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Immune response to bacteria induces dissemination of Ras-activated Drosophila hindgut cells

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 05 August 2011

Thank you for the submission of your research manuscript to EMBO reports. I have now had the opportunity to read it and I regret to say that I consider that your manuscript would not be suitable for EMBO reports.

In assessing your manuscript I note that you describe a model for cancer cell migration under bacterial infection conditions. You show that hindgut cells overexpressing an activated form of Ras acquire a migratory phenotype in vivo. This is enhanced by bacterial infection or activation of the Imd/JNK immune response pathway. Blocking the Imd/JNK pathway suppresses the migratory phenotype, suggesting that bacterial infection promotes the migratory behavior through activation of the immune response.

We appreciate that you describe a model for tumor cell migration and its relationship to bacterial infection in the Drosophila system in vivo. We think this will be of interest to other researchers in the field. However, I note that bacterial and fungal infections have been shown before to modulate tumor aggressive behavior, either to enhance metastatic progression or to repress it, and that innate immune response is involved in this modulation at least in some instances. These previous findings reduce the conceptual novelty of your study.

Also, it is well known that the metastatic phenotype observed in cancerous cells is only part of a very complex array of defects that normally include abnormal metabolism, genetic instability and aberrant activity of multiple intracellular signaling pathways. Thus, the physiological relevance of the system presented in this study, in which a single genetic defect promotes migration, as a model for cancer progression remains unclear. I have therefore decided no to proceed with the in-depth review process of you manuscript.

I am sorry to disappoint you on this occasion and hope that this negative decision does not prevent you from considering our journal for the publication of your future studies.

Yours sincerely,

Editor
EMBO Reports

Correspondence - authors' appeal 08 August 2011

I understand your critique and the points you are making. Nevertheless I have to note that it is exaclty the consfussion there is in the field regarding the role of bacteria, the positive or negative effect they might have on migration and our ignorance on which branches of innate immunity are involved that provides the novelty. For example NF-kB is considered to play a central role in tumor formation but in our experiment we notice that Toll immune pathway branches out to induce JNK instead while cooperating with Ras oncogene (onother link not previously shown).
And I couldn't agree more that cancer is a multifaceted process, but again the importance of our system is that we can focus on the migration part and have a simple tissue that can promote migration with Ras oncogene overexpression. What we want to study is not the whole cancer process but rather only cell migration and obviously all components are there to support it. Such a model system bares the qualities of any good Drosophila model system, incuding the border cell migration in the Drosophila ovary which is highly appreciated. I would thus greatly appreciate if you could reconsider our manuscript in the light of this perspective.
Thank you for your time and consideration.

Correspondence – editor’s reply 08 August 2011

Thank you for your email asking me to reconsider my decision on your manuscript. After discussing it with other members of our editorial team and receiving advice from one expert member of our Editorial Board, we have decided to send it out for peer-review.
Please note that we cannot predict the outcome of the reviewing process, which may turn out to be the same.
I will get in touch with you again as soon as I receive the comments from the referees.

Yours sincerely,

Editor
EMBO reports

2nd Editorial Decision 05 October 2011

Thank you for the submission of your research manuscript to our journal. I apologize for the delay in getting back to you with a decision but I was waiting for a third referee report that was never sent. I have received the reports from two referees that I copy below.

Both referees consider your findings interesting but share two major concerns. First, they agree in that the experimental system needs a much better description in terms of description of the behavior of the hindgut as compared to the midgut, the kind of cell types involved in the migration process or the identification of the migration / passive dissemination mechanism to which the cells are subjected. Second, they also consider that the link between the migration phenomenon and the Imd/JNK pathway should be more firmly established and suggest a number of additional experiments to further strengthen your conclusions. As you will see detailed in their reports, they also have other minor concerns that need to be addressed.

Given this comments from the referees, publication of your manuscript in our journal, as it stands, cannot be considered at this stage. However, as both referees state the potential interest of your findings, I would like to give you the opportunity to address the reviewers concerns and would be willing to consider a revised manuscript with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board.

Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Should you decide to embark on such a revision, revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Also, the length of the revised manuscript may not exceed 28,000 characters (including spaces) and maximally 5 figures may be presented in the main manuscript file. Supplementary figures should further ideally directly relate to one of the main figures in the manuscript and the number of supplementary figures should be restricted to a maximum of 5. Materials and Methods essential for the understanding of the experiments/analyses described in the main body of the manuscript must be included in the main manuscript file. Statistical analyses must be described either in the Materials and Methods section or in the legend of the figure to which they apply. Please include a definition of the error bars used and, when necessary, state the statistical significance of the results and the method used to calculate it.

I look forward to seeing a revised version of your manuscript when it is ready.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

In this article, Bangi and colleagues show that bacterial infection in the hindgut of Drosophila synergizes with a genetically induced pre-cancerous state to promote the delamination and migration of some hindgut cells. The authors first demonstrate that ectopic activation or the Ras oncogene in the hindgut of Drosophila induces morphological changes and promotes cell delamination. The authors provide evidence that this phenomenon correlates with the induction of the metalloprotease MPP1 by the JNK pathway. Interestingly, infection with virulent or avirulent strains P. aeruginosa promotes a stronger and faster "cell migration" phenotype. This effect was shown to be dependent on Imd pathway, specifically the Imd/TAK1 branch regulating the JNK pathway. Finally, the authors show that clearance of bacteria with antibacterial compounds reverts the "cell migration phenotype".
Experiments showing the synergy between a bacterial infection and a pro-oncogenic state are convincing but were already shown in their previous paper (Apidianakis PNAS 2010). The most interesting result is the observation that ras(V12) enterocytes migrates to the body cavity of the fly, mimicking one key aspect of cancerous cells. However, we feel that despite the originality of this finding, the current extent of experiments does not warrant publication as an original research paper

in EMBO R. Points that should be addressed are indicated below.

Major suggestions
1) This paper underlines the medical relevance of their findings but does not give enough indications on the biological context. More information should be provided on the hindgut tissue (by adding reference to previous papers in the field and not only their own work). The response of the hindgut to bacterial infection should be presented. Is it different to that described for the midgut? Does bacterial infection of the hindgut induced cell proliferation and epithelium renewal as in the midgut? The authors have previously shown the existence of a synergy between ras and infection in the case of the midgut. We should know whether bacterial infection also triggers midgut cell migration. If hindgut and midgut enterocytes differ, it would be interesting to propose hypothesis explaining the difference between these two tissues.

2) What are the cells that migrate into the body cavity? Cells that migrate are identified through the byn-Gal4, uas-GFP marker. This is not sufficient to characterize their nature. We should know i) what are they exactly (stem cells, enterocytes, enteroendocrines) using additional markers, ii) whether they are still dividing and, iii) what are their long-term fate.

3) The abstract states "The former converges with RasV12signaling on cJun-N'-Kinase (JNK) pathway activation, culminating in extracellular matrix degradation". This is a shortcut since the regulation of MMP1 by the JNK in the hindgut is not formally demonstrated. It seems to me that JNK activity and MMP1 expression patterns are different.

4/ Homozygous mutants of the imd pathway are available and viable. Experiments testing the interaction between Ras and the imd pathway should be repeated with null mutants of this pathway. Over-expression of bskDN can lead to ectopic effects. Experiments should be repeated using an another method to block the JNK pathway (ex. bsk/JNK RNAi).

5) The paper is difficult to read due to an inflation of redundant words and adjectives. Below are examples that reveal a lack of rigor in the terminology:
Page 2"in the process of basal delamination" do the author means that cells delaminates on the basal side? This should be clarified that this not the typical delamination process.
Page 2: "The hindgut showed reduced and intermittent laminin staining in the
gaps where hindgut epithelial" Intermittent suggest a interruption in time rather than space (punctuated may be more appropriate).

"In addition, coexpression of Ras1V12 with an activated form of Rel (relD) for 4 days in the absence of infection led to a low penetrant, predominantly weak migration phenotype that is drastically weaker than that of RasV12-Imd coexpression, and not statistically different from that of RasV12 alone (Fig. 4c)". This could be said in a simpler way!!!
Page 5: "can boost migration in early, weak migratory stages" idem
"We noticed that under these low RasV12 expression conditions flies are migration-free for at least 7 days, showing only weak migration at 14 days in a small percentage of the flies (Fig. 5a)" idem
"Such pathways may be utilized in an oncogenic background in order to divert part of the innate immune response towards tumor cell migration pathways, similarly to the Imd-dTab2-dTak1signaling feeding on JNK in the presence of RasV12 oncogene that facilitates MMP1 production(Fig. 5d)." This sentence is unclear.

"Instead, we find that pathogenic bacteria exert a sustained activation of an innate
immunity branch that cooperates with oncogenic Ras to facilitate the degradation of the basement membrane and promote migration." This sentence and other in the discussion: the term pathogenic bacteria is not adequate since non-pathogenic bacteria also induces the same stage.
Page 9: ileum: what means the term ileum in regard to the Drosophila digestive tract. There is a great difference between stating that the hindgut is analog to the ileum and calling the drosophila gut "ileum".

Referee #2:

Apidianakis and colleagues in this study propose that sustained infection with human intestinal colonizer Pseudomonas aeruginosa of adult flies expressing a Ras1V12 transgene in the hindgut induces enterocytes migration away from the hindgut to distant sites. They also propose that the synergy involves activation by the bacteria of the Imd-dTab2-dTak1 signalling pathway of the innate immunity. Though the synergistic effect of co-expressing Ras1V12 and Imd is remarkable, the claims that pathogens activate the endogenous Imd/JNK branch of the innate immune system to enhance the 'migration' phenotype is not compelling and in part inferred on what it is known in other systems.

The phenotypic read-out is novel and should be more thoroughly described due to its interest.
- byn-Gal4 pattern should be more extensively characterised. It is striking that some figure graphs show a weak or moderate 'migration' phenotype in controls.
- immunofluorescence analyses and DAPI staining are necessary to convince of the phenotypic read-out. For example, Fig. 1s-w 'migrating' cells. Are all these GFP-positive dots cells? Some dots might be apoptotic cells debris? The image in Fig 1q-r highlights this issue: Here one can see cell outlines and nuclei labelled with GFP and superimposed to them there are GFP-positive dots (debris??)

The expression of MMP1, the claims its induced by the JNK upon Ras1V12 expression (alone or with the pathogens), and that the migration is MMP-associated are weakly supported. though there are pieces of data suggesting this direction..
- MMP and pucZ double labelling is necessary. It should be shown in the bsk/hepACT conditions or with the inhibitor that expression of MMP is altered or that HepAct indeed induces MMP in this system. Actually, it should be shown also the cooperation between HepAct and Ras1V12
- It is not clear whether the invasive foci in the hindgut (arrowheads) co-localize with MMP1 and pucZ?
- A better MMP staining in wild type has to be presented to see endogenous domain of MMP1 in the whole hindgut.
- Please discuss the fact that pucZ positive cells (large nuclei), MMP overexpression, and protruding foci (small nuclei, e.g. fig.1k and also 3f-g) do not appear to happen together.

Reversibility of the migration phenotype is inaccurate. The bacterial clearance shows the necessity of sustained infection/inflamation and the clearance reduces dissemination back to Ras1V12 background levels ("reverse the enhancement" is formally correct but we find this expression misleading as it stands).

Throughout the text there is a repeated suggestion that Ras1V12 triggers tumour cell migration, but this is not verified. Passive tumour cell dissemination is an alternative possibility in which bacteria-induced injury (even though the strain is attenuated) and subsequent regeneration may account for this through extrusion of over-proliferating Ras1V12-cells; Indeed note in cross section images (1h-k) that local foci in the hindgut are between perfectly intact epithelium and visceral muscles with reduced laminin.

Cell migration is a dynamic process that requires continuous assembly and disassembly of cell-cell and cell-matrix adhesions that critically depends on interactions between tumour cells and their surrounding microenvironment, which the authors do not explore. Passive dissemination and benign metastasis are not uncommon events in human cancers.

A confirmation of some of the observations using clonal analyses (flp-out clones of Ras1V12) is paramount to more convincingly support their model of bacteria infection-Ras1V12 synergy. Imd+RasV12 synergy mechanistically explained by upregulation of MMP and laminin degradation: please show co-localization with MMP. Also rescue of MMP mediated dissemination by i.e. TIMP overexpression. MMP1 mutants, etc

I suggest the authors modify the part of the text in which they claim that the infection CF5 strain led

to a highly penetrant migratory phenotype after four days of expressing Ras1V12. The effect has to be described with percentages since a qualitative description might be misleading: the result can be also considered modest because what increases is primarily the group of flies with 1-3 GFP-foci, which is a very low expressivity phenotype given the robust and extended expression of Ras1V12 in the whole hindgut. Moreover, given that overtime the 'weak' phenotype occurs in controls, perhaps it would be useful to score more robust phenotype (>5 foci).

The moderate effect of the inhibitor and of bskDN does not sustain the claims that the severe migration phenotype is JNK gain promoted. Moreover, if any, it shows a requirement for JNK. To verify that the phenotype is indeed JNK promoted they should show that hepAct synergise with Ras1V12, and also show that it induces MMP. Again clonal analyses would resolve the important apparent discrepancy between pucZ positive cells (large nuclei) and invasive cells and 'free' GFP foci (small nuclei) as shown in the figures.

In sum, the proposed model that "pathogens accomplish the synergism with Ras1V12 by inducing JNK pathway activation in hindgut cells which in turn induces MMP1 expression required to degrade the basement membrane" requires more work to be compelling. The findings are of interest and potential relevance for human colon cancer, however interpretation of the phenotypes and genetic/bacterial infection interactions as shown in (fig5c-d) without direct evidence or clonal analyses seems an overstatement.

1st Revision - authors' response 09 February 2012

We revised our manuscript according to all comments of both reviewers. Essentially all comments helped us to improve our manuscript. The major concern about the comparison between midgut and hindgut is now thoroughly addressed in terms of damage and regeneration, as well as differences in immune response and migration potential. In addition, using a water immersion lens and confocal microscopy we provide now in vivo evidence that the large GFP foci that we observed in distant sites are composed of multiple cells. We also include examples of smaller foci with one or two cells only.

Regarding the other major concern to firmly establish Imd, JNK and MMP1 in the migration process we now provide strong genetic evidence for the necessity of all three factors for the full migratory potential of hindgut cells. Furthermore, we conducted the proposed colocalization studies of JNK activation with MMP1 expression, which helped us refine our proposed model to suggest the dynamic and non-autonomous expression of these factors.

A detailed point by point response is provided below for your assessment. We hope that you will find our responses satisfactory and we look forward to the acceptance of our manuscript.

Referee #1:

1) *"This paper underlines the medical relevance of their findings but does not give enough indications on the biological context. More information should be provided on the hindgut tissue (by adding reference to previous papers in the field and not only their own work)."*

We now add the two recent references describing hindgut stem cells and progenitors (Takashima et al., Nature 2008; Fox and Spradling, Cell Stem Cell 2009), two references showing the role of p38 in hindgut stress response (Seisenbacher et al. PLoS Genet. 2011; Chen et al. Proc Natl Acad Sci U S A. 2010) and two on *Drosophila* hindgut and midgut cell physiology (Murakami and Shiotsuki. J Morphol. 2001; Shanbhag and Tripathi. J Exp Biol. 2009).

2) *"The response of the hindgut to bacterial infection should be presented. Is it different to that described for the midgut?"*

We assessed the expression of antimicrobial gene reporters for Drosomycin, Diptericin and Cecropin. Cecropin is expressed strongly in the hindgut progenitors and enterocytes, but not in the midgut, upon infection. Diptericin is only induced in the very anterior midgut (cardia and adjacent region). Drosomycin is weakly and constitutively expressed in the very anterior midgut (cardia). We present the new results about Cecropin in supplementary figure 2 showing that *cecZ* is expressed strongly but non-uniformly in pylorus but also in the ileum region of the hindgut.

3) *"Does bacterial infection of the hindgut induced cell proliferation and epithelium renewal as in the midgut?"*

We have previously shown that bacterial infection with a virulent *P. aeruginosa* strain induces abundant proliferation in the midgut (Apidianakis et al., 2009). BRDU labeling experiments in the hindgut showed no BRDU positive cells in uninfected and *P. aeruginosa* infected hindguts, while midguts from the same animals showed large numbers of BRDU positive cells, particularly following virulent infection. Thus, infection does not directly lead to damage and regeneration in the hindgut as it does in the midgut. Nevertheless, when Ras^{V12} is overexpressed, we notice BRDU-positive cells in the pylorus, indicative of proliferation, but no differences either between uninfected and infected flies or between flies infected with the virulent versus the non-virulent strain. Therefore, we conclude that the enhancement of hindgut cell migration by infection is not due to regeneration as seen in the midgut, where the virulent and only the virulent strain is able to induce it (Apidianakis et al., 2009). We now dedicate supplementary figure 3 to the description of these new data.

4) *"The authors have previously shown the existence of a synergy between ras and infection in the case of the midgut. We should know whether bacterial infection also triggers midgut cell migration."*

To assess this we used the *esg-GAL4* expressing Ras^{V12} in the midgut progenitor cells, which in conjunction with infection induces midgut Ras^{V12}-expressing tumors in less than a week (Apidianakis et al., PNAS 2009). We followed these animals for 3 weeks after the induction of the Ras^{V12} transgene and found no evidence of migration using our migration assay. Furthermore, microbial infection did not induce migration in *esg-gal4>*Ras^{V12} animals (page 5).

5) *"If hindgut and midgut enterocytes differ, it would be interesting to propose hypothesis explaining the difference between these two tissues."*

We hypothesize that the absence of migration from the midgut as opposed to the hindgut might be related to the fast cell turnover of the midgut cells that might eliminate cells amenable to migration. Upon Ras^{V12} expression or infection the hindgut cells, rather than dying and being shed or growing additional layers towards the lumen like midgut cells, they might escape through the basal side into the abdominal cavity. We note now on page 5 that this might be because a) hindgut cells are more resistant to stress and apoptosis (Seisenbacher et al. *PLoS Genet.* 2011; Chen et al . *Proc Natl Acad Sci U S A.* 2010), b) they induce a stronger immune response (Supplementary Figure 2) and have a different physiology and metabolism (Murakami and Shiotsuki. J Morphol. 2001; Shanbhag and Tripathi. J Exp Biol. 2009).

6) *"What are the cells that migrate into the body cavity ? Cells that migrate are identified through the byn-Gal4, uas-GFP marker. This is not sufficient to characterize their nature. We should know i) what are they exactly (stem cells, enterocytes, enteroendocrines) using additional markers, ii) whether they are still dividing and, iii) what are their long-term fate."*

The cells that migrate are identified via the expression of *byn-GAL4*, which is a marker or hindgut enterocytes and progenitors. One limitation of our assay is that migrating cells should be monitored live because any staining protocol leads to their detachment and dispersal away from the tissues. This prevented a detailed molecular characterization of the GFP foci. Furthermore, since the GFP transgene labels both the cytoplasm and the nucleus, we were not able to discern whether these foci were composed of multiple cells. To bypass this limitation we co-expressed along with Ras^{V12} and GFP a nuclear dsRed and using a water immersion lens and confocal microscopy we visualized the presence and number of nuclei in the GFP foci within the abdominal cavity. We noticed that while most of the foci have one or more nuclei (Figure 1W,X), the larger foci contain multiple nuclei (Figure 1Y), indicating that foci contain live cells that might form clusters after they migrate or divide in remote sites. Because the number of foci increases over time (Figure 1Z), either migrating cells do not die or the rate of cell migration is higher than the rate of death.

7) *"The abstract states "The former converges with RasV12signaling on cJun-N'-Kinase (JNK) pathway activation, culminating in extracellular matrix degradation". This is a shortcut since the regulation of MMP1 by the JNK in the hindgut is not formally demonstrated. It seems to me that JNK activity and MMP1 expression patterns are different."*

To address the point whether JNK activation suffices to induce MMP1 we expressed the *Drosophila* JNK kinase *hep* in the hindgut using *byn-GAL4* and we detected uniform MMP1 expression throughout the hindgut.
To address the issue of overlap between JNK activity and MMP1 expression we assessed *pucZ* and MMP1 in the hindguts expressing Ras^{V12} and find a partial overlap between these two markers: Only a small fraction of Ras^{V12}-expressing cells activate JNK signaling and only some of the *pucZ*-positive cells show MMP expression. It is possible that there might be a delayed expression of MMP1 in cells that transiently induced *pucZ* and/or *pucZ* cells might induce MMP1 non-autonomously in their neighboring cells. We now refine our proposed model accordingly throughout the manuscript and in the illustration of Figure 5C.

8) *"Homozygous mutants of the imd pathway are available and viable. Experiments testing the interaction between Ras and the imd pathway should be repeated with null mutants of this pathway. Over-expression of bskDN can lead to ectopic effects. Experiments should be repeated using an another method to block the JNK pathway (ex. bsk/JNK RNAi)."*

We assessed migration in homozygous mutants for *Imd* or *Tak1* (another gene in the Imd pathway) and noticed that the enhancement of migration due to infection is almost abolished at 4 days following Ras^{V12} expression (Figure 4E)
In addition, we co-expressed Ras^{V12} together with JNK RNAi or Jun RNAi. We noticed that both reduce the induction of migration, similarly to JNK DN (Figures 2E, 4B).

9a) *"The paper is difficult to read due to an inflation of redundant words and adjectives. Below are examples that reveal a lack of rigor in the terminology:*
Page 2"in the process of basal delamination" do the author means that cells delaminates on the basal side? This should be clarified that this not the typical delamination process."

Basal delamination has been described for *Drosophila* and mammalian epithelia (Hogan et al., Nat. Cell. Biol. 2008) and we now substitute "basal delamination" in the text with "cells delaminating through the basal side of the epithelium".

9b) "*Page 2: "The hindgut showed reduced and intermittent laminin staining in the gaps where hindgut epithelial" Intermittent suggest a interruption in time rather than space (punctuated may be more appropriate)."*

We substitute now the word "intermittent" with "non-uniform".

9c) *"In addition, coexpression of Ras1V12 with an activated form of Rel (relD) for 4 days in the absence of infection led to a low penetrant, predominantly weak migration phenotype that is drastically weaker than that of RasV12-Imd coexpression, and not statistically different from that of RasV12 alone (Fig. 4c)". This could be said in a simpler way!!!"*

This sentence now reads: "Also co-expression of an activated form of Rel (relD) for 4 days failed to enhance Ras1V12 induced migration".

9d) *"Page 5: "can boost migration in early, weak migratory stages" idem*
"We noticed that under these low RasV12 expression conditions flies are migration-free for at least 7 days, showing only weak migration at 14 days in a small percentage of the flies (Fig. 5a)" idem"

The first sentence is now deleted. The second now reads: "Migration was not observed at 7 days, and only weak, low penetrant migration was seen at 14 days in uninfected flies".

9e) "Such pathways may be utilized in an oncogenic background in order to divert part of the innate immune response towards tumor cell migration pathways, similarly to the Imd-dTab2-dTak1signaling feeding on JNK in the presence of RasV12 oncogene that facilitates MMP1 production(Fig. 5d)." This sentence is unclear.

This sentence is now deleted.

9f) ""Instead, we find that pathogenic bacteria exert a sustained activation of an innate immunity branch that cooperates with oncogenic Ras to facilitate the degradation of the basement membrane and promote migration." This sentence and other in the discussion: the term pathogenic bacteria is not adequate since non-pathogenic bacteria also induces the same stage."

We now delete the term "pathogenic bacteria" throughout.

9g) *"Page 9: ileum: what means the term ileum in regard to the Drosophila digestive tract. There is a great difference between stating that the hindgut is analog to the ileum and calling the drosophila gut "ileum".*

Insects unlike vertebrates have a hindgut comprised of an anterior pylorus, which is followed by the main hindgut part called ileum which contains the differentiated enterocytes (please see Figure 1a) (Fox and Spradling, Cell Stem Cell 2009). In order to remain consistent with previously published papers describing the *Drosophila* hindgut we did not make any changes in our terminology.

Referee #2:

1) *"byn-Gal4 pattern should be more extensively characterised."*

byn-GAL4 has been characterized as directing expression in the hindgut ileum and the differentiating cells of the pylorus region (Fox and Spradling, Cell stem Cell 2009). Thorough examination of all adult tissues reveals that this GAL4 is also expressed in the salivary glands (as most GAL4 lines do). Since *esg-GAL4* also shows expression in the salivary glands and the midgut progenitor cells, and does not induce any migration when used to overexpess Ras^{V12}, we conclude that the GFP positive foci we observe in the abdominal cavity originate from the hindgut, not the salivary glands. We now make a pertinent note in the Materials and Methods section of our manuscript.

2) *"It is striking that some figure graphs show a weak or moderate 'migration' phenotype in controls."*

The penetrance of migration in the non-infected wild type flies mentioned by the reviewer is

1 out of 60 flies and this fly only showed 1 GFP focus. Overall, the penetrance of migration in our wild type animals was 1% (n=264) and in all cases we found only one GFP positive focus. And unlike Ras^{V12}-induced migration, this background phenotype in control animals never got stronger with time. Therefore, we believe this to reflect the noise of the migration assay. In infected wild type flies a weak (but never a moderate or strong) migration can be seen in 3-6 out of 60 flies. We now make a note about this in the Materials and Methods section of our manuscript. Nevertheless, this background migration is drastically different from the migration noticed in Ras^{V12} flies.

3) *"immunofluorescence analyses and DAPI staining are necessary to convince of the phenotypic read-out. For example, Fig. 1s-w 'migrating' cells. Are all these GFP-positive dots cells? Some dots might be apoptotic cells debris? The image in Fig 1q-r highlights this issue: Here one can see cell outlines and nuclei labelled with GFP and superimposed to them there are GFP-positive dots (debris??)"*

The images in Fig 1S-W are low magnification images (10x objective) taken using a dissecting microscope (compare magnification bars to higher magnification images of hindguts shown in other panels of Figure 1). Based on their size, we estimated these to be composed of multiple cells. However, since the GFP transgene used to label these foci is expressed throughout the cell, we could not discern whether these were intact cells or whether the foci were actually composed of multiple cells. As mentioned in the response to the comment #6 of Reviewer #1, our system is not amenable to immunohistochemistry of the migrating foci. To bypass these limitations, we added a nuclear dsRed construct into the background and used a water immersion lens and confocal microscopy to take higher magnification live images of these foci. We show now in Figure 1W-Y that the foci are comprised of 1 or more nuclei indicating the existence of live single cells as well as larger clusters of cells. As for the larger cell outlines the reviewer refers to as being in the background, these are large fragments of fat body and nephrocytes, unusually large podocyte-like cells that surround the heart tube.

4) *"It should be shown in the bsk/hepACT conditions or with the inhibitor that expression of MMP is altered or that HepAct indeed induces MMP in this system. Actually, it should be shown also the cooperation between HepAct and Ras1V12"*

We now present results that show that when *hep* is expressed in the hindgut, MMP1 expression is induced (Fig 2F,G). Furthermore, co-expression of *hep* with Ras^{V12} induced a significant increase in migration (Figure 4B).

5) *"MMP and pucZ double labeling is necessary. It is not clear whether the invasive foci in the hindgut (arrowheads) co-localize with MMP1 and pucZ? A better MMP staining in wild type has to be presented to see endogenous domain of MMP1 in the whole hindgut. Please discuss the fact that pucZ positive cells (large nuclei), MMP overexpression, and protruding foci (small nuclei, e.g. fig.1k and also 3f-g) do not appear to happen together."*

Please see also response to the reviewer #1 comment #7. As this reviewer notes, we do not see colocalization between *pucZ* and protruding hindgut cells. We did MMP1 *pucZ* double stainings and we revise our manuscript to show that there is a partial overlap between the MMP1 and *pucZ* expressing cells. JNK signaling is most likely transient and is followed by MMP1 expression in the same or the neighboring cells. We propose that JNK activation and MMP1 expression are not cell-autonomously required in the migrating cells themselves. These cells contribute to migration of their neighbors by secreting MMP1 and thereby leading to a weakening/degradation of the basement membrane. Infection enhances migration by increasing the number of JNK positive and MMP expressing cells, and increasing the migratory potential of their neighbors. Our observations that *hep* can induce MMP1 expression (Figure 2F,G) and *hep* expression increases migration (Figure 4B) are also consistent with this model. We incorporate these findings into the discussion of our model in the text.
Noticeably, MMP1 is not expressed in wild type hindguts. Therefore, the MMP channel in our control hindguts appears black. Under the same conditions MMP1 staining is evident in

the Ras^{V12}-expressing hindguts.

6) *"Reversibility of the migration phenotype is inaccurate. The bacterial clearance shows the necessity of sustained infection/inflamation and the clearance reduces dissemination back to Ras1V12 background levels ("reverse the enhancement" is formally correct but we find this expression misleading as it stands)."*

Per reviewer's suggestion we revised the text eliminating the use of "reversibility of the migration phenotype" substituting it with the phrase "no significant differences in migration were observed between previously infected and non-infected flies".

7) *"Throughout the text there is a repeated suggestion that Ras1V12 triggers tumour cell migration, but this is not verified. Passive tumour cell dissemination is an alternative possibility in which bacteria-induced injury (even though the strain is attenuated) and subsequent regeneration may account for this through extrusion of over-proliferating Ras1V12-cells; Indeed note in cross section images (1h-k) that local foci in the hindgut are between perfectly intact epithelium and visceral muscles with reduced laminin.*
Cell migration is a dynamic process that requires continuous assembly and disassembly of cell-cell and cell-matrix adhesions that critically depends on interactions between tumour cells and their surrounding microenvironment, which the authors do not explore. Passive dissemination and benign metastasis are not uncommon events in human cancers."

We thank the reviewer for this illuminating comment. To clarify this issue we assessed proliferation upon potential infection-induced damage along the hindgut epithelium (please see experiment description in response to reviewer#1 comment #3) and we find no evidence of cell proliferation due to infection. Therefore, bacteria-induced injury and subsequent local overproliferation or regeneration can not account for the enhanced migration of Ras^{V12} cells upon infection. While we value the comment that migration is different from passive dissemination, i) the basement membrane degradation, MMP1 expression and the basal delamination of cells rather than their luminal shedding, ii) the cytoplasmic processes seen during the cell delamination phase and those of foci cells, iii) the attachment of migrating cells in tissues and to each other and v) the progressive accumulation of migrating cells support the notion that Ras^{V12} cells are actually migrating towards the abdominal tissues rather than passively disseminating. Yet we agree that this migration might only be benign requiring additional mutations or factors (as commonly suggested) in order to establish secondary tumors and metastasis. We now comment on this issue in our discussion.

8) *"A confirmation of some of the observations using clonal analyses (flp-out clones of Ras1V12) is paramount to more convincingly support their model of bacteria infection-Ras1V12 synergy."*

Flip out experiments are usually valuable to determine the cell autonomous roles of signaling pathways. Nevertheless, they should be approached with some caution because the signaling circuits of genetically homogenous tissues might differ from those with genetic heterogeneity. In addition flip out clones are not helpful for the assessment of migration because clones arise everywhere (i.e. also in the abdominal tissues) and cannot be distinguished from migrating foci in the abdominal cavity. With these caveats in mind we generated flip out clones to look at MMP1 and *pucZ* expression in the hindgut. We present examples of these clones in the new supplementary figure 1. We found that *pucZ*-positive or MMP1-expressing cells were very rare in Ras^{V12} flip out cells and that in rare cases where MMP1 is expressed, it only partially overlaps with the borders of the clones. These data are compatible with our model that Ras induces JNK transiently, which in turn induces MMP1 autonomously and/or non autonomously.

The low number of *pucZ*-positive cells and MMP1 expression in Ras^{V12} FLP-out clones might not be surprising because JNK pathway activation in the context of wild type neighboring cells leads to rapid elimination of the JNK positive cells from the epithelium (McEwen and Peifer. Development 2005). On the other hand, uniform expression of Ras^{V12}

throughout the hindgut epithelium might allow the survival of more *pucZ*-positive cells and induction of MMP1 expression.

9) *"Imd+RasV12 synergy mechanistically explained by upregulation of MMP and laminin degradation: please show co-localization with MMP. Also rescue of MMP mediated dissemination by i.e. TIMP overexpression. MMP1 mutants, etc"*

Our results show that a-MMP1 staining is clearly enhanced synergistically by *Imd* and Ras^{V12}. MMP1 is a secreted protein and our new *pucZ*/MMP1 colocalization studies suggest that MMP1 expression might be transient or non autonomous (see response to comment #5 of this reviewer). Thus, we cannot localize it at the cellular level in comparison with invading cells.
To assess the effect of MMP on migration, we overexpressed TIMP together with Ras^{V12} and found that MMPs are necessary to induce full migration (Figure 2E).

10) *"I suggest the authors modify the part of the text in which they claim that the infection CF5 strain led to a highly penetrant migratory phenotype after four days of expressing Ras1V12. The effect has to be described with percentages since a qualitative description might be misleading: the result can be also considered modest because what increases is primarily the group of flies with 1-3 GFP-foci, which is a very low expressivity phenotype given the robust and extended expression of Ras1V12 in the whole hindgut. Moreover, given that overtime the 'weak' phenotype occurs in controls, perhaps it would be useful to score more robust phenotype (>5 foci)."*

We now refer to the 4 day induction of migration with percentages i.e. an increase in the penetrance of migration from 10-15% to ~50% (page 5), which is reproducible and statistically significant. Nevertheless, the scoring of weak migration phenotypes is essential too for the statistical assessments of differences at this early time point. Our findings that migration is induced earlier upon infection of Ras^{V12} flies are corroborated by the results of Figure 5A.

11) *"The moderate effect of the inhibitor and of bskDN does not sustain the claims that the severe migration phenotype is JNK gain promoted. Moreover, if any, it shows a requirement for JNK. To verify that the phenotype is indeed JNK promoted they should show that hepAct synergise with Ras1V12, and also show that it induces MMP. Again clonal analyses would resolve the important apparent discrepancy between pucZ positive cells (large nuclei) and invasive cells and 'free' GFP foci (small nuclei) as shown in the figures."*

Overexpression of hep^{ACT} alone in the hindgut causes lethality within 4 days after transgene induction. For this reason, we carried out the experiment the reviewer suggests using wild-type *hep*. We show that *hep* expression alone is sufficient to induce uniform MMP1 expression in the hindgut (Figure 2F,G) and that co-expression of *hep* and Ras^{V12} significantly enhances migration. (Figure 4B). In addition, we strengthen our data about requirement for JNK on migration, showing that JNK^{RNAi} and Jun^{RNAi} reduce migration similar to bsk^{DN} (Figures 2E, 4B).
Regarding the clonal analysis and *pucZ* and the invading cells, please see response to comment #8 of this reviewer.

3rd Editorial Decision 01 March 2012

Thank you for your patience while we have reviewed your revised manuscript.
Your manuscript has been sent again to both referees and, as you will see from the reports below, while referee #1 is reasonably content with your revision, referee #2 still raises some issues to which I would like to draw your attention.

Referee #2 agrees with referee #1 that your manuscript has been improved. However, two fundamental concerns still remain. First, the fact that the JNK pathway directly activates MMP-1 expression is not sufficiently substantiated by your experimental evidence in this context and, second, the migration phenotype of the disseminating cells has not been properly analyzed. After further exchange with this referee, s/he considers that further experimental work would not be necessary as long as the interpretations of the data in the manuscript are toned down. S/he particularly remarks that "the authors should describe the GFP positive cells in the abdomen as "disseminated cells" unless they are ready to characterize the behavior of these cells in vivo or in vitro by standardized tools to monitor migratory behavior". I would therefore like to ask you to thoroughly revise your manuscript text accordingly, including your title and abstract.

I look forward to seeing final version of your manuscript when it is ready.

Yours sincerely,

Editor
EMBO reports

REFEREE REPORTS:

Referee #1:

The revised version has been significantly improved. The authors have improved the writing all along the text and the language has been adequately tightened. They have well documented the hindgut response to infection and clarified differences with their previous work in the midgut. The data supporting the synergy between Rasv12 induction in the hindgut and immune activation following ingestion of pathogenic bacteria are convincing. I also find that their additional genetic work to precise the control of MMP1 by JNK is convincing. Altogether, I feel the paper is now acceptable in its current form for publication.

Referee #2:

To the authors
The revised version of the manuscript is improved, but still requires revisions in my opinion. There are still inconsistent parts in the manuscripts and the major criticism has not been satisfactorily clarified or firmly resolved in the revised text.
Thus, in the revised text the authors state: "Of note, delaminating enterocytes did not express detectable pucZ (0%, n=20) and pucZ staining only partially overlapped with MMP1 expression (Fig 2C,D and supplementary Fig S1)..... In spite of this restatement, the abstract states: "..... .... which converges with RasV12 signaling on JNK pathway activation, culminating in extracellular matrix degradation". In my opinion, this abstract is an over-simplification or over-interpretation of the evidence presented in the study.
The (only) attempt to correlate MMP1 activation (staining) and cell invasion (authors: migration) is figure 3G. However, as discussed in my previous report, the ectopic labelling of MMP1 is far away from the delaminating enterocytes (pointed by the arrowheads in the image). This image does not support the claim of the authors that "JNK induces MMP1.... to facilitate migration of hindgut cells. It is also arguably the use of 'migration' in this context. MMP1 may induce the invasive process downstream of the Jnk pathway. However, this would be a conjecture based on previous knowledge of MMP and Jnk but it cannot be wholly concluded from the authors' evidence. The authors show a broad activation of MMP1 upon hep overexpression and various examples hindguts with ectopic MMP1 but there is no firm confirmation that activation is associated with invasive behaviour.
In addition, whether infection or the overexpression of RasV12 induces hindgut cell migration remains formally untested. The authors present single time-point analyses or images of fixed material. Therefore, statements such as that: [.... a striking potentiation of migration (Fig. 3E) coupled with increased MMP1 expression (Fig.3F, G)] are inaccurate, unless they are confirmed with direct evidence such as in vitro cell migration assays or time-lapse analyses. What the authors show is an increase in the number of disseminated cells or increased MMP1 activation in the hindgut. The rest is an interpretation.

The response to comment (#7) is not entirely satisfactory as the authors use arguments such as MMP1 activation, cytoplasmic processes, attachment of 'migrating' cells in tissues, which are not shown or proven in the study (so cannot be verified by the reviewer). Finally, in the revised version the authors found single cell foci and foci formed by group of cells. This is a potentially interesting finding which is barely discussed. Collective cell migration shares some similarities but also fundamental differences to individual migrating cells. Does RasV12 induce both types of cell migration in the hindgut?? Is there precedent for such a thing in other systems?
Other minor comments:
• there is a lack of consensus in how the authors referred to Ras oncogene in the text and the various figures. For example; RasV12, Ras1V12, ras.V12). The FlyBase preferred name is Ras85D. In addition, RasV12 is an oncogenic form [isoform is better used for proteins produced by alternatively processed pre-messenger RNAs].
• methods are incomplete, some strains are not listed or described.
• what is anterior or posterior in the images. For example, images in figure 3F and G appear to be upside down (anterior is down?)

2nd Revision - authors' response 02 March 2012

Thank you for your efforts to clarify the remaining issues with the 2nd reviewer. In order to make sure that our modifications are the most appropriate for the purpose I will summarize the changes we intent to do in our manuscript. These changes will reflect the fact that JNK does induce MMP1 but we will point out (as we actually do quite extensively in the manuscript) that the induction might also be non-autonomous. Also we will modify the text to describe the phenomenon as "basal invasion" and "dissemination" as the 2nd reviewer points in his/her latest response instead of using the term "migration". Lastly the minor comments of reviewer #2 will be fixed, but we do not intent to make any changes in the figures.
1. The title will be: "Immune response to bacteria induces dissemination of Ras-activated Drosophila hindgut cells"
2. The abstract will be: "Although pathogenic bacteria are suspected contributors to colorectal cancer progression, cancer-promoting bacteria and their mode of action remain largely unknown. Here we report that sustained infection with the human intestinal colonizer Pseudomonas aeruginosa synergizes with the Ras1V12 oncogene to induce basal invasion and dissemination of hindgut cells to distant sites. Cross-talk between infection and dissemination requires sustained activation by the bacteria of the Imd-dTab2-dTak1 innate immune pathway, which converges with RasV12 signaling on JNK pathway activation, culminating in extracellular matrix degradation. Hindgut, but not midgut, cells are amenable to this cooperative dissemination, which is progressive and genetically and pharmacologically inhibitable. Thus, Drosophila hindgut provides a valuable system for the study of intestinal malignancies."
3. Reference style, Ras oncogene terminology and strains discription will be fixed.

4th Editorial Decision 06 March 2012

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,
Editor
EMBO Reports

