wandering third instar larvae, around one third of all hemocytes are present in the lymph glands, one third are circulating and one third are sessile (Lanot et al., 2001; Jung et al., 2005; Crozatier and Meister, 2007). The lymph gland does not normally release hemocytes except upon wounding or immune challenge by parasitoids or at metamorphosis (Lanot et al., 2001; Honti et al., 2010). We first asked whether a defect in lymph gland organization could explain the higher number of circulating hemocytes in eater<sup>1</sup> larvae. Dissecting the lymph glands, we observed that those of eater<sup>1</sup> larvae were not visibly different in size to those of w<sup>1118</sup> control larvae (Fig. 3B).

Recent studies show that hemocytes exchange between a circulating state and a static or sessile state in which they are body wall-bound (Babcock et al., 2008; Welman et al., 2010; Makhijani et al., 2011). The sessile hemocyte compartment is visible as a striped pattern of hemocyte patches along the length of the larva (Zettervall et al., 2004) and comprises plasmatocytes and crystal cells (Lanot et al., 2001). To explore hemocyte pattern in the absence of Eater, we combined the plasmatocyte reporter HmlAGAL4, UAS-GFP with the eater<sup>1</sup> mutation and imaged whole larvae. The sessile plasmatocyte striped pattern evident in w<sup>1118</sup> larvae was absent in eater<sup>1</sup> mutant larvae (Fig. 3C). All or almost all plasmatocytes in eater<sup>1</sup> larvae are in circulation and appear not to enter the sessile state. This ‘no sessile plasmatocyte’ phenotype was phenocopied in trans-heterozygous larvae carrying eater<sup>1</sup> over the deficiencies Df(3R)6206 or Df(3R)791 (data not shown), suggesting that absence of sessile hemocytes was indeed caused by the lesion in the eater gene. Together these data suggest that Eater is required for plasmatocytes to enter the sessile state, and that consequently, eater deficient larvae have close to double the number of freely circulating plasmatocytes that wild type larvae have.

eater is required cell-autonomously for plasmatocytes to enter the sessile state

Eater could either be required in plasmatocytes for them to enter the sessile state or in a different cell type. To address this, we knocked down eater transcripts in plasmatocytes using a UAS-eater RNAi. Knocking down eater in the Hml positive lineage alone was sufficient to cause a near absence of sessile plasmatocytes (see Fig. 5B). Next we used a clonal analysis to ask whether eater is required in individual plasmatocytes for them to become sessile. We generated gfp positive clones of eater<sup>1</sup> mutant hemocytes in mosaic larvae that were otherwise eater<sup>1/+</sup> heterozygous using MARCM (mosaic analysis with a repressible cell marker) (Lee and Luo, 1999). To image plasmatocytes in vivo, we immobilized third instar larvae to agar pads by gluing (see Materials and Methods). Imaging control gfp wild type hemocyte clones in third instar larvae showed that as the glued larva moves, many sessile plasmatocytes keep the same position within the elapsed time (supplementary material Fig. S1 and Movie 1). In contrast, imaging gfp positive eater<sup>1</sup> mutant plasmatocytes showed that the majority of gfp<sup>+</sup> hemocytes do not remain stationary with respect to the cuticle as the larva moved, indicating that the majority of eater deficient hemocytes lacked sessility (supplementary material Fig. S1 and Movie 2). These data together with the RNAi experiment indicate that Eater is required cell-autonomously in individual plasmatocytes for attachment to the sessile compartment.

Adherent eater deficient plasmatocytes are rounded and appear small on a glass surface

Eater localizes to the plasma membrane of Drosophila S2 cells (Chung and Kocks, 2011). We therefore hypothesized that the absence of attachment to the sessile compartment in eater deficient hemocytes could be due to a general requirement of Eater in hemocyte adhesion. A hallmark of impaired macrophage cell adhesion is a reduction in cell area on a substrate due to reduced cell spreading together with a decrease in the fraction of cells adhering (Fraser et al., 1993; Suzukii et al., 1997; Ribeiro et al., 2014). Spreading hemocytes on a cleaned glass slide, we observed that the cell areas of adherent eater<sup>1</sup> mutant hemocytes were small compared to those of control w<sup>1118</sup> hemocytes (Fig. 3D). We measured cell area and found that w<sup>1118</sup> adherent hemocytes have a mean cell area of 224 μm<sup>2</sup> while eater<sup>1</sup> mutant adherent hemocytes have a mean cell area of 129 μm<sup>2</sup> (Fig. 3E), consistent with a possible requirement of Eater in cell adhesion.

Sessile crystal cells are absent or almost absent in eater<sup>1</sup> mutant larvae

We next asked whether eater<sup>1</sup> larvae also have defects in the other sessile hemocyte type, the crystal cell (Lanot et al., 2001). Like plasmatocytes, crystal cells may either be in a sessile or circulating state (Lanot et al., 2001). Heating larvae in water for 30 min at 67°C causes spontaneous activation of the phenoloxidase zymogen within crystal cells and their subsequent
blackening, making them visible through the cuticle as black puncta (Rizki et al., 1980). Surprisingly, eater\textsuperscript{1} larvae showed very few black puncta, unlike Canton\textsuperscript{S} and Oregon\textsuperscript{R} wild types and w\textsuperscript{1118} controls (Fig. 4A,B). As heated larval tissues are opaque, the lack of black puncta in eater\textsuperscript{1} mutant larvae could be due to an absence of crystal cells or to a reduction in the numbers of visible, sub-epidermal crystal cells (i.e. sessile crystal cells). We therefore combined the crystal cell lineage marker lzGAL4, UAS-GFP with the eater\textsuperscript{1} mutation. Imaging whole larvae, we observed that sessile Lz\textsuperscript{>GFP} labelled crystal cells were almost absent in eater\textsuperscript{1} mutant larvae, compared to controls (Fig. 4C). We independently confirmed this result by combining eater\textsuperscript{1} with the mature crystal cell fate marker BcF6-CFP (Gajewski et al., 2007) (Fig. 4D). While sessile crystal cells appeared absent, crystal cells were still visible in circulation in eater\textsuperscript{1} mutant larvae (Fig. 4C,D).

To further examine the crystal cell population in eater\textsuperscript{1} mutants, we combined the eater\textsuperscript{1} mutation with Black cells (Bc), a mutation that causes blackening of crystal cells (Rizki et al., 1985; Lebestky et al., 2000; Lanot et al., 2001; Binggeli et al., 2014). The blackening of crystal cells in Bc homozygous larvae makes these cells readily visible through the cuticle as ‘black cells’ (Fig. 4E). Interestingly, though ‘black cells’ are dead crystal cells, they can still be sessile (Bc larva, Fig. 4E) and (Rizki et al., 1980). Observation of dissected hemocytes from the circulation of Bc control larvae revealed that melanised black cells often coincide with or have been ingested by plasmatocytes (Fig. 4F), as previously reported (Lanot et al., 2001; Gajewski et al., 2007). Examining larvae carrying both Bc and the eater\textsuperscript{1} mutation revealed a near absence of sessile black cells while the total number of black cells appeared to be similar to that seen in Bc control larvae (Fig. 4E).

Previously, larval crystal cell numbers have been quantified by counting black puncta in heated larvae, as in Fig. 4A typically revealing between 40 and 100 crystal cells per larva (Lanot et al., 2001). Since eater\textsuperscript{1} mutants lack sessile crystal cells, we used two alternative methods to count crystal cells. First, a fluorescence microscopy method based around counting live BcF6-CFP labeled cells.

Fig. 4. Sessile crystal cells are absent or almost absent in eater\textsuperscript{1} mutant larva. (A) Heating (67°C, 30 min) reveals the presence of superficial crystal cells in the posterior abdominal segments of Canton S, w\textsuperscript{1118} and eater\textsuperscript{1} null larvae. The ventral larval surface is shown. (B) Circumferential black puncta counts from the three posteriormost segments A6, A7 and A8 of heated Oregon\textsuperscript{R}, w\textsuperscript{1118} and eater\textsuperscript{1} third instar larva. (C) The crystal cell lineage marker lzGAL4, UAS-GFP reveals a near absence of sessile crystal cells in eater\textsuperscript{1} third instar larvae compared to controls. Dorsal view of the 5 posterior-most abdominal segments. (D) The mature crystal cell marker BcF6-CFP reveals a near absence of sessile crystal cells in eater\textsuperscript{1} mutant larva compared to control w\textsuperscript{1118} larvae. Vortexing (1 min, speed 10) is sufficient to release many sessile crystal cells. Genotypes: w, EaterGAL4, UAS-2xeYFP, BcF6-CFP (P1+); msn9-mCherry and w, EaterGAL4, UAS-2 xeYFP, BcF6-CFP (P1+); msn9-mCherry, eater\textsuperscript{1}. (E) The numbers of black cells in Bc; eater\textsuperscript{1} mutant larvae are similar to the numbers in control larvae carrying the Bc gain-of-function mutation alone. Sessile black cells are present in Bc control larvae but absent in Bc; eater\textsuperscript{1} larvae. (F) AF488-phalloidin and DAPI-stained hemocyte fields from Bc control and Bc; eater\textsuperscript{1} third instar larvae. Plasmatocytes ‘P’ and lamellocytes ‘L’ are visible. Black cells may be anuclear black cells (Bc) or may coincide with plasmatocytes (P-Bc). Lamellocytes are a type of hemocyte induced by activation of the Drosophila cellular immune response. (G) (i) Cell counts of live crystal cells from hemocyte samples of w\textsuperscript{1118} and eater\textsuperscript{1} mutant larvae carrying the BcF6-CFP reporter transgene. Genotypes: w, EaterGAL4, UAS-2xeYFP, BcF6-CFP (P1+); msn9-mCherry and w, EaterGAL4, UAS-2 xeYFP, BcF6-CFP (P1+); msn9-mCherry, eater\textsuperscript{1}. (ii) Hemocytometry counts of black cells numbers from hemocyte samples of larvae carrying the Bc gain-of-function mutation either alone or in combination with the eater\textsuperscript{1} mutation. Genotypes: Bc and Bc; eater\textsuperscript{1}.
crystal cells and, second, a hemocytometry method based around counting black cells from larvae carrying the Bc mutation. Sessile hemocytes can be released by mechanical stimulation of the larval cuticle (Makhijani et al., 2011). In order to count both sessile and circulating crystal cells, larvae were vortexed for 1 min at max speed which releases sessile hemocytes (Petrai et al., 2015), including sessile crystal cells (Fig. 4D). Counting crystal cells using the BcF6-CFP label revealed that eater mutant larvae had no lack of crystal cells; they appeared to have twice as many circulating crystal cells as w1118 control larvae (Fig. 4Gi). Similarly, counting black cells numbers as a surrogate for mature crystal cell numbers, we found Bc; eater mutant larval had similar numbers of circulating black cells as Bc control larvae (Fig. 4Gii). Interestingly, it appears that the number of black cells in larvae carrying the Bc mutation is ~4000, around 40–80 times more than the number of mature crystal cells in w1118 larvae (Fig. 4Gi) or wild type larvae (Lanot et al., 2001).

These findings, that eater mutant larvae lack all or almost all sessile crystal cells while retaining many crystal cells in circulation, suggests that the absence of eater does not impair crystal cell differentiation. Consistent with this, we observed that eater crystal cells rupture on exposure to air like mature wild-type crystal cells (supplementary material Movies 3, 4).

**eater is required in plasmatocytes for sessile crystal cells**

The near absence of sessile crystal cells was surprising as eater is expressed specifically in plasmatocytes but not in crystal cells (Kocks et al., 2005). We therefore asked how sessile crystal cells and sessile plasmatocytes are juxtaposed in the sessile compartment in third instar larvae. To do this, we used double live confocal imaging of the IGL4, UAS-GFP marker, which only labels crystal cells and the HmlDsRed.nls marker, which labels plasmatocytes but not mature crystal cells. Imaging revealed that most sessile GFP+ crystal cells are closely associated with sessile DsRed+ hemocytes (Fig. 5A). It is interesting to note that crystal cell and plasmatocyte tight associations are also frequently observed in hemolymph preparations from either wild-type or eater mutant larvae (supplementary material Movies 3, 4).

Following from our observation that sessile crystal cells are almost absent in eater mutant larvae, we asked whether lack of sessile crystal cells in eater larvae is due to a requirement of eater in the crystal cell lineage or the Hml lineage. To decipher between these two possibilities, we knocked down eater in the crystal cell lineage using HmlGAL4 driver or in the crystal cell lineage using IGL4 and used the heating assay to monitor the presence of sessile crystal cells. Interestingly, IGL4>eater-RNAi larvae still exhibited sub-epidermal crystal cells like wild type larvae, as observed by heating and black puncta visualization or by GFP fluorescence (Fig. 5C). However, sub-epidermal crystal cells were absent from HmlGAL4>eater-RNAi larvae, as viewed by heating and black puncta visualization (Fig. 5B). Thus, crystal cell sessility appears to require Eater non-cell-autonomously in hemocytes of the Hml+ lineage.
DISCUSSION

The transmembrane receptor Eater was initially described as a major phagocytic receptor, recognizing a broad range of bacteria or bacterial products (Kocks et al., 2005). This conclusion originated from ex-vivo analysis of phagocytosis in S2 cells, using RNAi knockdown of eater, and hemocytes from larvae ablated for eater by overlapping deficiencies. Although our study confirms that Eater contributes to the phagocytosis of Gram-positive bacteria, it does not reveal any role of Eater in the phagocytosis of Gram-negative bacteria. Thus, the use of a clean deletion of eater demonstrates that the phagocytic activity of this receptor as measured by ex vivo analysis is more restricted than expected. At this stage, we cannot exclude the possibility that Eater contributes to phagocytosis of Gram-negative bacteria in vivo, where secreted factors (e.g. opsonins) could assist Eater-mediated phagocytosis in the hemolymph. The very different surface chemistries of Gram-positive and Gram-negative bacteria (Mengin-Lecreulx and Lemaître, 2005; Lemaître and Hoffmann, 2007), namely peptidoglycan-based cell wall versus lipopolysaccharide-based outer membrane, respectively, could explain the different requirement of Eater in Gram-positive but not Gram-negative uptake. The results obtained with the eater mutant imply different phagocytic mechanisms for uptake of Gram-positive and Gram-negative bacteria. The phagocytosis of Gram-positive bacteria in Drosophila also requires the cell surface receptors Draper and Integrin βv (Hashimoto et al., 2009; Ulvila et al., 2011; Shiratsuchi et al., 2012). Whether Eater interacts with these proteins and how it contributes to phagocytosis remain an open question.

Our data suggest that eater deficient larvae do not have a gross defect in plasmatocyte maturation or differentiation. Indeed, eater deficient plasmatocytes are competent to phagocytose Gram-negative bacteria and express the mature plasmatocyte-specific marker NimC1 like wild-type hemocytes (Fig. 3D). Importantly, we have discovered a cell-autonomous requirement of eater in plasmatocytes for their association to the sessile compartment. To our knowledge, the eater deletion is the first loss-of-function mutation identified in Drosophila that causes an absence or a near absence of the sessile hemocyte state. Our RNAi and MARCM mosaic analysis confirm that eater is required specifically in individual plasmatocyte for sessility and not in another cell type, consistent with expression data (Kocks et al., 2005). We therefore suggest that the Eater receptor functions in some way to enable hemocyte adhesion at the sessile compartment. Our study does not distinguish whether Eater is required to promote hemocyte migration to the sessile compartment or maintain hemocytes at the sessile compartment. How a receptor such as Eater can contribute to two distinct functions, phagocytosis and sessility, is intriguing. The observation that eater deficient plasmatocytes fail to spread as much as the wild type on a glass slide suggests Eater could act as a cell adhesion receptor. A function in cell adhesion could explain why eater deficient hemocytes do not remain attached to the sessile niche as well as the defects observed in phagocytosis of Gram-positive bacteria. In the same line, mutation in the Drosophila integrin βv receptor also cause multiple defects in both phagocytosis and encapsulation (Howell et al., 2012; Shiratsuchi et al., 2012).

In eater mutant larvae, both plasmatocytes and crystal cells appear to be absent or almost absent from the sessile compartment. We have shown that sessile crystal cells require Eater in Hml+ plasmatocytes. This indicates that absence of sessile crystal cells in the eater mutant is a secondary consequence of the absence of sessile plasmatocytes. A first explanation is that crystal cells attach to sessile plasmatocytes to adhere to the sessile compartment. A second hypothesis is that sessile crystal cells derive from sessile hemocytes of the Hml+ lineage. The latter hypothesis is supported by observation that Lz-GAL4, UAS-GFP sessile crystal cells derive from Hml+dsRed. nls expressing sessile hemocytes (Leitao and Sucena, personal communication 2015). This is also consistent with recent observations showing that Drosophila hematopoiesis is not restricted to the embryonic stage or to the lymph gland but takes place in the circulation and in the sessile compartment with higher plasticity between hemocyte lineages than first thought (Lanot et al., 2001; Márkus et al., 2009; Avet-Rochex et al., 2010; Honti et al., 2010). The absence of sessile hemocytes in eater null larvae provide a unique tool to assess the function of the sessile compartment in the context of development, peripheral nervous system stimulation (Makihiani et al., 2011) or immune challenge (Márkus et al., 2009).

We have not addressed the ligand of the Eater receptor in this study. However, it is interesting to note that in absence of eater, essentially all plasmatocytes are absent from the sessile state. This suggests that all plasmatocytes use the same form of attachment site to attach to both lateral and dorsal patches of the sessile compartment. Clues as to what may form a hemocyte attachment site come from anatomy: invertebrate epithelial cells form junctions to the apical extracellular matrix (ECM) lining the larval cuticle and to the ECM lining the basal surface (Brown, 2011). Interestingly, electron microscopic cross sections through sessile plasmatocytes show attachment to the basal surface of cuticular epithelial cells (Lanot et al., 2001). Additionally, hemocytes appear to reside in the sessile compartment in close proximity to the endings of peripheral neurons and presumably their associated glial cells (Makihiani et al., 2011). Sessile hemocytes appear to cluster around oenocytes, secretory cells, which synthesize and secrete hydrocarbons onto the larval cuticle and may also contribute to endocrine regulation (Makki et al., 2014). Whether sessile hemocytes attach to a component of the ECM or directly to different cell types within the epithelial wall, remains to be determined.

Although the Nimrod gene family is thought to be an important component of insect innate host defense, few members of this family have been the focus of functional studies (Somogyi et al., 2008). The best characterized Nimrod gene is Draper, an atypical family member having only 1 Nim repeat and 15 EGF-like repeats, which is expressed in glia, hemocytes and other tissues and mediates engulfment of apoptotic cells during development and phagocytosis of bacteria during infection (Manaka et al., 2004; Awasaki et al., 2006; Shiratsuchi et al., 2012). An in vivo RNAi study has pointed to a role of NimC1 in the phagocytosis of the Gram-positive bacteria S. aureus (Kurucz et al., 2007). Here, we uncover a new role for Eater protein in hemocytes adhesion and localization. This raises the hypothesis that other Nimrod family members could also play a role in hemocyte homing by modulating their adhesion properties. Future genetic study should address the function of other Nimrod family member to better decipher the role of this family.

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Competing interests
The authors have no competing or financial interests to declare.

Author contributions
A.J.B., B.L., M.P., V.H., I.A. conceived and designed the experiments. A.J.B., V.H., O. Binggeli, M.P., E.K., J.Z., I.A. performed the experiments. A.J.B., B.L., O. Burri analysed the data. A.J.B. and B.L. wrote the paper.

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