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c-Src drives intestinal regeneration and transformation

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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.)

Editor: Thomas Schwarz-Romond

1st Editorial Decision 11 December 2013

Thank you very much for submitting your study on the role of src/src-family members in intestinal regeneration and tumor formation for consideration to The EMBO Journal editorial office.

I enclose comments of three referees that commented on the significance and conclusiveness of your work.

While appreciating the combined efforts in the Drosophila and mouse intestinal stem cell systems, all three remain hesitant regarding overall novelty. They therefore demand much stronger, integrating and conclusive molecular analyses to overcome such conceptual reservations.

For my reading, significant further molecular characterization particularly in the mouse system would be needed (please see ref#1 comments re
-robustness/characterization of the phenotypes caused by distinct genetic manipulations;
-link molecular aberrations with the presented survival data,
-address epigenetic ordering/consequences of src-deletion in ApcMin tumors
- clarify expression (and thus function) of p-Src in transient-amplifying cells versus (tumor) stem/progenitor populations
-study effects of SFK-alterations on normal and tumor stem cell functionality.

Similar concerns on the overall conclusiveness of the potentially interesting results are raised by the detailed comments from refs#2 and 3.

I recognize that these requests amount to significant further experimental efforts. I am certain that you see yourself in a strong position to address these in a relatively timely and satisfactorily manner.

Conditioned on such crucial amendments, I am therefore delighted to offer the opportunity to expand and amend the current dataset for a subsequent peer-review assessment at The EMBO Journal.

Please keep me informed in case you find these too demanding and with looming competition might rather decide to seek rapid publication in a less demanding title (please notice however that The EMBO Journal offers 'scooping protection' during formal revisions to facilitate scientifically substantiated investigations and to be able to address referee concerns with convincing experimental data).

For the moment, I remain with best regards. Please do not hesitate to get in touch with me (due to time constrains preferably via e-mail) in case further questions arise, to discuss anticipated timeline/feasibility of certain experiments or indeed proceedings in case o publication of relevant studies during your revisions.

REFEREE REPORTS:

Referee #1:

In this manuscript, Samson, Vidal and colleagues investigate the function of Src in the drosophila and mammalian intestine. They convincingly show that in drosophila, Src activation is required for ISC expansion during homeostasis and upon damage. They also provide evidence that hyperplasia of the drosophila midgut mediated by APC loss of function requires Src activity. From the mechanistic point of view, Src upregulates Egf receptor levels and activates Stat3 in ISCs. In addition, authors provide evidence that Src is also involved in tumor initiation in the mouse small intestine downstream of APC activation. I found the manuscript interesting. Experiments in drosophila are compelling and convincing. However, the analysis of Src function in the mammalian intestine is somewhat more preliminary. I detail this criticism below;

1. The phenotype of the mouse intestine deficient for Src, Fyn and Yes is poorly characterized. My impression from the pictures in figure 5 is that there are no major abnormalities or, if anything, an elongation of the crypts. Authors should explore more in depth the functions of Src in intestinal homeostasis.
2. Along the lines above, authors mention that mice compound for Src;Fyn;Yes deletions are in bad shape after 4 days of Cre induction. Is this because of intestinal alterations or rather because of a liver phenotype since Ah-Cre is also expressed in this tissue?
3. Authors show increased survival of mice with compound deletions in Apc (or carrying ApcMin/+) and Src. However, the reason behind this phenotype is not studied. Is there reduced tumor burden and/or size? What are the effects of Src deletion in ApcMin tumors after initiation? Do ApcMin adenomas collapse or stop proliferating? Finally, is there reduced pERK and pStat3 in Apc;Src tumors?
4. My impression is that p-Src does not mark Lgr5+ ISCs (Figure 6A) or tumor stem cells at the base of adenoma glands (Figure 1d) but rather the staining seems specific of transient amplifying cells and the upper regions of adenomas. p-ERK shows the same pattern (Figure 6H). Is this appreciation correct? Does Src or Src;Fyn;Yes deficiencies affect normal or tumor stem cell behavior? This is an important piece of information to strengthen the parallels with the results obtained in drosophila.

Referee #2:

The authors address the role of Src kinases in both the mouse and fly intestine models using genetic ablation (mouse), knock-down (fly) and overexpression (fly) techniques. Consistent with previous

reports in human colorectal cancer, the authors find an expansion of the number of Src+ cells in adenomas of APC mice and human samples. Turning to the fly model, they find that overexpression of Src family members in intestinal stem cells and progenitors leads to increased proliferation. Infection of fly guts with pathogenic bacteria known to drive stem cell proliferation causes an increase in pSrc immunoreactivity and a transcriptional increase of Src42 and knockdown of Src42 causes a fairly to mount the proliferative response. A similar transcriptional increase in Src42 is observed upon clonal inactivation of apc1 and RNAi knockdown of Src42 blocks proliferation in apc1 clones, suggesting that Src42 mediates this proliferative response. The authors previous found that upon apc1 inactivation EGFR and Jak/Stat signaling are upregulated and promote overproliferation. Here they show that upon overexpression of Src64, EGFR , pERK and the Stat target Socs36E are upregulated. Moreover, knockdown of EGFR and stat suppress the proliferation induced by Src overexpression in the fly intestine. Turning back to the mouse, they provide nice data that Apc loss requires Src for full tumorigenic effect.

This work nicely mixes the fly and mammalian system to provide new insights into the role of an important proto-oncogene in vivo. These findings suggest that upon Apc loss, Src is upregulated and somehow leads to activation of EGFR and Stat to drive proliferation in flies and mice. While they provide several lines of evidence supporting this, they do not show in one model system all of the data to prove this. They do not show in flies that pSrc is upregulated upon Apc1 inactivation, which could easily be tested. It is also unclear in mouse whether overactivity/expression of Src is sufficient to drive activation of EGFR and Stat to control proliferation. Addressing these points would strengthen the manuscript.

Major points:

1. In Figure 1- some numbers should be given for how many adenomas were assessed and what percent showed increased Src. All of them?

2. A better characterization of the clonal phenotypes of overexpression of Src64 and Src42CA should be done. Do they affect the number of stem cells or only the proliferation rate? In particular the authors should look at Delta staining to see if the number of stem cells are affected.

3. A related point: The characterization of "polyp-like hyperproliferative structures" are misleading as endoreplicating cells encorporate BrdU. Thus, it is more likely that the BrdU+ cells are newly produced enterocytes by faster dividing ISCs and not a cluster of proliferating stem cell-like cells. If this is really a cluster of dividing stem cells, this should be shown by PH3 staining. To me, this is an over-interpretation of the data that will be confusing for readers.

4. Is Src42 really essential for clone growth as stated on p. 9 and p.10 or does it simply promote clone growth? Fig. 2G-J should be complemented with clonal analysis and quantification. In 2J there are some clones that appear to grow and in 3F the average clone size is reduced, though it does not seem as if all cell division stops. It would be helpful to discriminate between being essential for cell division and acting to promote it. What happens over time to these clones? It would be useful to do this experiment in the MARCM context as only dividing cell clones are marked and not quiescent ones as seen in the esgF/O method.

5. Is pSrc upregulated in apc1 mutants in flies? This would be the prediction from the mouse data.

6. p.7 starts by asking whether elevated Src activity was a driver of intestinal proliferation. They say that they "first utilized the adult intestine of Drosophila". Is overexpression of Src sufficient in the mouse intestine to drive proliferation? Their statement suggests that they will test it.

7. The statement on p.11-"which does not involve canonical Jak/Stat signaling...?" is unclear: are they referring to published work, in which case they should cite it, or making a hypothesis based on their finding that dome RNAi did not suppress but stat RNAi did? How do they explain their lack of dome phenotype in this assay?

8. The previously published work in the Drosophila eye (Read, MCB, 2004) links Src with JnK and Jak/Stat. Is there also an effect on Jnk signaling here?

9. The requirement of SFK activity for homeostatic self-renewal (as concluded on p.12) is not rigorously demonstrated. Using the AhCre, which is expressed in a number of tissues, they examine 4d intestines and show that short term loss of SFK activity does not affect self-renewal (in contradiction to their statement). Presumably longer timepoints could not be examined due to the poor health. Their conclusion on homeostasis seems based on the failure of the Src Fyn and Yes triple KO to fail to grow in organoid culture. As this in not in vivo, they should not conclude on a homeostatic function in the mouse intestine. Could they use the Lgr5Cre to have longer-term data that would clarify the role of SFK activity (Src Fyn and Yes triple KO) in homeostasis?

Along similar lines, is there any effect on AhCre Srcfl/fl on the number of crypts without irradiation? Quantification of Fig 6E without irradiation would be informative.

10. In the Discussion- the authors mention a "therapeutic window" in which to affect Src without affecting normal homeostasis as they suggest that Src is not required for normal homeostasis, but is important after irradiation and upon APC loss. Problematically, the effects on homeostasis were not assessed at the longer timepoints as which regeneration and APC interaction were done: Homeostasis upon Src fl/fl was assessed at 4d, regeneration was assessed at 7d, and the affect on APC was tested at 50-120 days. To rigorously conclude about a therapeutic window between homeostasis and a regenerative or an oncogenic function, the Src fl/fl effect on homeostasis should be also assessed at 7d and over the timecourse of the Apc fl/fl experiment. Does Src fl/fl have reduced lifespan relative to controls?

11. It would be nice to compare an image of the tissue of the Apc-/- Src-/- with the apc-/-.

Minor points:

1. It is unclear to me how they picked the crypts with Src knockdown in Figure 6 H-M? Are they the serial sections that were stained one with pSrc and then with pERK and pStat3 or is this based on Rosa26R reporter expression?

2. Fig. 3B the genotypes are confusing- where is the LacZ? It is not listed in their fly stocks -is it in all lines or only the ones labeled "lacZ"?

3. Are the scale bars- 6DvsD' correct?

Referee #3:

Cordero et al characterize the role or Src family kinases in tissue regeneration and tumorigenesis in the fly and mouse intestine. The authors show that Src phosphorylation correlates with the formation of adenomas, that src42A is sufficient and required for ISC proliferation in the intestinal epithelium of wt and APC-deficient flies, that Src induced proliferation of ISCs can be rescued by reducing EGFR and Stat signaling, and that Src family kinases are required for intestinal organoid formation ex vivo, as well as for tumor formation in APC deficient models in vivo. Overall, the study confirms the not too surprising role for Src family kinases in promoting proliferation of intestinal epithelial cells. Mechanistically, the paper falls somewhat short, however, as the epistasis experiments can be interpreted in multiple ways and no clear molecular interactions between Src and Wnt, EGFR or Stat signaling have been studied here. Nevertheless, the evolutionary conservation of the role of Src family kinases shown here is interesting and could be recommended for publishing if the following concerns are addressed:

- the authors say that the 'polyp' like hyperproliferative structures shown in Fig 1 I' are never seen in wild-type flies, but these structure look very much like the aging phenotypes described by Biteau et al in 2008.

- the phenotypes shown in Figs 2G and I look almost identical, countering the author's statement that Src42 is required for homeostatic proliferation and not just for stress-induced proliferation. Indeed,

the 30D timepoint in this experiment is a timepoint where ISCs over-proliferate due to oxidative stress, and an interpretation about Src's role in homeostatic regeneration can thus not be made. The authors have to perform detailed analysis of Src mutant (not RNAi) MARCM clones in young, unstressed backgrounds to establish if Src is required for normal proliferation. The data shown in Fig 3D, where clones are nicely formed when Src is knocked down, would actually argue that Src is specifically NOT required for homeostatic regeneration, but only for stress-induced ISC proliferation.

- In Fig 3A the authors claim a 2-fold induction of Src42 levels, but barely a 1.5fold induction is seen.

- The regulation of EGFR/ERK signaling is not properly interpreted here. The authors argue that pERK is increased in Src gain-of-function conditions, but it is equally possible that Src gain of function simply leads to more progenitors that have high leveld of pERK. The images presented actually suggest that pERK levels do not change in individual ISCs, but that there are simply more ISCs. The same issue emerges in the mouse in Fig. 6. The authors need to develop a strategy to quantify the levels of pERK in individual Src gain-of-function ISCs. The same argument applies to the quantification of pStat.

- With respect to the epistasis experiments, the authors argue that EGFR/RAS acts downstream of Src because loss of EGFR/Ras can rescue the increase in proliferation induced by Src. However, EGFR/Ras deficient ISC do not proliferate under any conditions (see Jiang and Biteau papers on EGFR in fly ISCs) and the argument is thus not valid. The authors would need to show that increased ERK activity can rescue Src deficiency in ISC proliferation to confirm that ERK is downstream of Src.

- No mechanism for the induction of Src phosphorylation in response to injury has been explored (Fig. 6A). Src regulation has been studied extensively over the years and an attempt at explaining this induction should be made.

- I am mystified by the comment made with respect to pSrc in the Src knockout epithelium (Fig. 6D'). The authors discuss that these cells show less pSrc as evidence that epithelial Src is essential to drive regeneration. But since Src is knocked out, wouldn't one expect less pSrc?

1st Revision - authors' response 11 March 2014

We would like to start by thanking the Reviewers for their careful assessment of our manuscript and for their constructive criticism, which has helped us to significantly improve our work. Please find below a point-by-point answer to all the Reviewer’s comments.

Referee #1:

In this manuscript, Sansom, Vidal and colleagues investigate the function of Src in the drosophila and mammalian intestine. They convincingly show that in drosophila, Src activation is required for ISC expansion during homeostasis and upon damage. They also provide evidence that hyperplasia of the drosophila midgut mediated by APC loss of function requires Src activity. From the mechanistic point of view, Src upregulates Egf receptor levels and activates Stat3 in ISCs. In addition, authors provide evidence that Src is also involved in tumour initiation in the mouse small intestine downstream of APC activation. I found the manuscript interesting. Experiments in drosophila are compelling and convincing. However, the analysis of Src function in the mammalian intestine is somewhat more preliminary. I detail this criticism below;

1. The phenotype of the mouse intestine deficient for Src, Fyn and Yes is poorly characterized. My impression from the pictures in figure 5 is that there are no major abnormalities or, if anything, an elongation of the crypts. Authors should explore more in depth the functions of Src in intestinal

homeostasis.

Response: We now present a detailed analysis of crypt length, cell proliferation, migration, apoptotis and differentiation in control, *AhCre; Src$^{fl/fl}$* and *AhCre; Src$^{fl/fl}$; Fyn$^{-/-}$; Yes$^{-/-}$* intestines at 4 days after Cre induction. Our results presented in new Figures 6, S4 and S5 indicate that Src deletion only does not affect normal intestinal homeostasis, while combined loss of Src, Fyn and Yes leads to villae apoptosis and Paneth cell loss in the mouse intestine, with no obvious liver defects. Paneth cells are known to be an essential component of the intestinal stem cell niche. Therefore, altogether data explains the poor survival of the *AhCre; Src$^{fl/fl}$; Fyn$^{-/-}$; Yes$^{-/-}$* mice and the inability of crypts from such genotype to form intestinal organoids in culture.

2. Along the lines above, authors mention that mice compound for Src;Fyn;Yes deletions are in bad shape after 4 days of Cre induction. Is this because of intestinal alterations or rather because of a liver phenotype since Ah-Cre is also expressed in this tissue?

Response: As mentioned in the previous point, *AhCre; Src$^{fl/fl}$; Fyn$^{-/-}$; Yes$^{-/-}$* mice display no-detectable liver defects. Data presented in new Figure S5 presents a detail analysis of overall tissue weight, cell proliferation, apoptosis and fibrosis in livers from control, *AhCre; Src$^{fl/fl}$* and *AhCre; Src$^{fl/fl}$; Fyn$^{-/-}$; Yes$^{-/-}$* mice. Furthermore, mice subject to a reduced Cre induction regime, which leads to complete Cre-dependent recombination in the liver but only partial recombination in the intestine, live over long periods of time with no signs of poor health. New Figure S5K presents an example of liver and intestine form a 30-day old mouse induced with such regime. Altogether these data indicates that the poor survival of *AhCre; Src$^{fl/fl}$; Fyn$^{-/-}$; Yes$^{-/-}$* mice is likely due to intestinal rather than liver defects.

3. Authors show increased survival of mice with compound deletions in Apc (or carrying ApcMin/+) and Src. However, the reason behind this phenotype is not studied. Is there reduced tumour burden and/or size? What are the effects of Src deletion in ApcMin tumours after initiation? Do ApcMin adenomas collapse or stop proliferating? Finally, is there reduced pERK and pStat3 in Apc;Src tumours?

Response: we now present in new Figures 8 and S7 a detailed characterization of the phenotype of *Apc$^{Min/+}$* (control) versus *Apc$^{Min/+}$; AhCre Src$^{fl/fl}$* tumours. Our data show that *Min; AhCre Src$^{fl/fl}$* mice have reduced tumour burden and proliferation when compared with control counterparts. These results suggest that preventing Src function alone is sufficient to reduce proliferation of established intestinal tumours. We have also attempted to characterize *Apc$^{Min/+}$* and *Apc$^{Min/}$; AhCre Src$^{fl/fl}$* in the context of pStat3 and pErk1/2 levels. However, unlike in the regeneration paradigm, these markers do not appear to provide a robust staining pattern in *Apc$^{Min/+}$* tumours, which precluded further analysis in that direction.

4. My impression is that p-Src does not mark Lgr5+ ISCs (Figure 6A) or tumour stem cells at the base of adenoma glands (Figure 1d) but rather the staining seems specific of transient amplifying cells and the upper regions of adenomas. p-ERK shows the same pattern (Figure 6H). Is this appreciation correct? Does Src or Src;Fyn;Yes deficiencies affect normal or tumour stem cell behaviour? This is an important piece of information to strengthen the parallels with the results obtained in Drosophila.

Response: the Reviewer's interpretation is correct. Our data clearly show that p-Src is not restricted to stem cells within the intestinal crypts but rather marks the entire proliferative domain (Figure 1A). Importantly, our functional data clearly shows that Src is required within the stem/progenitor cell population: data from *AhCre; Src$^{fl/fl}$* mice demonstrate Src's requirement in intestinal regeneration and *Apc^{Min}*-dependent tumourigenensis and new data on the *AhCre; Src$^{fl/fl}$; Fyn$^{-/-}$; Yes$^{-/-}$* mice show the redundant requirement for the three SFKs in intestinal homeostasis. Furthermore, our

results show that Src is at least partly required within the Lgr5^{+ve} stem cell population: *Lgr5 Cre; Apc$^{fl/fl}$; Src$^{fl/fl}$* mice have increased survival when compared to *Lgr5 Cre; Apc$^{fl/fl}$* controls. Analysis of Apc-dependent tumourigenesis could not be done on a *Src$^{fl/fl}$; Fyn$^{-/-}$; Yes$^{-/-}$* background due to the short survival of mice with that genotype. Due to the existence of a single stem cell population in the fly midgut and the fact that stem cells are the only proliferative cells (there is no equivalent to the transit amplifying compartment), the fly system is much more amenable to stem cell-specific genetic manipulations with unambiguous outcomes. This is not the case of the mouse intestine were multiple stem cell populations and even quiescent stem cells appear to have redundant roles (Tian et al, 2011). Therefore the use of AhCre is likely to be the most appropriate to address gene function in mouse intestinal stem/progenitor cells. In any case, our work reveals a great deal of conservation regarding the role of Src in the intestine, which we now discuss (pages 17-18 of revised manuscript) and include in a working model presented in New Figure 9.

Referee #2:

The authors address the role of Src kinases in both the mouse and fly intestine models using genetic ablation (mouse), knock-down (fly) and overexpression (fly) techniques. Consistent with previous reports in human colorectal cancer, the authors find an expansion of the number of Src+ cells in adenomas of APC mice and human samples. Turning to the fly model, they find that overexpression of Src family members in intestinal stem cells and progenitors leads to increased proliferation. Infection of fly guts with pathogenic bacteria known to drive stem cell proliferation causes an increase in pSrc immunoreactivity and a transcriptional increase of Src42 and knockdown of Src42 causes a fairly to mount the proliferative response. A similar transcriptional increase in Src42 is observed upon clonal inactivation of apc1 and RNAi knockdown of Src42 blocks proliferation in apc1 clones, suggesting that Src42 mediates this proliferative response. The authors previous found that upon apc1 inactivation EGFR and Jak/Stat signaling are upregulated and promote overproliferation. Here they show that upon overexpression of Src64, EGFR , pERK and the Stat target Socs36E are upregulated. Moreover, knockdown of EGFR and stat suppress the proliferation induced by Src overexpression in the fly intestine. Turning back to the mouse, they provide nice data that Apc loss requires Src for full tumourigenic effect.

This work nicely mixes the fly and mammalian system to provide new insights into the role of an important proto-oncogene in vivo. These findings suggest that upon Apc loss, Src is upregulated and somehow leads to activation of EGFR and Stat to drive proliferation in flies and mice. While they provide several lines of evidence supporting this, they do not show in one model system all of the data to prove this. They do not show in flies that pSrc is upregulated upon Apc1 inactivation, which could easily be tested. It is also unclear in mouse whether overactivity/expression of Src is sufficient to drive activation of EGFR and Stat to control proliferation. Addressing these points would strengthen the manuscript.

Major points:

1. In Figure 1- some numbers should be given for how many adenomas were assessed and what percent showed increased Src. All of them?

Response: 10 mouse adenomas and 7 human adenomas were assessed and 100% of them showed increased pSrc levels. This information is now included within the corresponding result section in the text (page 6 of revised manuscript).

2. A better characterization of the clonal phenotypes of overexpression of Src64 and Src42CA should be done. Do they affect the number of stem cells or only the proliferation rate? In particular the authors should look at Delta staining to see if the number of stem cells are affected.

Response: This information is now provided in new Figure 1M-P. Our new data suggest that, in addition to driving ISC proliferation, Src overexpression leads to increase in the total number of ISCs assessed by Delta staining.

3. A related point: The characterization of "polyp-like hyperproliferative structures" are misleading as endoreplicating cells encorporate BrdU. Thus, it is more likely that the BrdU+ cells are newly produced enterocytes by faster dividing ISCs and not a cluster of proliferating stem cell-like cells. If this is really a cluster of dividing stem cells, this should be shown by PH3 staining. To me, this is an over-interpretation of the data that will be confusing for readers.

Response: The reviewer is correct. We now provide in new Figure 1J-K' immunofluorescence data showing co-labelling between *esg-gfp* and pH3 in midguts overexpressing Src. These results support our conclusions on the role of Src as a driver of ISC proliferation in the midgut. However, pH3 staining is always restricted to small nuclei *esg-gfp*$^{+ve}$ cells, which correspond to ISCs. Therefore, we must conclude that the BrdU^{+ve} cells with big nuclei present in Figure 1I' represent endoreplicating ECs. These results are now clearly described in the revised text (page 8) to avoid confusion and over interpretation.

4. Is Src42 really essential for clone growth as stated on p. 9 and p.10 or does it simply promote clone growth? Fig. 2G-J should be complemented with clonal analysis and quantification. In 2J there are some clones that appear to grow and in 3F the average clone size is reduced, though it does not seem as if all cell division stops. It would be helpful to discriminate between being essential for cell division and acting to promote it. What happens over time to these clones? It would be useful to do this experiment in the MARCM context as only dividing cell clones are marked and not quiescent ones as seen in the esgF/O method.

Response: we now include an intermediate time point to our *esg F/O* analysis in new Figure 2H, K, which demonstrate that in midguts subject to *Src* knockdown (*esg F/O> Src42-IR*) there is no lineage derived from the esg>gfp^{+ve} stem cells between 14 and 30 days of tracing. Furthermore, we have performed a detailed clonal analysis to assess the proliferation of control and *Src42-IR MARCM* clones over time. This data is presented in new Figure 2M-S. Our results show that, unlike their control counterparts, MARCM *Src42-IR* clones failed to grow and even decreased in size over time. Therefore we conclude that Src is essential to sustain homeostatic ISC proliferation over time.

5. Is pSrc upregulated in apc1 mutants in flies? This would be the prediction from the mouse data.

Response: Consistent with our tissue staining data in the mouse intestine, pSrc is upregulated in *Apc1*$^{-/-}$ fly midguts as well as in midguts overexpressing Wg/Wnt or activated β-Catenin/Armadillo. This data is presented in new Figure 3.

6. *p.7 starts by asking whether elevated Src activity was a driver of intestinal proliferation. They say that they "first utilized the adult intestine of Drosophila". Is overexpression of Src sufficient in the mouse intestine to drive proliferation? Their statement suggests that they will test it.*

Response: Directly testing Src sufficiency to drive intestinal proliferation is a relatively simple experiment in *Drosophila* due to the amenable genetics and easily available transgenic lines. We were persuaded to address this question given the clear upregulation of pSrc in intestinal adenomas. Unfortunately, doing this type of experiment in the mouse intestine would take years of work and can certainly not be achieved within the time frame of this revision. In fact, we turned to *Drosophila* to address this particular question because of the difficulties of doing it in vivo in the mouse. Our statement at the beginning of the paper is perhaps unintentionally misleading in that regard. We have now been more careful to explain that we take advantage of the *Drosophila* model to address

sufficiency and then assess the in vivo requirement of Src in intestinal proliferation by performing loss of function experiments in the mouse system.

7. The statement on p.11-"which does not involve canonical Jak/Stat signaling...?" is unclear: are they referring to published work, in which case they should cite it, or making a hypothesis based on their finding that dome RNAi did not suppress but stat RNAi did? How do they explain their lack of dome phenotype in this assay?

Response: It has been previously reported in mammalian systems that Src can directly activate Stat Our results show that Stat signalling is activated upon Src overexpression. Using previously validated *stat* and *dome* RNAi lines we find that *stat* but not *dome* knockdown suppresses the Src overexpression phenotype. This is consistent with an activation of Stat by Src, which does not require activation of the cytokine receptor. In the revised manuscript we make sure that the hypothesis leading to this set of experiments is better postulated, results and conclusions are more clearly explained and the appropriate literature is cited (page 12 of revised manuscript).

8. The previously published work in the Drosophila eye (Read, MCB, 2004) links Src with JnK and Jak/Stat. Is there also an effect on Jnk signaling here?

Response: Data addressing this point is presented in new Figure S3R-T. qRT-PCR to assess levels of the JNK target gene *puckered* (*puc*) shows that Src overexpression results in JNK upregulation in the midgut. However, our genetic interaction assays shows that JNK within stem/progenitor cells does not mediate Src-dependent hyperproliferation in the fly midgut. Similar outcomes have been reported in the analysis of JNK activation and requirement during intestinal regeneration in *Drosophila* (Jiang et al, 2009).

9. The requirement of SFK activity for homeostatic self-renewal (as concluded on p.12) is not rigorously demonstrated. Using the AhCre, which is expressed in a number of tissues, they examine 4d intestines and show that short-term loss of SFK activity does not affect self-renewal (in contradiction to their statement). Presumably longer time points could not be examined due to the poor health. Their conclusion on homeostasis seems based on the failure of the Src Fyn and Yes triple KO to fail to grow in organoid culture. As this in not in vivo, they should not conclude on a homeostatic function in the mouse intestine. Could they use the Lgr5Cre to have longer-term data that would clarify the role of SFK activity (Src Fyn and Yes triple KO) in homeostasis?

Response: Lgr5-Cre is known to yield less penetrant recombination when compared with other intestinal cre recombinases such as AhCre or VillinCreER (Sansom OJ et al., unpublished results). Therefore, knocking out SFK using the Lgr5-Cre is unlikely to produce a more reliable or different result from the one observed with AhCre. The Src expression domain in the intestine is not restricted to stem cells at the base of the crypt and it rather labels the whole crypt (Figure 1A). Therefore, we believe that, for loss of gene function, the use of AhCre is more appropriate in this case. Importantly, as detailed in our response to Reviewer 1 we now present a detailed analysis of crypt length, cell proliferation, migration apoptosis and differentiation in control, *AhCre; $Src^{fl/fl}$* and *AhCre; $Src^{fl/fl}$; $Fyn^{-/-}$; $Yes^{-/-}$* intestines at 4 days after Cre induction. Our results presented in new Figures 6, S4 and S5 indicate that combined loss of Src, Fyn and Yes leads to villae apoptosis and Paneth cell loss in the mouse intestine, with no obvious liver defects. These data explains the poor survival of the *AhCre; $Src^{fl/fl}$; $Fyn^{-/-}$; $Yes^{-/-}$* mice and the inability of crypts from such genotype to form intestinal organoids in culture.

Along similar lines, is there any effect on AhCre Srcfl/fl on the number of crypts without irradiation? Quantification of Fig 6E without irradiation would be informative.

Response: Unirradiated *AhCre; Src$^{fl/fl}$* intestines showed normal crypt number. This result is presented in new Figure 7E.

10. In the Discussion- the authors mention a "therapeutic window" in which to affect Src without affecting normal homeostasis as they suggest that Src is not required for normal homeostasis, but is important after irradiation and upon APC loss. Problematically, the effects on homeostasis were not assessed at the longer timepoints as which regeneration and APC interaction were done: Homeostasis upon Src fl/fl was assessed at 4d, regeneration was assessed at 7d, and the affect on APC was tested at 50-120 days. To rigorously conclude about a therapeutic window between homeostasis and a regenerative or an oncogenic function, the Src fl/fl effect on homeostasis should be also assessed at 7d and over the timecourse of the Apc fl/fl experiment. Does Src fl/fl have reduced lifespan relative to controls?

Response: As previously described we are now presenting a much more detailed assessment of the homeostatic phenotype of *AhCre; Src$^{fl/fl}$* intestines after 4 days of transgene induction (new Figures 6, S4 and S5), which confirms normal intestinal homeostasis in those mice. Please note that this time point is classically used to assess the role of genes on homeostatic self-renewal in the mouse intestine (Sansom et al, 2004). Furthermore, as described in the answer to the previous comment by the Reviewer, we now present crypt number of non-irradiated mice, which are matched in age with irradiated ones (new Figure 7E, F). Regarding the lifespan of *AhCre; Src$^{fl/fl}$* mice: we have being ageing a cohort of such mice for the last 90 months and they show no signs of poor health. While these mice have not yet reached the longest lifespan observed in Min; AhCre-Src $^{fl/fl}$ mice it is important to point out that knocking out Src extend the survival of *Min* and *Lgr5-Apc $^{fl/fl}$* mice, which supports the therapeutic potential of targeting Src in the context of *Apc* loss. Critically, previous work on the characterization of the phenotype of constitutive *Src KO* mice report that these animals show only bone defects and can live for up to a year (Lowell & Soriano, 1996).

11. It would be nice to compare an image of the tissue of the Apc-/- Src-/- with the Apc-/-.

Response: This data is now presented in new Figure 8.

Minor points:

1. It is unclear to me how they picked the crypts with Src knockdown in Figure 6 H-M? Are they the serial sections that were stained one with pSrc and then with pERK and pStat3 or is this based on Rosa26R reporter expression?

Response: pErk and pStat3 staining has being done on serial sections of *AhCre; Src$^{fl/fl}$* regenerating mouse intestines in which impaired regeneration and Src knockdown has been previously confirmed by scoring of crypt survival and pSrc staining, respectively as shown in new Figure 7 D, D'.

2. Fig. 3B the genotypes are confusing- where is the LacZ? It is not listed in their fly stocks -is it in all lines or only the ones labeled "lacZ"?

Response: LacZ represents a control transgene recombined into the same FRT line (FRT82B), which is the FRT recombined into the *Apc1^{Q8} allele*. The LacZ transgene is only present in the control (*MARCM LacZ*) and in the *MARCM Src42-IR* line. In the later, the presence of this transgene is essential to generate the clones since the *Src42-IR* is no recombined into an FRT site. The full genotype of these lines is indicated within the 'Fly Genotypes' section of the Supplementary Information.

3. Are the scale bars- 6DvsD' correct?

Response: We have reviewed the original images from the microscope and these scale bars appear correct.

Referee #3:

Cordero et al characterize the role or Src family kinases in tissue regeneration and tumorigenesis in the fly and mouse intestine. The authors show that Src phosphorylation correlates with the formation of adenomas, that src42A is sufficient and required for ISC proliferation in the intestinal epithelium of wt and APC-deficient flies, that Src induced proliferation of ISCs can be rescued by reducing EGFR and Stat signaling, and that Src family kinases are required for intestinal organoid formation ex vivo, as well as for tumour formation in APC deficient models in vivo. Overall, the study confirms the not too surprising role for Src family kinases in promoting proliferation of intestinal epithelial cells. Mechanistically, the paper falls somewhat short, however, as the epistasis experiments can be interpreted in multiple ways and no clear molecular interactions between Src and Wnt, EGFR or Stat signaling have been studied here. Nevertheless, the evolutionary conservation of the role of Src family kinases shown here is interesting and could be recommended for publishing if the following concerns are addressed:

Response: We now provide a more complete analysis of the role and mechanism of action of Src in the intestine. We would like to point out that Src family kinases (SFK) have been reported to act redundantly in most biological contexts (Lowell & Soriano, 1996; Stein et al, 1994). Therefore, we believe that our reports showing that conditional knockout of Src alone from the intestinal epithelium is sufficient to prevent intestinal regeneration and tumorigenesis is indeed relevant and not necessarily an obvious outcome. Such outcome suggests an important therapeutic window for specific inhibition of Src in colorectal cancer.

- The authors say that the 'polyp' like hyperproliferative structures shown in Fig 1 I' are never seen in wild-type flies, but these structure look very much like the aging phenotypes described by Biteau et al in 2008.

Response: Please note that the *esg>Src* midgut presented in Figure 1 and across the whole study represents tissues from 7-day old animals. Therefore, our conclusions are based on the comparison between age-matched *esg>gfp* (control) and *esg>Src* midguts (controls midguts also being from 7-day old animals). Age-associated midgut hyperplasia reported by Biteau et al., 2008 is observed in 30 to 60-day old midguts depending on the temperature of incubation. The age of the midguts used in our studies has been more clearly described in each of the relevant Figure legends in the revised manuscript. We state that these phenotypes were “never observed in aged-matched controls” (page 7 of revised manuscript).

- the phenotypes shown in Figs 2G and I look almost identical, countering the author's statement that Src42 is required for homeostatic proliferation and not just for stress-induced proliferation. Indeed, the 30D timepoint in this experiment is a timepoint where ISCs over-proliferate due to oxidative stress, and an interpretation about Src's role in homeostatic regeneration can thus not be made. The authors have to perform detailed analysis of Src mutant (not RNAi) MARCM clones in young, unstressed backgrounds to establish if Src is required for normal proliferation. The data shown in Fig 3D, where clones are nicely formed when Src is knocked down, would actually argue that Src is specifically NOT required for homeostatic regeneration, but only for stress-induced ISC proliferation.

Response: Its chromosomal location in close proximity to the centromere has precluded the creation of *Scr42 FRT* loss of function lines. Therefore, we have used two independent RNAi lines and *Src42* heterozygote mutants in our study to assess the role of endogenous *Src42* in the most rigorous way possible. Consistent with previous reports, it takes approximately two weeks to completely self-renew the fly midgut epithelium. Therefore, the midguts in new Figure 2G and J depict only the initial time point of the lineage tracing but is not sufficient to make a conclusion regarding self renewal since the control midguts have clearly not completely self-renewed. Our quantification of the MARCM analysis presented in new Figure 4 clearly shows that Src42-IR clones have less number of cells and are less proliferative (by pH3 quantification) than their control (LacZ) counterparts (new Figure 4E, F). To address the Reviewers' concern we now include an intermediate time point to our *esg F/O* analysis in new Figure 2H, K. These data demonstrate that in midguts subject to *Src* knockdown (*esg F/O> Src42-IR*) there is no lineage derived from the esg>gfp^{+ve} stem cells between 7 and 14 days of tracing, which should discard age-associated effects. Furthermore, we have performed a detailed clonal analysis to assess the proliferation of control and *Src42-IR MARCM* clones over time. This data is presented in new Figure 2M-S. Our results show that, unlike their control counterparts, MARCM *Src42-IR* clones failed to grow and even decreased in size over time. Therefore we conclude that Src is essential to sustain homeostatic ISC proliferation over time.

- In Fig 3A the authors claim a 2-fold induction of Src42 levels, but barely a 1.5fold induction is seen.

Response: The relative Src42 levels we measured in the *Apc1^{Q8}* guts versus wild type, was 1.7-fold. We have now precisely indicated this in the results. Importantly, Src is mostly regulated at the protein expression and kinase activity levels rather than the mRNA levels, and we now provide data showing that pSrc is upregulated in *Apc1$^{-/-}$* fly midguts as well as in midguts overexpressing Wg/Wnt or activated β-Catenin/Armadillo. This data is presented in new Figure 3.

- The regulation of EGFR/ERK signaling is not properly interpreted here. The authors argue that pERK is increased in Src gain-of-function conditions, but it is equally possible that Src gain of function simply leads to more progenitors that have high leveld of pERK. The images presented actually suggest that pERK levels do not change in individual ISCs, but that there are simply more ISCs. The same issue emerges in the mouse in Fig. 6. The authors need to develop a strategy to quantify the levels of pERK in individual Src gain-of-function ISCs. The same argument applies to the quantification of pStat.

Response: ISCs normally have high levels of pErk. The Reviewer is correct in that Src overexpression does not lead to an increase in the levels of pErk per cell. However, *esg>Src* midguts show clear ectopic pErk1/2 activation: while in control midguts pErk1/2 staining is restricted to small nuclei esg>gfp^{+ve} cells, *esg^{ts}>Src64^{WT}* midguts show pErk1/2 staining in big nuclei esg>gfp^{+ve} cells. A similar phenomenon has been observed in midguts overexpressing activated Ras (*esg>Ras.V12*) (Jiang et al, 2011). These data is now presented in new Figure 5E-F' and quantified in new Figure 5G. We also provide a quantitative analysis of pErk and pStat levels in the mouse intestine in new Figure 7. We have now made sure we properly describe the results to avoid misinterpretation.

- With respect to the epistasis experiments, the authors argue that EGFR/RAS acts downstream of Src because loss of EGFR/Ras can rescue the increase in proliferation induced by Src. However, EGFR/Ras deficient ISC do not proliferate under any conditions (see Jiang and Biteau papers on EGFR in fly ISCs) and the argument is thus not valid. The authors would need to show that increased ERK activity can rescue Src deficiency in ISC proliferation to confirm that ERK is downstream of Src.

Response: Please note that our conclusions regarding the epistatic relationship between Src and EGFR/Ras and Stat have not been merely drawn upon genetic interactions but also based on the fact that EGFR and Stat signalling are both upregulated upon Src activation in the midgut. Similar to the issues with the loss of function studies on EGFR/Ras knockdown, increased ERK activity does cause intestinal hyperplasia on its own (Jiang et al. 2011 and Cordero et al, unpublished results). Alternatively, we now provide genetic interaction data on *esg>Src* midguts in which a single copy of EGFR has been removed through combination with a heterozygote loss of function allele of EGFR leading to partial suppression of ISC hyperproliferation (new Figures 5L and S3I).

- *No mechanism for the induction of Src phosphorylation in response to injury has been explored (Fig. 6A). Src regulation has been studied extensively over the years and an attempt at explaining this induction should be made.*

Response: We have now extended our analysis to show that Src activation in response to damage requires Wg. Activation of Wg signalling by overexpression of Wg/Wnt or activated β-Catenin/Armadillo results in pSrc upregulation in the midgut. Complementary, blocking regeneration by Wg knockdown impairs pSrc upregulation in response to damage in the midgut. These results present the first direct evidence on a role of Wg upstream of Src activation in vivo. This data is presented in new Figure 3.

- *I am mystified by the comment made with respect to pSrc in the Src knockout epithelium (Fig. 6D'). The authors discuss that these cells show less pSrc as evidence that epithelial Src is essential to drive regeneration. But since Src is knocked out, wouldn't one expect less pSrc?*

Response: The pSrc antibody used throughout our studies cross-reacts with multiple SFK members in the mouse intestine (this is clarified at the beginning of out result section to avoid confusion). In fact, removal of Src, Fyn and Yes from the intestine is necessary to reliably ablate the staining in the intestine in homeostatic conditions (Figure 1B). Consistently, the upregulation of staining observed in control regenerating midguts (new Figure 7B) could in principle reflect upregulation in the activation of any of the three aforementioned SFKs. Therefore, the observation that Src knockdown in regeneration does indeed show decreased pSFK staining (new Figure 7D') implies that Src is the one SFK member that is mostly activated in response to damage. We have made an effort to clarify this section of the results in the revised text to avoid confusions.

References:

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Sansom OJ, Reed KR, Hayes AJ, Ireland H, Brinkmann H, Newton IP, Batlle E, Simon-Assmann P, Clevers H, Nathke IS, Clarke AR, Winton DJ (2004) Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. *Genes Dev* 18: 1385-1390

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2nd Editorial Decision 03 April 2014

Thank you very much for the revised study.

Two of the original referees assessed your revised paper with the enclosed comments below.

Conditioned on constructive integration/positioning yourself in the discussion to the remaining remarks, I am happy to proceed with formal acceptance/publication and take the liberty to congratulate you already at this point to this publication.

To enable rapid proceedings, I would be delighted to receive:

-a finalized text-file incorporating/addressing the final referee comments;

-a 2 up to 4 'bullet point' SYNOPSIS, that summarizes the major novelty/advance provided by your study.

Upon receipt of these items, the editorial office will soon be in touch about formal acceptance of your study.

REFEREE REPORTS:

Referee #1

The authors have addressed my main criticisms satisfactorily and as a result the manuscript is now convincing. The only remaining issue is the reason behind the death of triple mutant mice. Authors have convincingly shown that there is no liver phenotype yet why these mutant mice die remains undetermined. Authors argue in the rebuttal that this may be due to loss of Paneth cells yet Paneth Cells are apparently dispensable for life (e.g. Math1 conditional KO mice and other mutant mice lack Paneth cells without showing a lethal phenotype). I suggest leaving this aspect opened and discussing the different possibilities in the text.

Referee #2

The authors have responded appropriately to my concerns and have added new data that support their model and interpretations. The paper has significantly improved and presents a nice integration of invertebrate and vertebrate models to establish the regulation of stem cell proliferation and intestinal regeneration by src family kinases. I have one remaining concern that could be dealt with in the text: In their response to my concern regarding the interpretation of EGFR signaling as acting downstream of Src, the authors argue that the proposed experiment, namely to show that increased EGFR signaling rescues Src loss of function phenotypes, is not going to resolve the question because activation of EGFR by itself induced proliferation. I do not agree with this response, since such a rescue would demonstrate that src deficiency does not lead to disruption of other downstream

signaling pathways, but that lack of EGFR/Ras signaling is the only cause for the proliferation defect. However, I do accept the argument that their immunostaining data show that EGFR signaling is indeed activated by Src. Since the authors have added so much additional data, I do believe that this concern could be dealt with in the discuss, highlighting the different possible interpretations of the epistasis experiments.