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c-Src drives intestinal regeneration and transformation

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Abstract

The non-receptor tyrosine kinase c-Src, hereafter referred to as Src, is overexpressed or activated in multiple human malignancies. There has been much speculation about the functional role of Src in colorectal cancer (CRC), with Src amplification and potential activating mutations in up to 20% of the human tumours, although this has never been addressed due to multiple redundant family members. Here, we have used the adult Drosophila and mouse intestinal epithelium as paradigms to define a role for Src during tissue homeostasis, damage-induced regeneration and hyperplasia. Through genetic gain and loss of function experiments, we demonstrate that Src is necessary and sufficient to drive intestinal stem cell (ISC) proliferation during tissue self-renewal, regeneration and tumourigenesis. Surprisingly, Src plays a non-redundant role in the mouse intestine, which cannot be substituted by the other family kinases Fyn and Yes. Mechanistically, we show that Src drives ISC proliferation through upregulation of EGFR and activation of Ras/MAPK and Stat3 signalling. Therefore, we demonstrate a novel essential role for Src in intestinal stem/progenitor cell proliferation and tumourigenesis initiation in vivo.

Keywords Apc; intestinal stem cells; regeneration; Src; tumourigenesis

Subject Categories Cancer; Signal Transduction; Stem Cells

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Introduction

Since its discovery in the 1970s, the non-receptor tyrosine kinase Src has been implicated in multiple types of human cancers (Irby & Yeatman, 2000). There is rationale for a driver role for Src within CRC, and putative Src activating mutations were reported specifically within CRC, with amplification found in up to 20% of the advanced human CRC tumours (Irby et al., 1999; The Cancer Genome Atlas Network, 2012; www.cbioportal.org/public-portal). There is also increased Src activity in progressive stages of CRC (Talamonti et al., 1993; Termuhlen et al., 1993; Jones et al., 2002). Consistent with this, multiple studies have shown high percentage Src activation in CRC and suggested that this upregulation and/or hyperactivation of Src contributes to CRC progression and metastasis (Yeatman, 2004). Studies using Src family kinases (SFKs) inhibitors within CRC cell lines have predominantly shown an impact on invasion rather than proliferation (Serrels et al., 2006). One of the difficulties in interpreting the results with SFK inhibitors is the broad number of targets they inhibit. Given the strong evidence for a role for Src in CRC, it is somewhat surprising that Src inhibitors do not inhibit tumour cell proliferation. Therefore, we here performed genetic studies to more definitely address Src’s role in the intestine.

CRC is one of the most common cancers and the third most common cause of cancer deaths in the western world. Inactivating mutations in the gene encoding for the negative regulator of Wnt signalling, adenomatous polyposis coli (Apc), are detected in 80% of hereditary and sporadic forms of CRC (Kinzler et al., 1991; Korinek et al., 1997). Mouse models have shown that inactivation of Apc is sufficient to drive intestinal hyperplasia (Sansom et al., 2004; Andreu et al., 2005). Moreover, Apc deletion within the murine intestinal stem cells (ISCs) results in rapid adenoma formation suggesting they can act as cells of origin in CRC (Barker et al., 2009). Wnt signalling is also required during intestinal regeneration, and this process mimics many of the features of Apc loss, further suggesting that stem cell proliferation, for example during regeneration, and cancer initiation are linked and co-regulated by key signalling molecules (Cordero & Sansom, 2012; Cordero et al., 2012a,b).

The adult Drosophila midgut resembles the vertebrate intestine (Casali & Batlle, 2009), and it has proven to be an extremely useful model to study intestinal homeostasis, regeneration and disease (Biteau et al., 2011; Jiang & Edgar, 2012; Seton-Rogers, 2013). The fly intestinal epithelium is self-renewed by dedicated ISCs

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was observed within the core of intestinal polyps from mice (100%; n = 10; Fig 1D). To visualize Src activation at the early stages of human CRC, we stained non-invasive human adenomas for pSrc and found it consistently upregulated (100%; n = 7; Fig 1E and F). Therefore, ectopic activation of SFK is detected from the earliest stages of transformation following Apc loss, prior to the formation of invasive tumours and dissemination. This suggested that SFKs might have important roles in the initial stages of tumourigenesis in addition to their recognized roles in invasion and metastasis (Yeatsman, 2004).

**Src overexpression is sufficient to drive intestinal hyperproliferation**

We next asked whether elevated SFK activity was a driver of intestinal hyperproliferation, or a passive event. To address this, we used the adult intestine of *Drosophila melanogaster*, which was previously validated as a model system to study aspects of CRC (Cordero et al., 2009, 2012a; Lee et al., 2009). Unlike the mouse intestine, which contains proliferating stem- and transit-amplifying (TA) cells, only the stem cells are proliferative in the adult *Drosophila* midgut (Mitchelli & Perrimon, 2006; Ohlstein & Spradling, 2006). Therefore, assessment of mitotic proliferation in the fly midgut represents a direct measure of ISC proliferation. We used the temperature-sensitive driver *escargot-gal4, UAS-GFP; tubulin-gal80* (esggt > gfp; Mitchelli & Perrimon, 2006) to induce overexpression of Src within ISCs/EBs (stem/progenitor cells) in the adult *Drosophila* midgut. The ectopic activation of Src kinase was achieved by overexpression of independent UAS-Src transgenes—wild-type *Src64B (esggt > Src64WT)* and constitutively active form of *Src42A (esggt > Src42C-K)*—or by overexpression of an RNA interference transgene to knockdown Csk (Vidal et al., 2006; esggt > Csk-IR). All three approaches resulted in substantial ISC hyperproliferation and hyperplasia of the adult *Drosophila* intestine (Fig 1G and H and Supplementary Fig S1). Histological analysis of sections of paraffin-embedded midguts from 7-day-old esggt > Src64WT animals revealed ‘poly-ply’ structures containing multiple big and small BrdU+ve cell nuclei, which were not observed in age-matched control intestines (Fig 1I and I’ and Supplementary Fig S1A and B). To further characterize the phenotype of esggt > Src64WT midguts, we stained tissues with anti-ph3 (Fig 1J-K) and anti-Delta (Fig 1M-O) antibodies to specifically label cells undergoing active mitosis and ISCs, respectively. Src-overexpressing midguts showed significant upregulation in the number of ph3+ve and Delta+ve cells when compared with control counterparts (Fig 1J-P and Supplementary Fig S1C and C’). Consistent with previous reports (Mitchelli & Perrimon, 2006; Ohlstein & Spradling, 2006), ph3+ve staining was restricted to small nuclei ISCs (Fig 1J-K’) indicating that big BrdU+ve cell nuclei (Fig 1I’) are likely to represent newly made, endoreplicating enterocytes (ECs; Mitchelli & Perrimon, 2006). Importantly, Src-dependent ISC hyperproliferation in the midgut was largely suppressed by concomitant overexpression of the human homologue of Csk, Chk (Vidal et al., 2006; Fig 1L and Supplementary Fig S1G-J), highlighting the conserved nature of SFK regulation by upstream inactivating kinases. Altogether, these data demonstrate that enhanced expression of Src in stem/progenitor cells is sufficient to drive hyperproliferation and increase numbers of ISCs in the adult *Drosophila* midgut.
Figure 1. Src upregulation drives ISC proliferation.

A–D Immunohistochemistry to detect the activated form of Src (pSrc) in tissue sections from mouse and human intestines. pSrc is detected in the proliferative 'crypt' region (indicated with dashed line) at the base of the mouse small intestinal epithelium in control animals (A). Conditional knockout of Src (Srcfl/fl) combined with full knockout of related kinases Fyn and Yes, resulted in no staining within the intestinal epithelium (B). Intestines with conditional Apc knockout (Apcfl/fl) depict the expected 'crypt-like' progenitor phenotype (dashed line) and expansion of the pSrc domain (C). Example of a small intestinal polyp from an ApcMin/+ mouse, showing high p-Src staining within the core of the polyp (arrow), is shown in (D). Note normal tissue around polyp showing pSrc localized at the crypt base (dashed line). Scale bars, 100 μm.

E, F pSrc is upregulated within benign human intestinal adenoma lesions (arrows) when compared with normal surrounding tissue (asterisks). Scale bars, 100 μm.

G, H Adult Drosophila midguts overexpressing gfp (G; control) or Src (H) for 7 days under the stem/progenitor cell (ISCs/EBs) driver escargot-gal4 (esg>gfp and esg>Src64wt, respectively). Unless otherwise noted green marks esg > gfp cells and Dapi (blue) stains all cell nuclei. Scale bars, 100 μm.

I, I' Paraffin-embedded sections from 7-day-old Src-overexpressing midguts (esg>Src64wt) analysed by Haematoxylin and Eosin H+E (I) and BrdU (I') staining. Arrows point to 'polyp-like' structures containing BrdU+ve cells.

J–K' 7-day-old esg64 > Src64wt posterior midguts stained with anti-pH3 (red) to visualize proliferating ISCs (arrows). Scale bars, 20 μm.

L ISC proliferation quantified as the number of cells which stained positive for phosphorylated histone 3 (pH3) in posterior midguts from control animals (esg64 > gfp) or animals overexpressing Src (esg64 > Src6442CA or esg64 > Src42CA) or RNA interference for the Src inhibitor Csk (esg64 > Csk-IR). Note that co-expression of human Chk (esg64 > Src64wt, Chk) suppresses Src-driven hyperproliferation in the Drosophila midgut.

M–O 7-day-old adult posterior midguts from animals of the indicated genotypes stained with anti-Delta (red) to label ISCs. Scale bars, 20 μm.

P Quantification of the number of Delta+ve ISCs per field from posterior midguts as in (M–O).

Data information: Data in (L) and (P) represent average values ± SEM (***P < 0.0001 one-way ANOVA with Bonferroni’s multiple comparison test).
Src is required to drive stem cell proliferation during intestinal homeostasis and regeneration

We next tested whether endogenous Src was required for proliferation within the Drosophila intestine. To do this, we examined the role of Src in homeostatic self-renewal and damage-induced regeneration of the adult midgut.

Like its vertebrate counterpart, the adult Drosophila posterior midgut is replenished by dedicated ISCs and displays a remarkable regenerative response to damaging agents (Cordero & Sansom, 2012). Damage-induced intestinal regeneration is characterized by an acute expansion of the stem/progenitor cell population and increase in ISC proliferation, which is required to regenerate the damaged intestinal epithelium (Amcheslavsky et al., 2009; Buchon et al., 2009; Jiang et al., 2009; Fig 2A and B). We first assessed the regulation of Src during intestinal regeneration after feeding flies with the pathogenic bacteria Pseudomonas entomophila (Pe; Buchon et al., 2009; Fig 2A–C).

While immunostaining for pSrc showed barely detectable signal in the midgut epithelium from unchallenged animals, pSrc levels became more evident within the intestinal epithelium of animals subject to damage by Pe (Fig 2A–B'). RT–qPCR from whole midguts confirmed a threefold transcriptional upregulation of Src42 in regenerating tissues (Fig 2C). We next tested the functional role of Src42 during intestinal regeneration. We used RNAi transgenes to knockdown Src42 within stem/progenitors cells of the adult midgut (esgΔ > Src42-IR). Knockdown of Src42 by using two independent RNAi lines resulted in almost complete suppression of ISC proliferation in regenerating midguts (Fig 2D–F and Supplementary Fig S2A; compare with Supplementary Fig S2B, C'), showing that Src42 is rate-limiting during regeneration. RNAi knockdown of Src46 did not significantly affect intestinal regeneration (data not shown).

We also noted that esgΔ > Src42-IR midguts looked ‘thinner’ than their control counterparts (Fig 2E; compare with Fig 2D and Supplementary Fig S2B; compare with Supplementary Fig S2A), even when unchallenged. We therefore tested whether Src42 was required for ISC proliferation during homeostatic self-renewal of the adult midgut. We first overexpressed a Src42-IR transgene within the intestinal epithelium using the inducible ‘escargot flip out’ system (esgΔ F/O > gfp; Jiang et al., 2009), in which every progenitor cell and its new progeny will express Gal4 and UAS-gfp in addition to the UAS-Src42-IR RNAi transgene. We then visualized the newly produced esg cell lineage 7, 14 and 30 days after transgene induction (Fig 2G–L). Knockdown of Src42 from the epithelium of undamaged midguts impaired homeostatic self-renewal in the adult posterior midgut (Fig 2G–L) as evidenced by the reduced esg lineage labelling in esgΔ F/O > Src42-IR midguts after 14 and 30 days of tracing (Fig 2K and L; compare with Fig 2H and I). We next used the MARCM system (Lee & Luo, 1999) to create adult midgut clones from a control transgene (MARCM LacZ; Fig 2M–O) or with Src42 knockdown (MARCM Src42-IR; Fig 2P–R) and follow their growth at 7, 14 and 30 days after clonal induction (ACI). No significant difference was observed in the size of control and Src42-IR during the initial phase of clonal growth (Fig 2M, P and S). However, unlike control clones, 14- and 30-day-old Src42-IR clones failed to progressively grow over time and even decreased their size (Fig 2Q–S; compare with 2N, O and S). These results indicate that endogenous Src42 activity is essential to sustain ISC proliferation during homeostatic self-renewal as well as to drive ISCs during regeneration of the adult Drosophila midgut.

Src is activated downstream of Wg/Wnt signalling in the adult Drosophila midgut

c-Src is hyperactivated in response to Apc loss in mouse and human intestinal adenomas (Fig 1C–F). Work from us and others has shown that Wnt/Wg signalling drives ISC hyperproliferation and is required for regeneration of the adult Drosophila midgut (Lin et al., 2008; Lee et al., 2009; Cordero et al., 2012a,b). We therefore tested whether Src activation was dependent on Wnt signalling in the midgut. Src42 mRNA showed a 1.7-fold upregulation in whole midguts from animals homozygous for the Apc1 null allele Apc1G8 (Ahmed et al., 1998; Fig 3A). Immunofluorescence staining revealed pSrc upregulation within stem/progenitor cells from Apc1G8 midguts (Fig 3B–C') and in midguts overexpressing Wg (esgΔ > wg) or an activated form of β-catenin/Armadillo (esgΔ > armS10; Fig 1D–F'). Importantly, upregulation of pSrc upon Pe infection was impaired in midguts unable to regenerate due to wg knockdown in stem/progenitor cells (esgΔ > wg-IR; Fig 3G–I'). Altogether these results suggest that Wnt signalling is necessary and sufficient for Src activation in the Drosophila midgut.

Figure 2. Src is required for ISC proliferation during homeostasis and regeneration of the adult Drosophila midgut.

A–B' Immunofluorescence staining to detect pSrc (red) in midguts from esg > gfp (green) animals after feeding with Sucrose (Suc) (A, A') or subject to intestinal damage by feeding bacteria (Pe) (B, B'). Arrows point to examples of cell membranes stained with anti-pSrc. Scale bars, 20 μm.

C qRT–PCR from whole midguts as in (A–B') to detect transcript levels of Drosophila Src42 and Src64. Only Src42 was significantly upregulated in damaged midguts. Data represents average values ± SEM.

D–E' Posterior midguts from 14-day-old Suc- or Pe-fed control animals (D, D'; esgΔ > gfp) or animals subject to RNAi knockdown of Src42 in ISCs/EBs (E, E'; esgΔ > Src42-IR).

F Quantification of ISC proliferation in regenerating posterior midguts from animals of the indicated genotypes and treated as in (D–E'). Data represent average values ± SEM (**P < 0.0001; one-way ANOVA with Bonferroni’s multiple comparison test).

G–L Homeostatic self-renewal in control and Src42-IR posterior midguts using the escargot ‘flip out’ system (esgΔ F/O). The lineage from gfp (control) and Src42-IR esgΔ cells (green) was analysed 7, 14 and 30 days after transgene induction. Scale bars, 50 μm.

M–R Adult posterior midguts carrying 7-, 14- and 30-day-old MARCM clones (green) from a control transgene (M–O; LacZ) or from Src RNAi (P–R; Src42-IR). Scale bars, 20 μm.

S Quantification of the number of cells per clone in posterior midguts as in (M–R). Note that, unlike their control counterparts, MARCM Src42-IR clones failed to grow and even decreased in size over time. Clonal size distribution is presented as a dot plot with the mean clonal size ± SEM (**P < 0.0001; **P < 0.001 one-way ANOVA with Bonferroni’s multiple comparison test).
Src is required for tumourigenesis after Apc loss in Drosophila

We next tested the functional role of Src42 in ISC hyperproliferation driven by loss of Apc1 in the adult Drosophila midgut (Cordero et al., 2012a). We created adult midgut clones of cells deficient for Apc1 only (MARCM Apc1ΔC; Fig 4B) or combined with Src42 knockdown (MARCM Src42-IR; Apc1ΔC; Fig 4D). As previously reported, posterior midgut Apc1Δ/C0 mutant clones contained significantly more cells and displayed increased ISC proliferation when compared with control (MARCM Lac-Z) clones (Cordero et al., 2012a; Fig 4A, B, E and F). Consistent with our previous results (Fig 2M–S), Src42-IR clones (MARCM Src42-IR) were smaller than control clones (Fig 4A, C, E and F). Importantly, knocking down Src42 inhibited hyperproliferation in Apc1Δ/C0 clones (Fig 4B, D, E and F). These data demonstrate an essential role for Src42 in Apc1-dependent ISC hyperproliferation in the adult Drosophila midgut.
Src drives ISC hyperproliferation through regulation of the EGFR/MAPK and Stat signalling pathways

The EGFR/Ras/MAPK and Jak/Stat signalling pathways are required for Drosophila intestinal homeostasis and regeneration (Buchon et al., 2009; Jiang et al., 2009, 2011; Beebe et al., 2010; Biteau & Jasper, 2011) and are essential mediators of Apc1-driven intestinal hyperproliferation (Cordero et al., 2012a). We therefore tested whether these pathways were potential mediators of the role of Src in ISC proliferation in vivo. Src overexpression (esgts>Src64WT and esgts>Src42CA) resulted in ectopic activation of MAPK/Erk1/2 (pErk1/2; Fig 5A, A', C, C', E-G and not shown). Such phenotype resembled the one resulting from overexpression of activated Ras in stem/progenitor cells (Jiang et al., 2011) and correlated with...

Figure 3. Src is activated downstream of Wnt signalling in the adult Drosophila midgut.

A  qRT-PCR of whole midguts from 7-day-old control and Apc1−/− (Apc1Q8) animals to assess transcript levels of Src42 and Src64. Only Src42 was significantly upregulated in the midgut in response to Apc1 loss. Data represent average values ± SEM.

B–C' 7-day-old control (B, B') and Apc1Q8 midguts (C, C') co-stained with anti-pSrc (red) and anti-Delta (green). Apc1Q8 midguts display significant pSrc upregulation within Delta+/+ ISCs (arrows). Scale bars, 20 µm.

D–F' pSrc immunofluorescence (red) in 14-day-old midguts from control animals or animals subjected to Wg signalling upregulation by overexpressing Wg or activated β-catenin/Armadillo within ISCs/EBs (esgts>gfp; esgts>wg and esgts>armS10, respectively). Note pSrc upregulation in ISCs/EBs in response to Wg signalling activation (arrows). Scale bars, 20 µm.

G–I' pSrc staining (red) in 14-day-old midguts from control animals or animals subject to wg knockdown by overexpressing a wg RNAi within ISCs/EBs (esgts>wg-IR) and fed with Suc (G, G') or Pe (H–I'). Note that pSrc upregulation in ISCs/EBs upon Pe feeding (H, H', arrows) is suppressed by wg knockdown (I, I'). Scale bars, 20 µm.
upregulation of total levels of EGFR within the same cells (Fig 5B, B’, D and D’ and not shown). Transcriptional upregulation of egfr by Src overexpression was confirmed through qRT–PCR from whole midguts (Fig 5H). Importantly, knocking down EGFR or Ras completely suppressed ISC hyperproliferation in esgts>Src midguts (Fig 5J–L and Supplementary Fig S3A–I). Altogether, our results suggest that EGFR expression and downstream pErk activation are key mediators of intestinal hyperproliferation driven by Src.

qRT–PCR of Src-overexpressing midguts also indicated a significant upregulation of the Jak/Stat target Socs36E (Fig 5I). To test the functional relevance of Jak/Stat signalling activation in esgts>Src midguts, we combined Src overexpression with RNAi knockdown for stat92E/marrelle (esgts>stat-IR; Src42CA) or the Upd/IL-6 receptor domeless (esgts>dome-IR; Src42CA; Fig 5M). Unlike in intestinal regeneration and Apc1-dependent hyperproliferation (Buchon et al, 2009; Jiang et al, 2009; Cordero et al, 2012a), Stat but not dome knockdown suppressed Src-dependent ISC hyperproliferation in the adult Drosophila midgut (Fig 5M and Supplementary Fig S3J–M). These results support a direct activation of Stat by Src (Yu et al, 1995; Cao et al, 1996), which does not involve cytokine receptor. Previous work on the Drosophila eye imaginal disc links Src-dependent overgrowth to parallel activation of Stat and JNK signalling (Read et al, 2004). qRT–PCR to assess levels of the JNK target gene puckered (puc) shows that Src overexpression results in JNK upregulation in the midgut (Supplementary Fig S3R). However, blocking JNK signalling within stem/progenitor cells does not mediate Src-dependent hyperproliferation in the fly midgut (Supplementary Fig S3S and T). Similar outcomes have been reported in the analysis of JNK activation and requirement during intestinal regeneration in Drosophila (Jiang et al, 2009). Together these results place EGFR/MAPK and Stat activation as key in vivo mediators of Src-driven stem cell hyperproliferation in the Drosophila intestine.

Mammalian SFKs are redundant for intestinal homeostasis

An important question posed by our results on the role of Src in the Drosophila intestine was whether these would translate to the mammalian system. We first assessed whether there was an exclusive role of Src in the adult mammalian intestine. To achieve this, we bred mice carrying a LoxP-flanked Src allele (Srcfl/fl; Marcotte et al, 2012) with mice carrying a Cre recombinase driven under the control of the Cyp1A1 aryl hydrocarbon-responsive promoter (AhCre). Induction of AhCre recombinase results in high penetrance Cre expression within the intestinal epithelium and liver (Ireland et al, 2004; Reed et al, 2008; Supplementary Fig S5K). Deletion of Src from the mouse intestinal epithelium (AhCre Srcfl/fl; Src KO) resulted in no apparent defects in homeostatic self-renewal of unchallenged intestines (Fig 6A and B). We next conditionally knocked out all SFKs expressed in the intestine: Src, Fyn and Yes (Fig 1B; AhCre Srcfl/fl Fyn−/− Yes−/−; Src Fyn Yes KO; Fig 6C). Those
mice displayed multiple signs of poor health 4 days post-Cre induction, unless when subject to a reduced induction regime, which resulted in partial recombination within the intestinal epithelium (Supplementary Fig S5K). We next assessed levels of cell proliferation, migration, apoptosis and lineage differentiation in control, Src KO and Src Fyn Yes KO intestines 4 days after Cre induction.
Interestingly, intestinal cell proliferation and migration (Supplementary Fig 5A–I) as well as the differentiation of Goblet and enteroendocrine cell lineages (Supplementary Fig S4J–O) were unaffected in Src Fyn Yes KO mice. On the other hand, Src Fyn Yes KO intestines displayed significant levels of villus apoptosis (Fig 6D–F’ and J) and a complete ablation of Paneth cells (Fig 6G–I and K). No liver phenotypes were detected (Supplementary Fig S5A–K). Consistent with the absence of Paneth cells, which are essential components of the mouse intestinal stem cell niche (Sato et al., 2011), isolated crypts from Src Fyn Yes KO intestines were unable to form organoids in culture (Sato et al., 2009; Fig 6L–P). These data suggest that, in parallel to our observations in the fly midgut, the activity of SFKs (Src, Fyn and Yes) is required for homeostatic self-renewal of the mammalian intestinal epithelium with the three kinases acting redundantly in such context.

**Src is required for mouse intestinal regeneration**

We next tested whether Src was required for either intestinal regeneration in the mammalian intestine. Our previous work had identified that Wnt/MYC signalling is essential for intestinal regeneration and tumourigenesis downstream of Apc loss, and that this is conserved from mammals to Drosophila (Sansom et al., 2004; Ashton et al., 2010; Cordero et al., 2012a,b).

pSrc was upregulated within the crypt membranes of intestines regenerating following DNA damage by gamma irradiation (Fig 7A and B). Conditional deletion of Src from the intestinal epithelium (Fig 7C and D) was sufficient to impair intestinal regeneration as determined by the decreased number of surviving crypts scored 72 h after damage when compared with equally treated control intestines (Fig 7E and F and Supplementary Fig S5M). Surviving Src KO crypts were significantly smaller than their control counterparts (Fig 7C and Supplementary Fig SSL and M; arrows) but displayed normal Paneth cell numbers (Supplementary Fig S6A–D). Given that available anti-pSrc antibodies cross-react with multiple SFK members, the reduced p-Src staining observed in irradiated Src KO intestines (Fig 7D’) suggests that Src is responsible for the majority of SFK activity upregulated during intestinal regeneration. Altogether, these results uncover an essential role of epithelial Src driving mouse intestinal regeneration in response to damage.

We next tested whether mechanisms mediating the role of Src in the Drosophila midgut were conserved in the mammalian intestine by examining activation of Erk1/2 and Stat3, the closest orthologue of Drosophila stat92E/marrelle, in those regenerating Src KO intestines, which showed robust gene knockdown (Fig 7D’). We observed that while pErk1/2 and pStat3 strongly stained throughout the regenerating crypts in control intestines (Fig 7H, K, M and N and Supplementary Fig S6F), small regenerating Src KO crypts mimicked those from unirradiated control intestines displaying only a few cells positive for either protein (Fig 7G, J, I, L, M and G). Taken together, these results suggest that activation of EGF/MAPK/Erk and Stat3 signalling is likely to be conserved outcomes downstream of Src activation in the intestine.

**Src is required for mouse intestinal tumourigenesis following Apc loss**

Finally, we addressed whether Src was important for intestinal tumourigenesis following Apc loss in the mouse. We therefore bred SrcE7/R animals into two models of intestinal tumourigenesis driven by Apc loss: the ApcΔMIN/+ and Lgr5CreER ApcE7/Δ mice. Both of these models mimic intestinal tumourigenesis; however, the timing of Src deletion is different. The ApcΔMIN/+ mouse, which forms adenomas sporadically on loss of the remaining copy of Apc, was bred into the AhCre SrcE7/R mice, and Cre was induced post-weaning. The Lgr5CreER ApcE7/Δ mice, which form tumours rapidly following Cre induction (Barker et al., 2009), were interbred with SrcE7/R mice and Cre induction therefore leads to simultaneous loss of Apc and Src.

Conditional deletion of Src significantly increased the survival of Lgr5CreER; ApcE7/Δ and ApcΔMIN/+ (Min) mice (Fig 8A and B, respectively). We next analysed the intestines of mice from the Min cohorts, which present discrete and therefore scorable tumours to further characterize the effect of Src deletion on pre-established intestinal adenomas. Min; AhCre SrcE7/Δ mice exhibited reduced tumour burden when compared with control ones (Fig 8C). Furthermore, even though the overall distribution of tumour types (n = 40 Min; n = 25 Min; AhCre SrcE7/Δ) was similar (Fig 8D and Supplementary Fig S7), Min; AhCre SrcE7/Δ small, medium and big adenomas displayed significantly reduced proliferation as determined by BrdU staining (Fig 8E–H and Supplementary Fig S7). Unlike during regeneration (Fig 7G–L), pStat3 and pErk1/2 levels do not appear to provide a robust staining pattern within ApcΔMIN/+ tumours (not shown), which precluded further analysis of the effect of Src on such pathways in tumourigenesis. Importantly, our results indicate that preventing Src activity is sufficient to slow down intestinal tumourigenesis in mammals.

**Discussion**

Using two genetic model systems, our work provides definitive evidence for a conserved functional role for Src in intestinal tumourigenesis.
Figure 6. SFKs are redundantly required for mouse intestinal homeostasis.

A–C H&E staining of small intestines from a control mouse (A) or mice subject to 4 days of intestinal epithelial knockout of Src only (AhCre; Src<sub>fl/fl</sub>) (B) or in combination with constitutive knockout of Fyn and Yes (AhCre; Src<sub>fl/fl</sub>; Fyn<sup>C0/C0</sup>; Yes<sup>C0/C0</sup>) (C). Scale bars, 100 µm.

D–I Small intestines from mice as in (A–C) stained with anti-cleaved caspase-3 (D–F′) and anti-lysozyme (G–I). Close up views (F′, F″) from boxed areas in (F). Combined loss of Src, Fyn and Yes leads to villae apoptosis (F″; arrows) and loss of Paneth cells (I). Scale bars, 100 µm.

J Quantification of villae apoptosis from intestines as in (D–F). Data represent average values ± SEM (**) P < 0.001 one-way ANOVA with Bonferroni’s multiple comparison test).

K Quantification of the percentage Paneth cells per crypt from intestines as in (G–I). Data represent mean values ± SEM (***) P < 0.0001 one-way ANOVA with Bonferroni’s multiple comparison test).

L–O In vitro organoid formation from intestinal crypts of mice of the indicated genotypes. Note that combined loss of Src, Fyn and Yes from the intestinal epithelium prevents organoid formation.

P Quantification of the percentage of organoids formed per 100 crypts seeded. Organoids were scored 1 week after seeding. Data represent average values from two independent experiments ± SEM (***) P < 0.0001 one-way ANOVA with Bonferroni’s multiple comparison test). N.S.: statistically not significant.)
regeneration and oncogenic transformation (Fig 9). Moreover, using the adult Drosophila intestine, we demonstrate that Src is sufficient to drive intestinal hyperproliferation. Our work elucidates a key new role for Src during initial stages of tumourigenesis.

A conserved role of SFKs in intestinal stem/progenitor cells

Our results show that Src activation within the mouse intestinal epithelium is restricted to the stem/progenitor cell population,
Src in regeneration and tumourigenesis

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**p=0.0064

***p=0.0008

Min; AhCre Src^{fl/fl}

Min; AhCre Src^{fl/+}

Min; AhCre Src^{fl/+}

Min; AhCre Src^{fl/+}

Min; AhCre Src^{fl/fl}

Min; AhCre Src^{fl/fl}

Brdu

Min; AhCre Src^{fl/+}

Min; AhCre Src^{fl/fl}
which includes proliferating stem and transit-amplifying cells (Fig 1A). Consistently, functional data on the use of AhCre recombinase demonstrate Src’s requirement in intestinal regeneration and Apc<sup>Min</sup>-dependent tumourigenesis. A comparable scenario can be drawn from our studies on the adult <i>Drosophila</i> midgut where Src<sup>dS42</sup> is activated in response to damage and upregulation within stem/progenitor cells of the adult <i>Drosophila</i> midgut and mouse small intestine leading to tissue hyperproliferation. Src knockdown prevents intestinal regeneration and tumourigenesis in both model systems. dSrc<sup>dS42</sup>/cSrc: activated in response to damage and upon Wg/Wnt signalling upregulation within stem/progenitor cells of the adult <i>Drosophila</i> midgut and mouse small intestine leading to tissue hyperproliferation. Src knockdown prevents intestinal regeneration and tumourigenesis in both model systems. ISC/EB: intestinal stem cell/enteroblast; EC: enterocyte; ee: enteroendocrine cell; TA: transit-amplifying cell; Paneth: Paneth cell.

Non-redundant roles of SFKs

Work in cell lines has previously shown distinct properties of SFKs (Sandilands <i>et al</i>, 2007). However, the evidence regarding non-redundant roles of SFK <i>in vivo</i> is rather limited. Examples include classical work on the role of Src in bone formation (Stein <i>et al</i>, 1994; Lowell & Soriano, 1996) and recent work indicating a non-redundant role of the kinase in mouse mammary carcinogenesis (Marcotte <i>et al</i>, 2012). Our results show that loss of Src is sufficient to slow down intestinal regeneration and tumourigenesis <i>in vivo</i> without affecting overall tissue homeostasis. Surprisingly, constitutive loss of Fyn and Yes (Fyn<sup>−/−</sup>; Yes<sup>−/−</sup>) was also sufficient to prevent intestinal regeneration (Supplementary Fig S5N and O). The overall poor survival of Fyn<sup>−/−</sup>; Yes<sup>−/−</sup> mice prevented us from assessing the contribution of those kinases in our intestinal tumourigenesis models. Therefore, there is a therapeutic window, which would allow targeting of Src within transformed intestinal epithelia without affecting the normal tissue.

A redundant role of SFKs in the mammalian intestine

The deletion of Src, Fyn and Yes from the adult intestine using the AhCRE transgene led to ill health so that all mice required euthanasia. Src Fyn Yes triple KO intestines displayed two main phenotypes, which were not observed upon loss of Src alone: significant levels of villae apoptosis and ablation of Paneth cells (Fig 6). While the latter is consistent with the inability to obtain intestinal organoids from isolated Src Fyn Yes KO crypts, it does not explain animal viability. The precise cause of the ill health is unknown; however, perturbed intestinal epithelial barrier dysfunction, which is in part...
characterized by exacerbated villae apoptosis, could cause similar phenotypes. This is currently under further investigation.

**Src as a therapeutic target in early stage colorectal tumourigenesis**

The complete rescue of Src-dependent hyperproliferation in the fly midgut by human ChK overexpression (Fig 1 and Supplementary Fig S1) suggests a main involvement of Src kinase activity in this process and highlights the level of functional conservation in these kinases. Treatment with the kinase inhibitor dasatinib, a widely used Src inhibitor, has been effective to target Src in multiple cell lines and mouse models of tumour invasion and metastasis (Serrels et al., 2006; Morton et al., 2010). However, dasatinib and other Src inhibitors have proven ineffective when used in mouse models of CRC initiation (data not shown) as well as in the clinic (Serrels et al., 2006). Key issues with such inhibitors are their broad spectrum of targets and unclear in vivo efficacy (Brandvold et al., 2012). The current scenario emphasizes on the importance of developing more selective Src inhibitors and highlights the need for genetic experiments to validate results from pharmacological targeting of molecules.

**Distinct molecular events mediate Src-dependent proliferation in the intestine**

Mechanistically, we have defined the action of Src in the *Drosophila* intestine as working via EGFR/Erk1/2 and Stat pathway activation (Fig 5). EGFR/Ras activation is a limiting step in stem/progenitor cell proliferation in the fly midgut (Jiang et al., 2011). Consequently, our genetic experiments are not sufficient to unambiguously establish the epistatic relationship between Src and EGFR/MAPK signalling. However, our expression data suggest that EGFR levels and pErk activation are increased in response to Src overexpression in the fly midgut. Furthermore, we see impaired activation of Erk1/2 and Stat3 in Src-deficient mammalian intestines during regeneration (Fig 7). Therefore, our evidence suggests that EGFR/MAPK and Stat signalling activation are key events downstream of Src activation in the intestine. However, there may be other pathways downstream of Src aside EGFR/MAPK important for the phenotypes we observed that warrant future testing.

Previous work on *Drosophila* suggests that Src drives hyperproliferation of the developing eye epithelium through parallel activation of Stat and JNK signalling (Read et al., 2004). However, such role of Src does not appear to involve EGFR activation. Reciprocally, our results indicate that JNK signalling is, at least not directly, involved in Src-dependent ISC hyperproliferation in the fly midgut. Importantly, our work presents the first direct evidence demonstrating that Wnt signalling is required to activate Src in vivo (Brandvold et al., 2012). Inhibitors that could specifically target Src may be very useful in those patients that are of high risk of developing CRC.

**Materials and Methods**

**Fly stocks**

The following fly stocks were kindly provided to us by our colleagues: the null allele *Apc1Q8* (Yashi Ahmed), *escargot-gfp* (Shigeo Hayashi), UAS-egfr^{D2N} (Mathew Freeman), EGFR^{D2O}/Cyo (Marek Mlodzik), UAS-JNK^{1N} (Aron DiAntonio), esg^{E8}F/O (Bruce Edgar), MARCM 82B line (David Bilder). The rest of the lines used were obtained from VDRC and the Bloomington Stock collection.

**VDRC ID numbers**

- UAS-stat-IR (43866), UAS-dome-IR (2612), UAS-Src42-IR^{KK} (100708), UAS-egfr-IR^{KK} (107130), UAS-Ras-IR^{KK} (106642), UAS-Ras-IR^{CG} (28129).

**Bloomington stock numbers**

- UAS-Src4UCA (6410), UAS-Src64^{p96} (8477), Src4^{E10108}/Cyo (10969), UAS-Src42-IR^{CG} (44039).

**Fly and mouse genetics**

A complete list of fly genotypes and mouse strains used in this study can be found as part of the Supplementary Materials and Methods.

**Fly maintenance**

Crosses were maintained at 18°C in standard medium. Animals of the desired genotypes were collected within 48–72 h of eclosion and then switched to the desired temperature. All experiments involving the activation of a transgene under the control of *gal4*/*gal80*

were switched from 18 to 29°C to allow transgene activation for as long as necessary. Animals were kept in incubators with controlled 12-h light-dark cycles. Flies were changed into new food every 2 days.

**Regeneration assays**

Experimental flies were collected within 48 h of eclosion at 18°C and moved to 29°C for 14 days on standard media. Flies were then transferred to either 5% sucrose (vehicle) or 10× overnight (Pe) culture on filter-paper discs (Whatman) for the last day of the incubation period. Midguts were then dissected and analysed using immunofluorescence and confocal imaging.
Analysis of intestinal homeostasis

Three- to five-day-old animals carrying the inducible ‘escargot flip out’ system (esg<sup>f</sup>O>gfp; Jiang et al., 2009) were switched from 18 to 29°C and their midguts analysed 7, 14 and 30 days after transgene induction to visualize the esg cell lineage over time.

Clonal analysis

Recombinant clones were generated using the MARCM technique as previously described (Lee & Luo, 1999). Crosses were carried out at 25°C. Two- to five-day-old adults of the desired genotypes were selected and subject to three 30-min heat shocks at 37°C in 1 day. Flies were then incubated at 25°C for 7, 14 and 30 days.

RNA quantification

Total RNA was extracted from at least 10 midguts using RNAeasy mini kit (Qiagen) followed by DNase treatment (Qiagen). cDNA synthesis was performed using the High-Capacity cDNA reverse transcription kit (Applied Biosystems). Transcript levels were measured using the primers pairs shown underneath. RNA extractions were performed from three biological replicates. DNA was analysed in triplicate using the Applied Biosystems 7500. Expression of the target genes was measured relative to that of RpL32 (rp49). A series of 10-fold dilutions of an external standard was used in each run to produce a standard curve. MAXIMA SYBR GREEN Master Mix (Fermentas) was used for qPCR following manufacturer’s instructions. Data were extracted and analysed using Applied Biosystems 7500 software version 2.0 and Prism GraphPad software. Primer sequences are indicated in Supplementary Table S1.

Sample number and statistical analysis for mouse data

Only posterior midguts of female flies were analysed in this study. Between 7 and 15 midguts were analysed in each experiment. Data were plotted using GraphPad Prism 5 software. Statistical methods used for the analysis of each experiment are detailed in the corresponding figure legends.

Quantification of pErk1/2 and Delta staining

pErk1/2 staining (Fig 5G) was quantified with ImageJ by measuring total esg-gfp<sup>−ve</sup> and pErk1/2<sup>−ve</sup> areas normalized by the total number of cells (identified by Dapi staining) within that area. Five posterior midguts were scored per genotype.

The number of ISC (Fig 1M–P) was scored manually by counting the total number of Delta<sup>−ve</sup> within a consistent region of the posterior midgut, which was imaged with a 40× objective and comprised a field of 0.04 mm<sup>2</sup>. Six to seven posterior midguts were analysed per genotype.

Mouse experiments

All experiments were performed under the UK Home Office guidelines. Mouse strains were backcrossed into a C57Bl6J background for 5–10 generations.

Mice carrying the AhCre recombinase were induced by single injection (Supplementary Fig S5K) or 3 daily intraperitoneal (i.p.) injection of 80 mg/kg β-Napthoflavone for 1 day. Intestinal phenotypes were analysed 4, 6 or 7 days after transgene induction. Mice carrying the Lgr5-CreER recombinase were given one i.p. injection of 120 mg/kg tamoxifen, followed by one daily i.p. injection of 80 mg/kg tamoxifen for 3 days.

Intestinal regeneration was induced by irradiating mice with 14 Gy gamma irradiation 4 days after recombinase induction. Mice were sacrificed 72 h post-irradiation and the small intestine isolated and flushed with tap water. 10 × 1 cm portions of small intestine were bound together with surgical tape and fixed in 4% neutral buffered formalin. Haematoxylin-and-Eosin-stained sections were used for analysis. Crypts were scored as regenerating if they contained six or more consecutive cells. The average number of regenerating crypts across cross sections of the 10 gut pieces was used for statistical analysis. Intestines from 12 control and nine Src<sup>+/−</sup> mice were analysed. Age-matched (7-day post-induction) unirradiated mice where used as controls (Fig 7E).

Quantification of villae apoptosis

Villae apoptosis was scored by counting the number of cells stained for activated caspase-3 from small intestinal gut rolls.

Quantification of tissue staining

Staining with anti-lysozyme, anti-pErk1/2 and anti-pStat3 was quantified by scoring the percentage of crypt cells, which stained with each of the markers.

Organoid formation assay

Data were scored as the percentage of crypts that formed intestinal organoids in culture 1 week after seeding.

Sample number and statistical analysis for mouse data

In all cases, data represent average values ± SEM from at least three mice. Cell migration values for AhCre; Smn<sup>C0/C0</sup>; Fyn<sup>−/−</sup>; Yes<sup>−/−</sup> mice (Supplementary Fig S4I) are from a single mouse due to the low availability of surviving mice from such genotype.

Data were plotted using GraphPad Prism 5 software. Statistical methods used for the analysis of each experiment are detailed in the corresponding figure legends.

Histology and tissue analysis

Immunofluorescence

Tissues were dissected in PBS and fixed 30 min in 4% paraformaldehyde (Polysciences, Inc.). Midguts processed for pErk1/2 staining were subject to additional fixation in ethanol. After fixation, samples were washed three times in PBS + 0.1% Triton X-100 (PBST) and incubated in primary antibodies overnight at 4°C. Samples were then washed as described and subjected to secondary antibody staining for 2 h at room temperature followed by washing and mounting on Vectashield containing DAPI (Vector Laboratories, Inc.). Primary and secondary antibodies were incubated in PBST + 0.5% BSA.
**Primary antibodies used (Drosophila)**

Chicken anti-GFP 1:4,000 (ab13970; Abcam); mouse anti-dEGFR 1:10 (C-273; Sigma); rabbit anti-ph3 S10 and S28 1:100 (9701 and 9713; Cell Signalling); rabbit anti-p-Src 1:100 (44660G; Invitrogen); rabbit anti-pErk1/2 1:100 (9101; Cell Signalling).

**Secondary antibodies used (Drosophila)**

Alexa 488 1:200 and Alexa 594 1:100 (Invitrogen). Nuclei were counterstained with DAPI. Confocal images were collected using a Zeiss 710 confocal microscope and processed with Adobe Photo-shop CS.

**BrdU labelling**

300 µg/ml BrdU (RPN 201V1; GE Healthcare) was mixed into the fly food, and animals were fed ad libitum ON prior to midgut dissection. Mouse intestines were labelled by i.p. injection of 250 µl BrdU 2 and 48 h prior to tissue dissection.

**Immunohistochemistry of fly midguts**

Whole midguts were dissected and fixed at 4°C ON using a 10% formalin solution including 2% toluidine blue to improve visualization of the midguts during the embedding and sectioning process. Tissues were then mounted between two layers of 1% agar with the posterior midguts appropriately oriented. Agar-embedded tissues were further incubated in 10% formalin ON and then subjected to paraffin embedding. The paraffin blocks were trimmed in at 10 µm until the beginnings of the orientated midguts were revealed. Serial 4-µm sections of posterior midguts were cut, placed onto polysine slides and processed for staining.

**Immunohistochemistry of mouse intestines**

Briefly, tissues were flushed with water and fixed in 4% neutral buffered formalin and then subjected to paraffin embedding and processing for histological analysis.

**Primary antibodies used (Mouse tissue)**

rabbit anti-p-Src 1:100 (2101; Cell Signalling); rabbit anti-pErk1/2 1:400 (9101; Cell Signalling); rabbit anti-pStat3 1:50 (9131; Cell Signalling); mouse anti-BrdU 1:200 (347580; BD Biosciences); rabbit anti-anti-cleaved caspase-3 1:50 (9661; Cell Signalling); rabbit anti-Ki67 1:100 (RM-9106, Thermo); rabbit anti-lysozyme 1:150 (A099, Dako).

**Tissue dyes used**

Alcian Blue, Grimelius and Sirius Red staining solutions were used to visualize Goblet and enteroendocrine cells in the intestine and liver fibrosis, respectively.

**Secondary antibodies used (Mouse tissue)**

Mouse and rabbit EnVision (K4001 and K4003; Dako). Images were collected using an Olympus BX51 microscope and images taken with a DP70 camera and processed with Adobe Photoshop CS.

**Supplementary information** for this article is available online:

http://emboj.embopress.org

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**Author contributions**

JBC designed, performed and analysed experiments and wrote the manuscript. RAR performed and analysed experiments. NV contributed to the analysis of mouse tumours. CN performed immunohistochemistry. MCF analysed experiments and wrote the manuscript. WJWM provided Srcfl/fl mice and analysed experiments. MV and OJS designed and analysed experiments and wrote the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

**References**


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