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Drosophila Perlecan Regulates Intestinal Stem Cell Activity via Cell-Matrix Attachment

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SUMMARY

Stem cells require specialized local microenvironments, termed niches, for normal retention, proliferation, and multipotency. Niches are composed of cells together with their associated extracellular matrix (ECM). Currently, the roles of ECM in regulating niche functions are poorly understood. Here, we demonstrate that Perlecan (Pcan), a highly conserved ECM component, controls intestinal stem cell (ISC) activities and ISC-ECM attachment in *Drosophila* adult posterior midgut. Loss of Pcan from ISCs, but not other surrounding cells, causes ISCs to detach from underlying ECM, lose their identity, and fail to proliferate. These defects are not a result of a loss of epidermal growth factor receptor (EGFR) or Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling activity but partially depend on integrin signaling activity. We propose that Pcan secreted by ISCs confers niche properties to the adjacent ECM that is required for ISC maintenance of stem cell identity, activity, and anchorage to the niche.

INTRODUCTION

Stem cell niches are specialized local microenvironments that are able to house and maintain stem cells (Fuchs et al., 2004; Morrison and Spradling, 2008). Previous studies have shown that stem cell niches are composed of supporting cells and their associated extracellular matrix (ECM) (Chen et al., 2013; Jones and Wagers, 2008; Lander et al., 2012). Supporting cells can regulate stem cells by secreting diffusible factors or through adheren junctions (Chen et al., 2013; Jones and Wagers, 2008; Xie and Spradling, 2000). However, roles of ECM in niches are less understood.

The ECM is thought to be an important component of niche because in many cases, stem cells directly contact the ECM (Chiarini-Garcia et al., 2003; Collins et al., 2005; Kanatsu-Shinohara et al., 2008; Kuang et al., 2008; Shen et al., 2008; Watt, 2002). Particularly, some stem cells, including mouse skeleton muscle satellite cells, mouse skin basal keratinocytes, and *Drosophila* intestinal stem cells (ISCs), are not associated with any specialized supporting cells but are located adjacent to the basement membrane (Kuang et al., 2008; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Watt, 2002). So far, niches for these stem cells are difficult to define. One possibility is that stem cell niches are formed around stem cells and are established by stem cells through stem cell-ECM interaction.

Drosophila adult posterior midgut is an ideal system to investigate the roles of ISC-ECM interaction. In this system, ISCs are individual small cells that reside on the basement membrane and are surrounded by mature epithelial cells (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Under the basement membrane is a layer of muscle cells. In normal homeostasis, ISCs produce new mature epithelial cells, including abundant big enterocytes (ECs) and rare small enteroendocrine (ee) cells, to replenish the gut every 1–2 weeks. In response to the damage of gut epithelium caused by ingestion of cytotoxic agent dextran sodium sulfate (DSS), ISCs overproliferate and thus help gut epithelium to regenerate itself (Amcheslavsky et al., 2009; Lucchetta and Ohlstein, 2012). Although the functions of signaling pathways in ISC regulation have been intensively studied, what is an ISC niche and how it is established are not understood.

Perlecan (Pcan) is a highly conserved basement membrane-specific heparan sulfate proteoglycan (HSPG) and is composed of a core protein with heparan sulfate chains attached (Cohen et al., 1993; Lin, 2004). Pcan is deposited to the ECM by producing cells and crosslinks with many ECM components (Friedrich et al., 2000). It is encoded by *HSPG2* in mammals and *trol* in *Drosophila* (Kallunki et al., 1991; Voigt et al., 2002). Here, we demonstrated that Pcan plays critical roles in the regulation of ISC activity by mediating stem cell-ECM attachment. Our results suggest that ISC secrets Pcan to form an activated ECM and, therefore, establish a niche for itself.

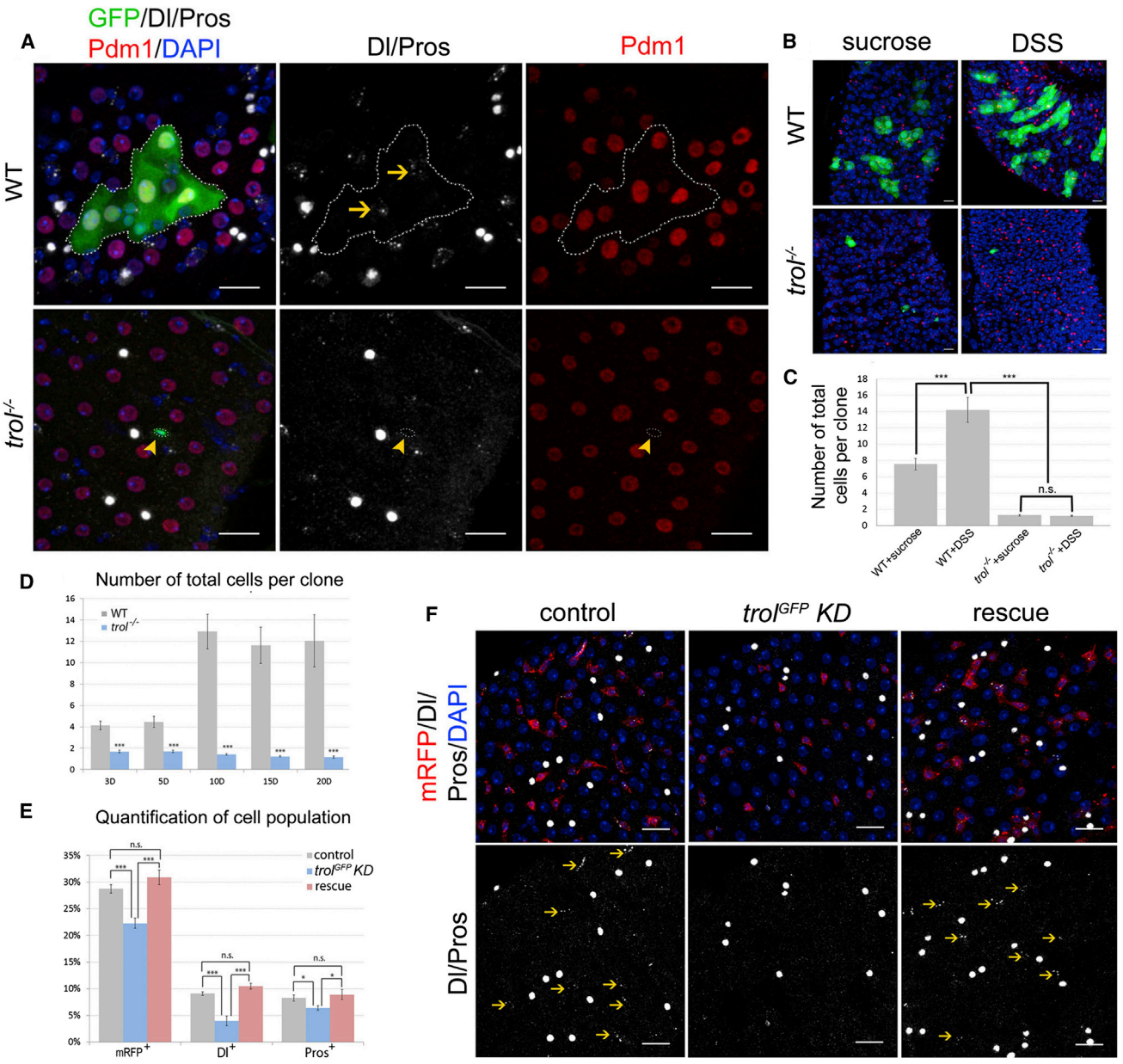

Figure 1. Loss of Pcan Led to Loss of Stem Cell Activity and Identity

(A) A WT MARCM clone and a $trol^{-/-}$ clone (arrowheads).

(B and C) WT and $trol^{-/-}$ clones in DSS- or sucrose-treated gut.

(D) Quantification of the number of the total cells per clone in WT and $trol^{-/-}$ MARCM clones from 3 to 20 days ACI.

(E and F) Progenitor cells (monomeric red fluorescent protein [mRFP] in red) and ISCs (arrows) in the control, $trol^{GFP}KD$, and rescue group. Scale bars, 20 μm. Dotted lines outline clones. ***$p < 0.001$; *$p < 0.05$; n.s., nonsignificant difference. Error bars show SEs. In (C)–(E), more than 15 flies were analyzed for each group. See also Figure S1.

RESULTS

Loss of Pcan Leads to Loss of ISC Activity and Identity

To determine the roles of Pcan in ISC regulation, we generated positively marked *trol* homozygous mutant ($trol^{-/-}$) clones with a $trol^{null}$ allele using the MARCM (mosaic analysis with a repressible cell marker) technique (Lee and Luo, 1999). Ten days after clone induction (ACI), a GFP-marked wild-type (WT) ISC was able to generate 10–15 GFP-labeled cells to form a clone (Figure 1A). In contrast, $trol^{-/-}$ clones usually only contained one to two cells (Figure 1A, arrowhead), suggesting that $trol^{-/-}$ ISCs lost their ability to produce new cells.

From 3 to 20 days ACI, *trol*$^{-/-}$ clones were always significantly smaller than WT clones (Figure 1D). In addition, *trol*$^{-/-}$ clones did not contain any cell that was positive for the stem cell marker Delta (Dl), whereas WT clones usually contained one to two Dl^{+} cells (Figure 1A, arrows). Normally, loss of Dl leads to loss of Notch signaling activity and, therefore, induces tumor-like cell mass (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Zeng et al., 2013). Our data showed that loss of Pcan led to loss of Dl but did not result in tumor-like cell mass, suggesting that *trol*$^{-/-}$ ISCs had lost their identity and their activity to produce new cells as well. *trol*$^{-/-}$ cells were not positive for the ee cell marker, Prospero (Pros) or the EC marker, Pdm1, indicating that *trol*$^{-/-}$ cells were not able to differentiate to ee cells or ECs.

To further confirm the phenotypes of *trol* loss-of-function (LOF) clones, we used two additional *trol* hypomorphic alleles to generate clones. Both *trol*$^{G0271/G0271}$ and *trol*$^{G0023/G0023}$ clones contained only one to two cells, which were similar to *trol*$^{-/-}$ clones (Figures S1A–S1C available online). In addition, when *trol* was knocked down in progenitor cells (ISCs and enteroblast [EB] cells) by the expression of *trol* RNAi driven by *esg-gal4*, the number of progenitor cells and ISCs was obviously reduced (Figures S1D and S1E). Together, these results demonstrated that loss of Pcan led to loss of stem cell identity and activity.

Because gut epithelium is able to regenerate when it is damaged by DSS (Amcheslavsky et al., 2009; Lucchetta and Ohlstein, 2012), we examined the roles of Pcan in regeneration. Ten days ACI, WT clones in DSS-treated flies were obviously larger than clones in sucrose-treated control flies (Figures 1B and 1C). However, *trol*$^{-/-}$ clones in DSS-treated flies contained only one to two cells and were significantly smaller than WT clones in DSS-treated flies (Figures 1B and 1C). These results demonstrated that Pcan is required for ISC activities during gut regeneration.

We also performed rescue experiments to show that expression of *trol* can rescue loss of ISC identity and activity. Because *trol* is larger than 100 kb and has more than 20 transcripts, it is difficult to ectopically express it using the GAL4/upstream activating sequence (UAS) system. A study suggested that in homozygous *trol*^{GFP} (*trol*$^{GFP/GFP}$) flies, expression of GFP RNAi could knock down *trol*^{GFP} and therefore deplete normal Pcan function (Pastor-Pareja and Xu, 2011). Here, in *trol*$^{GFP/GFP}$ flies, when *trol*^{GFP} was knocked down in progenitor cells by the expression of GFP RNAi, the number of progenitor cells and ISCs was obviously reduced (Figures 1E and 1F). In rescue experiments, with the involvement of a copy of WT *trol* whose expression is not affected by GFP RNAi, the number of progenitor cells and ISCs was increased and had no significant difference from that in control (Figures 1E and 1F). These results demonstrated that Pcan plays critical roles in the regulation of ISC activity and identity.

Pcan-Deficient ISCs Lost Their Ability to Proliferate

We further asked whether loss of Pcan led to cell death. We examined this possibility by determining the levels of cleaved caspase-3 (Casp3) using fluorescence immunostaining. In normal conditions, the levels of cleaved Casp3 were very low, and no cleaved Casp3 could be detected in *trol*$^{-/-}$ clones (Figures S2A and S2B). When guts were damaged by DSS treatment, the overall levels of cleaved Casp3 were increased. In *trol*$^{-/-}$ clones, the levels of cleaved Casp3 were not higher than surrounding cells (Figures S2C and S2D). These data argue that loss of Pcan did not lead to cell death.

Next, we determined whether *trol*$^{-/-}$ ISCs lose their activity due to loss of proliferation. We examined the levels of phosphorylated histone H3 (pH3) by fluorescence immunostaining in guts with WT or *trol*$^{-/-}$ clones. Usually, around 5% of WT clones contained a pH3^{+} cell(s), whereas less than 1% of *trol*$^{-/-}$ clones did (Figures 2A and 2B). We also performed a 5-ethynyl-2′-deoxyuridine (EdU) incorporation assay and found that, compared to WT clones, *trol*$^{-/-}$ clones contained significantly less EdU^{+} cells, suggesting that *trol*$^{-/-}$ ISCs lost their proliferation ability (Figures 2C and 2D).

Janus kinase/signal transducer and activator of transcription (JAK/STAT) and epidermal growth factor receptor (EGFR) signaling are two major signaling pathways that control the ISC proliferation in both normal homeostasis and epithelium regeneration (Beebe et al., 2010; Jiang et al., 2009, 2011; Liu et al., 2010; Ren et al., 2010; Xu et al., 2011). Also, HSPGs are able to interact with many ligand proteins to regulate signaling activities (Hayashi et al., 2012; Lin, 2004; Zhang et al., 2013). Therefore, one possibility is that Pcan may control JAK/STAT and EGFR signaling activities to regulate ISC proliferation. To test this possibility, we examined the activities of JAK/STAT signaling with 10XSTAT92E-GFP and pSTAT staining, and EGFR signaling with dpERK staining in WT and *trol*$^{-/-}$ clones (Bach et al., 2007; Gabay et al., 1997; Zhang et al., 2013). From 3 to 15 days ACI, the percentage of 10XSTAT92E-GFP^{+} cells in *trol*$^{-/-}$ clones was not obviously reduced compared to that in WT clones (Figures 2E and 2G). The percentage of pSTAT^{+} cells in *trol*$^{-/-}$ clones showed no significant difference from that in WT clones (Figures 2F and 2H). In addition, the percentage of dpERK^{+} cells in *trol*$^{-/-}$ clones was not significantly different from that in WT clones (Figures 2I and 2J). Based on these findings, we conclude that the loss of proliferation in *trol*$^{-/-}$ ISCs is not the consequence of loss of JAK/STAT or EGFR signaling activity.

Pcan Regulates ISCs in a Cell-Autonomous Manner

Because Pcan is a major component of the ECM, we speculated that Pcan may act to maintain the integrity of the

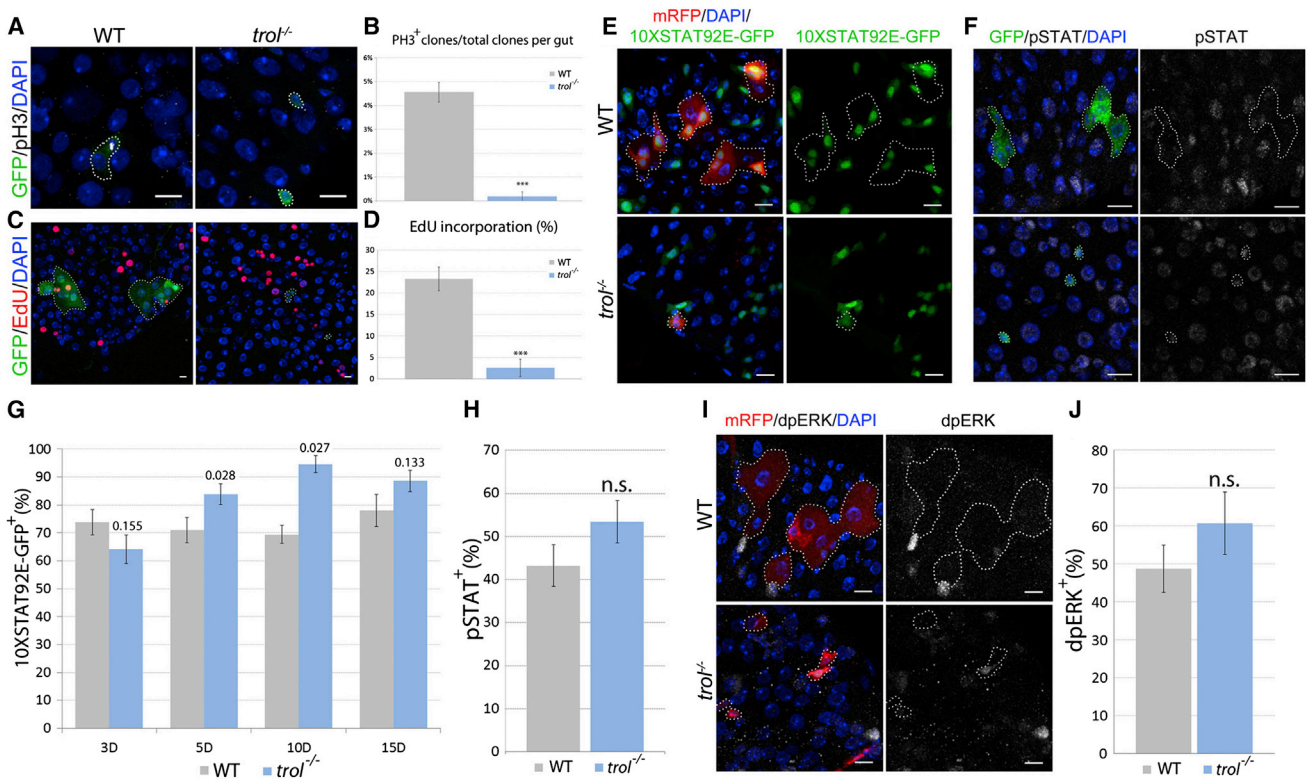

Figure 2. *trol*$^{-/-}$ ISCs Lost Their Proliferation, but Not JAK/STAT or EGFR Signaling Activities

(A and B) pH3 staining (white) showed mitosis cells in clone.

(C and D) EdU incorporation (red) showed proliferating cells in clone.

(E–H) 10XSTAT92E-GFP (green) and pSTAT staining (white) showed that JAK/STAT signaling remained activated in *trol*$^{-/-}$ clones. p values are shown on top of bars in (G).

(I and J) dpERK staining (white) showed that EGFR signaling remained activated in *trol*$^{-/-}$ cells.

Scale bars, 10 μm. Dotted lines outline clones. ***$p < 0.001$; n.s., nonsignificant difference. Error bars show SEs. In (B), (D), (G), (H), and (J), more than 15 flies were analyzed for each group. See also Figure S2.

ECM and thus help ISCs to maintain their identity and activity via interaction with the ECM. In the posterior midgut, high levels of Pcan were detected in the basement membrane, which is located on top of muscle cells (Figure 3A), and low levels of Pcan can be detected around muscle cells. *trol* knockdown in muscle cells by *trol* RNAi driven by *24B-gal4* or *mef2-gal4* led to the depletion of Pcan in the muscle layer and reduction of Pcan in the basement membrane (Figures 3B and 3C). When *trol* was knocked down in ECs using *NP1-gal4*, the levels of Pcan in the basement membrane were also obviously reduced (Figure 3D). However, in all cases, the number of ISCs was not reduced (Figures S3A–S3C and S3F), suggesting that the overall levels of Pcan in the basement membrane are not essential for the maintenance of ISC identity and activity.

Importantly, we observed a significantly reduced number of ISCs when *trol* was knocked down in ISCs (Figures S3E and S3F). Although the number of ISCs was not reduced when *trol* was knocked down in EB cells (Figures S3D and S3F). These results are consistent with the data showing that *trol*$^{-/-}$ clones only contained one to two cells and suggest that Pcan secreted by surrounding WT cells cannot rescue the deficiency in *trol*$^{-/-}$ ISCs. We also examined the levels of Pcan in the basement membrane and did not find obvious differences between the levels of Pcan under WT ISCs and that under *trol*$^{-/-}$ ISCs (Figures 3E and 3F). Consistent with this result, when *trol* was knocked down in ISCs or EB cells, the levels of Pcan in the basement membrane were not changed (Figures S3G–S3J). Together, these results demonstrate that Pcan regulates ISCs in a cell-autonomous manner.

Pcan Is Required for ISC-ECM Attachment

How does Pcan regulate ISC activity cell autonomously? One possibility is that Pcan may confer its producing ISCs to the local microenvironment required to maintain ISC activities. To test this possibility, we examined *trol*$^{-/-}$ ISC behavior and interaction with the basement membrane.

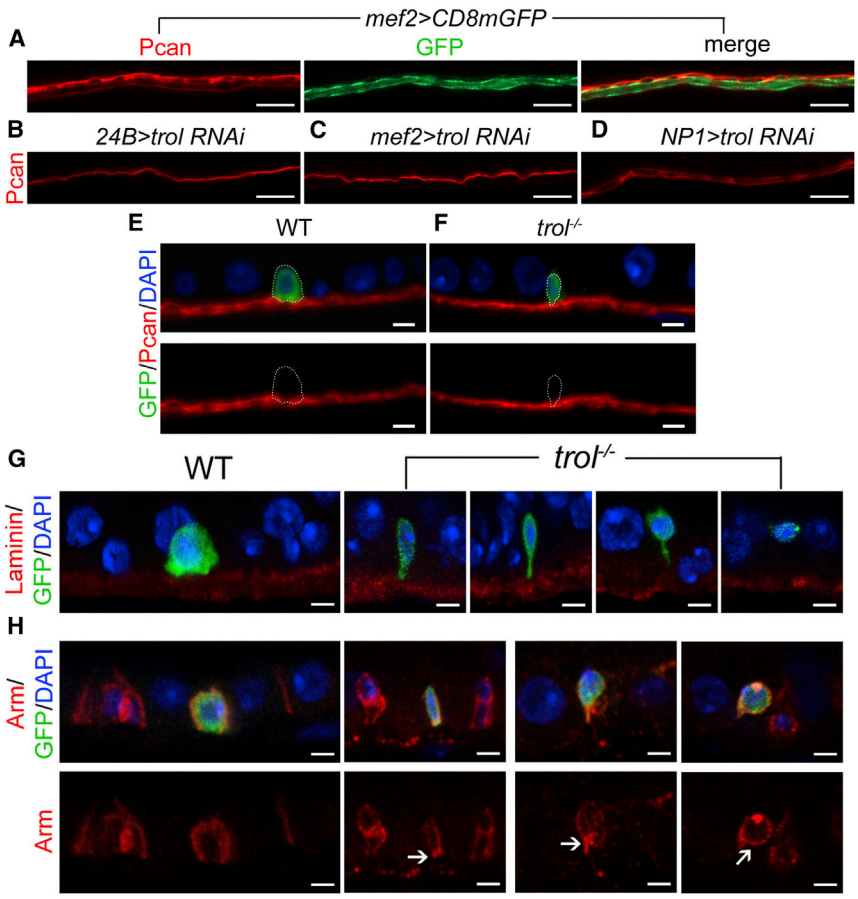

Figure 3. *trol*$^{-/-}$ ISCs Lost Their Attachment to the Basement Membrane

(A) Immunostaining of Pcan (red) and muscle cells (CD8mGFP; green).

(B–D) Levels of Pcan were reduced when *trol* was knocked down in muscle cells (B and C) or in EC cells (D).

(E and F) Levels of Pcan under a *trol*$^{-/-}$ ISC (F; marked by GFP) are similar to that under a WT ISC (E).

(G) A WT ISC (green) stayed at the most basal side and tightly attached to the basement membrane (marked by Laminin; red). *trol*$^{-/-}$ ISCs (green) moved to the apical side and lost attachment to the basement membrane.

(H) High levels of Arm (red) could be detected at the apical and lateral sides of a WT ISC (green). Arm mis-localized to the basal side (arrows) in *trol*$^{-/-}$ ISCs (green).

Scale bars, 10 μm (A–D) and 5 μm (E–H). See also Figure S3.

As shown in Figure 3G, we marked WT or *trol*$^{-/-}$ ISCs with GFP and the basement membrane with Laminin. Ten days ACI, a WT ISC stayed at the basal side of the gut epithelium and tightly attached to the basement membrane (Figure 3G). However, *trol*$^{-/-}$ ISCs elongated along the apical-basal axis, moved to the apical side, and finally lost their attachment to the basement membrane (Figure 3G). We further examined the polarity of ISCs with immunostaining of adherens junction components Armadillo (Arm; *Drosophila* β-catenin) and DE-cadherin (E-CAD; *Drosophila* E-cadherin). Normally, Arm and E-CAD only stayed in the apical and lateral side and were absent from the basal side (Figures 3H and S3K). High levels of Arm and E-CAD can be detected at the interface membrane between the ISCs and their adjacent EB cells (Figure S3L, arrows) (Maeda et al., 2008; Ohlstein and Spradling, 2006). However, in *trol*$^{-/-}$ ISCs, Arm mis-localized to the basal side and could be detected all around ISCs (Figure 3H). Similar to Arm, E-CAD also mis-localized to the basal side of ISCs (Figures S3K and S3L). These results demonstrated that *trol*$^{-/-}$ ISCs lost their polarity and their attachment to the basement membrane, indicating that Pcan might help create the proper microenvironment for normal ISC attachment and polarity.

Functions of Pcan Are Partially Dependent on Integrin Signaling Pathway

We further examined the mechanism(s) by which Pcan regulates ISC activity and cellular properties (polarity and cell-ECM attachment). Integrin signaling pathway plays important roles in mediating cell-ECM attachment (Ellis and Tanentzapf, 2010; Hynes, 2002). To determine whether integrin signaling activities were impaired in *trol*$^{-/-}$ ISCs, we examined the levels of phosphorylated FAK (pFAK) with anti-pFAK immunostaining (Ivankovic-Dikic et al., 2000). In WT ISCs, pFAK usually accumulated at the attachment point (Figure S4A, arrows), whereas in *trol*$^{-/-}$ ISCs, such attachment points with pFAK staining cannot be detected (Figure S4A). To further confirm that Pcan is required for normal integrin signaling activity, we generated *trol*$^{-/-}$ clones in wing discs and examined the levels of pFAK as well. Levels of pFAK in *trol*$^{-/-}$ clones were obviously reduced compared to those in surrounding WT cells (Figure S4B). Therefore, without Pcan, integrin signaling activity was impaired.

Next, we examined the roles of integrin signaling in the regulation of ISC activity. In *Drosophila*, *mew* encodes for the α1-integrin receptor, and *mys* encodes for the β-integrin

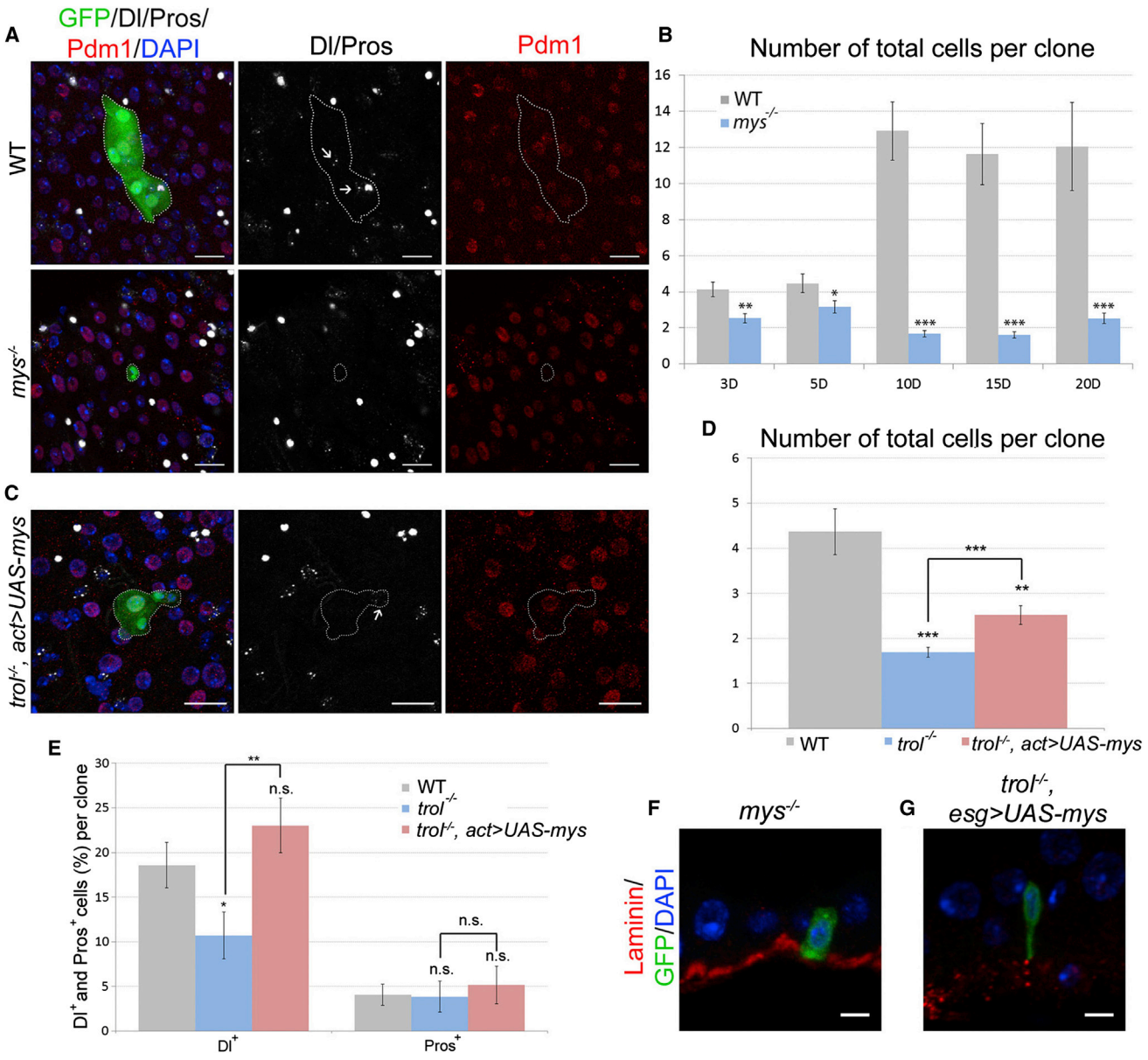

Figure 4. Functions of Pcan Partially Depend on Integrin Signaling Activity

(A and B) A WT clone and a *mys*$^{-/-}$ clone 10 days ACI (A). Quantification of the number of total cells per clone from 3 to 20 days ACI is shown (B).

(C and D) The *mys* rescue clone 5 days ACI (C). Quantification of the number of total cells per clone is shown (D).

(E) The percentage of Dl^{+} cells and Pros^{+} cells in WT, *trol*$^{-/-}$, and *mys* rescue clones.

(F) A *mys*$^{-/-}$ ISC (marked by GFP; green) was tightly attached to the basement membrane (showed by Laminin staining; red).

(G) A *trol*$^{-/-}$ ISC with *mys* expression (green) was detached from the basement membrane.

Scale bars, 20 μm (A and C) and 5 μm (F and G). Dotted lines outline clones. ***p < 0.001; **p < 0.01; *p < 0.05; n.s., nonsignificant difference. Error bars show SEs. In (B), (D), and (E), more than 15 flies were analyzed for each group. See also Figure S4.

receptor (Brown, 2000). High levels of both Mew and Mys were detected around progenitor cells (Figures S4C and S4D). Similar to *trol*$^{-/-}$ clones, *mys*$^{-/-}$ clones were significantly smaller than WT clones (Figures 4A and 4B). Moreover, cells in *mys*$^{-/-}$ clones were not positive for Dl, Pros, or Pdm1 staining, suggesting that *mys*$^{-/-}$ ISCs lost their activity and identity. Together with the data showing that integrin signaling activity was reduced in *trol*$^{-/-}$ clones (Figures S4A and S4B), our data suggest that *trol*$^{-/-}$ ISCs may lose their identity and activity due to loss of integrin

signaling activity. To test this possibility, we ectopically expressed *mys* in *trol*$^{-/-}$ clones to see if *trol* LOF phenotypes could be rescued. The levels of pFAK in *trol*$^{-/-}$ cells with *mys* expression were similar to those in neighboring WT cells, indicating that integrin signaling was activated in the clone (Figure S4E). Five days ACI, *trol*$^{-/-}$ clones with *mys* ectopic expression (*mys* rescue clones) were significantly larger than *trol*$^{-/-}$ clones but still smaller than WT clones (Figures 4C and 4D). The percentage of Dl^{+} cells in *mys* rescue clones was higher than that in *trol*$^{-/-}$ clones and had no significant difference from that in WT clones (Figure 4E). Ten days ACI, the size of *mys* rescue clones was not increased compared to that in 5 days ACI (Figures S4E and S4F). But the percentage of Dl^{+} cells in *mys* rescue clones was higher than that in *trol*$^{-/-}$ clones and had no significant difference from that in WT clones (Figure S4G). Together, these results indicated that overexpression of *mys* in *trol*$^{-/-}$ clones can partially restore the ISC identity and rescue the proliferation defect of *trol*$^{-/-}$ ISCs.

Importantly, we found that *mys*$^{-/-}$ ISCs still attached to the basement membrane and maintained their polarity (Figure 4F). Expression of *mys* in *trol*$^{-/-}$ ISCs did not rescue the detachment phenotype (Figure 4G). These data suggest that the ISC-ECM attachment is not controlled by the integrin signaling pathway. Pcan can regulate ISC attachment to the basement membrane in a manner independent of the integrin signaling pathway.

DISCUSSION

Our data presented here demonstrate that the ECM protein Pcan plays critical roles in the regulation of ISC activity by mediating ISC-ECM attachment. Because Pcan is a secreted protein that is distributed in ECM (Friedrich et al., 2000), it is surprising to find that Pcan regulates ISCs in a cell-autonomous manner. How could Pcan have cell-autonomous functions? We observed that *trol*$^{-/-}$ ISCs detached from the basement membrane and lost their polarity, suggesting that ISC-ECM interaction was disrupted. This defect is independent of the integrin signaling pathway because *mys*$^{-/-}$ ISCs did not show this detachment phenotype. Consistent with this view, we show that the activation of integrin signaling could not rescue the detachment phenotype in *trol*$^{-/-}$ ISCs. On the basis of these data, we propose that Pcan forms a complex with a cell surface and transmembrane protein(s) before being secreted. Once this complex is presented to the cell surface, Pcan crosslinks to ECM components and forms an activated ECM around the ISC. This activated ECM is required for the ISC anchorage to the basement membrane and, thus, helps to establish the ISC niche. Previous studies show that Pcan binds to Dystroglycan (Dg), a widely expressed ECM receptor, to mediate ECM-cytoskeleton linkage in *Drosophila* follicle cells (Schneider et al., 2006). Therefore, Dg might be a good candidate for binding Pcan to control the ISC-ECM attachment.

Importantly, the detached *trol*$^{-/-}$ ISCs lost their ability to proliferate, suggesting that the ISC-ECM attachment is important for the regulation of ISC proliferation. Previous studies show that ISC-ECM attachment is required for keeping stem cells in the correct position to receive diffusible factors such as bone morphogenetic protein, EGF, and cytokines to maintain the stem cell activity (Ellis and Tanentzapf, 2010; Marthiens et al., 2010; Morrison and Spradling, 2008). However, in detached *trol*$^{-/-}$ ISCs, JAK/STAT and EGFR signaling pathways remained activated, demonstrating that the ISC does not need to be anchored to the basement membrane to receive JAK/STAT or EGFR signaling. Also, even though JAK/STAT and EGFR signaling pathways remained activated, the detached *trol*$^{-/-}$ ISCs still cannot proliferate, indicating that ISC-ECM attachment regulates ISC proliferation in a manner independent of JAK/STAT and EGFR signaling. Interestingly, we observed that integrin signaling activity is reduced in *trol*$^{-/-}$ ISCs. *mys*$^{-/-}$ ISCs lost their ability to proliferate. Moreover, the activation of integrin signaling can partially rescue the proliferation defect in *trol*$^{-/-}$ ISCs. These results suggest that *trol*$^{-/-}$ ISCs lose their ability to proliferate partially due to loss of integrin signaling activity. Our results suggest that ISC-ECM attachment is required for the activation of integrin signaling, and point out that integrin signaling plays a major role in the regulation of *Drosophila* ISC proliferation.

Together, we propose that ISC secrets Pcan to create an activated ECM, which is required for the niche establishment. The activated ECM is critical for both ISC-ECM attachment and the maintenance of stem cell activity and identity. Therefore, our data support a view that stem cells can generate their niches by themselves.

EXPERIMENTAL PROCEDURES

Clonal Analysis and Feeding Experiments

For clonal analysis, flies of appropriate genotypes were given heat-shock treatment at 3 days after eclosure and then kept at 25°C. For feeding experiments, 3 days ACI, flies were transferred to an empty vial with six pieces of round chromatography paper (Fisher Scientific). A total of 400 μl 5% sucrose solution or 3% DSS (MP Biomedicals) in 5% sucrose solution was added to the paper. Flies were transferred to new vials every day and were dissected 4 days after feeding with DSS.

Immunofluorescence Staining and Microscope

Antibody staining was performed as described (Belenkaya et al., 2002). After antibody staining, samples were washed in PBS with DAPI (Sigma-Aldrich) for 1 hr. Samples were observed with a Nikon

A1R si laser-scanning confocal microscope on a Nikon Ti inverted microscope. All images were taken at multiple optical sections and converted to volume view by NIS element version 4.0.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2014.04.007.

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