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The CSF-1 Receptor Fashions the Intestinal Stem Cell Niche

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Abstract Gastrointestinal (GI) homeostasis requires the action of multiple pathways. There is some controversy regarding whether small intestine (SI) Paneth cells (PC) play a central role in orchestrating crypt architecture and their relationship with Lgr5+ve stem cells. Nevertheless, we previously showed that germline CSF-1 receptor (*Csf1r*) knock out (KO) or *Csf1* mutation are associated with an absence of mature PC, reduced crypt proliferation and lowered stem cell gene, *Lgr5* expression. Here we show the additional loss of CD24, *Bmi1* and *Olfm4* expression in the KO crypts and a high resolution 3D localization of CSF-1R mainly to PC. Induction of GI-specific *Csf1r* deletion in young adult mice also led to PC loss over a period of weeks, in accord with the anticipated long life span of PC, changed distribution of proliferating cells and this was with a commensurate loss of *Lgr5* and other stem cell marker gene expression. By culturing SI organoids, we further show that the *Csf1r*$^{-/-}$ defect in PC production is intrinsic to epithelial cells as well as definitively affecting stem cell activity. These results show that CSF-1R directly supports PC maturation and that in turn PCs fashion the intestinal stem cell niche.

Key Words: Paneth cells, Colony Stimulating Factor-1 (CSF-1), CD24, Lgr5, Olfm4, Bmi-1, CSF-1 Receptor, organoids

Introduction

The maintenance of gastrointestinal homeostasis and the response to tissue damage requires the orchestration of intestinal stem and progenitor cell populations. Several signaling pathways regulate the maintenance and differentiation of intestinal stem cells (ISC) which is also dependent on other cell types, including ISC-derived Paneth cells (PC) (Barker et al., 2007). PC development within the crypt is dependent on colony stimulating factor-1 (CSF-1) (Huynh et al., 2009), the primary regulator of the development of macrophages and osteoclasts. CSF-1 effects are mediated by the CSF-1 receptor (CSF-1R), which is expressed on PC, macrophages and osteoclasts (Chitu and Stanley, 2006).

In mice deficient in either CSF-1 (*Csf1*$^{op/op}$) or CSF-1R (*Csf1r*$^{-/-}$) the PC fail to develop and the SI exhibited cell proliferation and differentiation defects associated with reduced expression of the ISC marker Lgr5. This phenotype can be rescued in *Csf1*$^{op/op}$ mice by a membrane-spanning cell-surface isoform of CSF-1 that acts locally and does not contribute to the pool of circulating CSF-1, suggesting juxtacrine or paracrine regulation of PC by CSF-1 (Huynh et al., 2009). Consistent with such local regulation, the *Csf1* promoter is active in cells in the crypt that closely neighbor PC (Huynh et al., 2009). In single Lgr5+ ISC-derived cultures, there is a close physical association of ISC with PC which express multiple ligands important to ISC maintenance and co-culture of ISC with PC markedly improves organoid formation (Sato et al., 2011). Together, these studies indicated that locally expressed CSF-1 is required for PC development and that PC directly support the maintenance of ISC.

Despite the circumstantial evidence at least two important questions remain unresolved concerning the role of CSF-1 and PC in the regulation of ISC. The first is whether there is a direct or indirect role of macrophages in the lamina propia and/or those below the crypt base that, with PC, are also dramatically reduced in *Csf1*$^{op/op}$ and *Csf1r*$^{-/-}$

mice. The second is whether the CSF-1R is required for both PC development and/or PC maintenance in adult mice. To address these questions, we used intestinal epithelial specific inducible deletion of *Csf1r* and *in vitro* organoid cultures. We find that the defects evident in mice with germline deletion of *Csf1r* were recapitulated by the conditional KO studies but the phenotype took several weeks to manifest consistent with the long half life of PC. The use of organoid cultures with either germline or inducible deletion of *Csf1r* led to reduced colonies and size as well as defective ISC gene expression indicating that CSF-1R is essential and cell intrinsic to the ISC niche.

Material and Methods

Mice

Csf1r^{-}/*Csf1r*$^{-}$ (*Csf1*$^{-/-}$) mice on a FVB/NJ background were generated by heterozygote intercrossing and sacrificed at two weeks of age (Dai et al., 2002). *VillinCre*^{ERT2} (el Marjou et al., 2004) and *Csf1r*^{flox}/*Csf1r*^{flox} (*Csf1r*$^{fl/fl}$) (Li et al., 2006) on a C57Bl/6 background were housed under SPF conditions. Experiments were conducted with animal experimentation ethics committee approval. Tamoxifen (Sigma-Aldrich, Australia) was administered (100 mg/kg) in the chow.

Immunohistochemistry

Mouse pups (P14) were perfused with periodate-lysine-2%paraformaldehyde-0.05% glutaraldehyde, pH 7.4, and processed as described (Huynh et al., 2009). Anti-PCNA and anti-CD24 antibodies were used followed by processing with the Dako Envison+ mouse detection kit (Campbellfield, Vic, Australia). For H & E staining, intestine sections were fixed in methacarn for 2 hours and transferred to 70% ethanol, embedded and sectioned. Full crypts exposing a lumen and identifiable base were scored. Crypt position counting was performed as described (Malaterre et al., 2007). HRP reactions were developed with Pierce Metal-enhanced detection reagent. Antibodies and titres used are listed in **Table 1**.

Immunofluorescence

Crypts were fixed for 10 minutes in 4% paraformaldehyde and subsequently 0.4% overnight, prior to blocking with 1% fetal bovine serum or rabbit serum for 1 hour. Primary antibodies to the CSF-1R, β-catenin and Lysozyme were applied overnight at ambient temperature and processed as described (Ramsay, 2004). Nuclei were identified by DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride). Crypts were imaged using an Olympus Fluoroview FV1000 Confocal microscope. Imaris software (Bitplane Inc St Paul, MN, USA) was used to generate 3D images for movies of whole crypts.

Organoid cultures

The SI were excised from 2 to 3 weeks old WT and *Csf1r*$^{-/-}$ or 4-5 weeks old, WT, *VillinCre*^{ERT2} and *Csf1r*$^{fl/fl}$; *VillinCre*^{ERT2} mice. Briefly, mice were euthanized, the entire SI was removed. Crypts were isolated and dissociated as described (Sato et al., 2009). One thousand crypts were seeded in 50 μl Phenol Red-free Matrigel (BD Bioscience North Ryde, Australia) overlayed with 500 μl of DME medium/F12 (Sigma, St. Louis, MO) containing 20 ng/ml EGF (BD Biosciences), 10 ng/ml basic FGF (Roche, Kew, Vic, Australia), 500 ng/ml R-Spondin (R&D Systems, Minneapolis, MN), 100 ng/ml Noggin (Peprotech, Rocky Hill, NJ), and B27supplement (Invitrogen, Mulgrave, Vic, Australia) in 24-well plates. Crypt nests derived from *Csf1r*$^{fl/fl}$; *VillinCre*^{ERT2} and wt; *VillinCreE*^{RT2} mice were treated with 4-hydroxy-tamoxifen (4OHT, Sigma-Aldrich, 0.1 μM) with medium changes every 2-3 days and after 7 days the organoids were counted. MTT (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assays were performed after 7 days of culture.

Gene expression and qRT-PCR primers

SI crypt epithelium was prepared, and its purity was confirmed by phase contrast microscopy of crypts. Villi were mostly excluded from these preparations. Real-time reverse transcription–polymerase chain reactions (RT-PCRs) were conducted on genomic DNA-depleted RNA using a StepOnePlus real time PCR system machine (Applied Bisosystems, CA. USA) and the appropriate primers. Gene expression was normalized to *Gapdh* expression. Amplification parameters were: 95^{o}C for 10 minutes, followed by 40 cycles of 95^{o}C for 15 seconds and 60^{o}C for 1 minute and a final cycle for the melt curve at 95^{o}C for 15 minutes, 60^{o}C for 1 minute and 95^{o}C for 15 seconds. Primer sequences are tabulated in **Table 2.**

Results

CD24 is lost in *Csf1r* null SI

We have previously shown that *Csf1r*$^{-/-}$ crypts are deficient in PC by staining for antigens which are indicative of the products they generate, including Lysozyme and Cryptdins (Huynh et al., 2009). With the discovery that PC may have a direct role in support in intestinal stem cells (ISC) we also evaluated the effect on the ISC gene, *Lgr5* finding it was similarly lost along with PCs (Huynh et al., 2009). To advance this further here we investigated the expression of an additional cell surface marker employed to enrich PC and ISC, CD24 (Gracz et al., 2010; Sato et al., 2011; von Furstenberg et al., 2011) in *Csf1r*$^{-/-}$ crypts by IHC and found to be mostly lost compared to WT crypts (**Figs. 1A-B**). This implied that both categories of cells that collectively constitute the intestinal stem cell niche were lost.

Notch and Wnt target gene expression is affected in *Csf1r* null SI crypts

As others have reported Paneth cell production is distorted by the deletion of Notch pathway gene *Atoh1/Math1* (Durand et al., 2012; Kim et al., 2012). Accordingly, when *Math1* expression was examined in SI crypts from *Csf1r*$^{-/-}$ mice we observed a trend towards increased expression (**Supplementary Fig. 1**) entirely consistent with an increase in PAS and Alcian Blue histochemistry (Huynh et al., 2009) as well as a commensurate loss of *Math-1* negative regulator *Hes-1* (Akazawa et al., 1995; Ishibashi et al., 1995) mRNA levels (**Supplementary Fig. 1**). This suggests that Notch signalling is affected when PCs are lost. Similarly, we reported previously that Wnt target genes *Lgr5* and *CyclinD1* expression was also significantly reduced in *Csf1r*$^{-/-}$ SI crypts consistent with the observed reduced proliferation (Huynh et al., 2009) and an effect on Wnt signalling. These data suggest that the PC deficit reported here and that shown in *Sox9* KO mice (Bastide et

al., 2007; Gracz et al., 2010; Mori-Akiyama et al., 2007) differs from that reported in *Atoh1/Math1* KO mice; a view also held by others (Rizk and Barker, 2012).

CSF-1 receptor is expressed most strongly by Paneth cells in isolated crypts

To more precisely localize where CSF-1R was expressed in crypts we first isolated crypts using an established EDTA method which in the SI leads to villi loss through shearing (**Fig. 1C**) and visualized CSF-1R and Lysozyme by 3D confocal microscopy in the crypts alone (**Fig. 1D**). (Corresponding movies are also provided as **Supplementary Data** online). When CSF-1R is deleted a substantial reduction in Lysozyme staining is evident (**Fig. 1E**). Further confocal images of β-catenin basal-lateral staining and CSF-1R are shown (**Fig. 1F-H**). Collectively these data highlight the basal-lateral expression of Csf-1R on PC in SI crypts where the pericryptal fibroblasts have been definitively left behind with the mucosa during the processing of the samples and that CD24 is essentially completely lost in the KO SI.

***Csf1r* null crypts show impeded capacity to form organoids**

It is considered that ISC genes and PC play an important role in the generation of crypts and with the advent of SI organoid cultures now it is feasible to directly test the role of other pathways such as the CSF-1R signaling. When organoid cultures were initiated from *Csf1r*$^{-/-}$ mice it was very clear that they were substantially impeded in this capacity visually (**Fig. 2A**) and in terms of organoid number and size (**Fig. 2B**). Notably, those *Csf1r*$^{-/-}$ organoids that did form were invariably associated with the early appearance of granulated cells (PC). These data show that the decrease in ISC marker expression and progenitor proliferation previously observed in *Csf1r*$^{-/-}$ SI *in vivo* (Huynh et al., 2009) can be replicated *in vitro,* demonstrating an epithelial cell-intrinsic defect.

Expression of Intestinal stem cell genes is lost in *Csf1r* null crypts

Indicative of the absence of CD24 in the PC and ISC it would be the predicted that there should also be a commensurate loss of ISC stem cell gene expression in organoid cultures established from *Csf1r*$^{-/-}$ mice. This is indeed the case whereby *Bmi-1*, *Olfm4* and *Lgr5* are expressed at significantly reduced levels (**Figs. 2C-E**).

Intestinal-specific deletion of *Csf1r* demonstrates intrinsic defect in PC maturation

To directly address the question of whether any of these effects were mediated by non-PC cell types, *Csf1r*$^{f/fl}$ mice (Li et al., 2006) were crossed to mice expressing a Tamoxifen-inducible *Cre*-transgene driven by an intestine-specific promoter (*VillinCre*^{ERT2}) (el Marjou et al., 2004). Epithelial cell *Csf1r* gene deletion was apparent, as assessed by loss of CSF-1R staining in the SI crypts, but not in adjacent lamina or muscularis propria (**Figs. 3A-B**). PC, assessed by lysozyme staining visualized by 3D imaging shows some Lysozyme staining persisted at the base of the crypts (**Fig. 3C**) but most of this was remnant Lysozyme within the crypt lumen or that associated with, and expected of, macrophages. Overall the amount of Lysozyme were substantially decreased after 4 weeks of Tamoxifen administration (**Fig. 3D**), This relatively slow PC ablation suggests that CSF-1R signaling is not essential for short-term survival of PC, but rather that it is required for their replacement and is consistent with estimates of the PC lifespan of greater than 30 days (Ireland et al., 2005). Commensurate with the loss of the PC was a concomitantly reduced expression of *Lgr5* (**Fig. 3E**). Interestingly, an increased abundance of proliferating cell nuclear antigen (PCNA)+ve cells at the crypt base suggested that these cells were occupying the address of the disappearing PC (**Fig. 3F**). This phenomenon of crypt cells occupying the vacated positions of the lost PCs has been noted in the case of the *Sox9* KO mouse (Sato et al., 2011).

Sustained reduction of ISC gene expression in conditional *Csf1r* deleted mice

In view of the extended life span displayed by PC we conducted a further study whereby *Csf1r*$^{fl/fl}$;*VillinCre*^{ERT2} mice were provided Tamoxifen in their chow *ad libitum* for 4 weeks and then followed in the absence of steroid for a further 4 weeks. When the SI was examined their appearance was similar to that observed at 4 weeks (with partial to complete absence of PC: as measured by Lysozyme) but some crypts had PCs and in these the PCNA positive cells were now normally redistributed (*data not shown*). These data suggested that in ISC where *Csf1r* was not ablated restoration of the crypt cell composition had occurred. Such escaper crypts are commonly observed in these types of models (Sato et al., 2011). Nevertheless, to examine the gene expression status within the SI crypts of these mice subjected to 4 week with Tamoxifen and 4 week chase we performed qRT-PCR to show that *Csf1r* remained significantly reduced as was the expression of *Lgr5* and *Olfm4* compared to *Csf1r*$^{fl/fl}$;*VillinCre*^{ERT2} mice not exposed to Tamoxifen (**Fig. 4**). Other cell cycle genes (*c-Myb*, *CyclinD1* and *c-Myc*) were slightly but not significantly reduced in these samples. Collectively these observations indicate that by 8 weeks the crypts were progressively recovering but nevertheless the deletion of CSF-1R has a sustained impact on ISC gene expression.

Conditional ablation of *Csf1r in vitro* reduces organoid formation

To address the effect of directly deleting *Csf1r in vitro* rather than assessing ISC in crypts that had reached a steady-state in the global absence of CSF-1R we isolated SI crypts from *Csf1r*$^{fl/fl}$;*VillinCre*^{ERT2} mice and cultured these in the presence of 4-HydroxyTamoxifen (4-OHT) during the process of organoid formation. We thus found that organoid formation was significantly impeded compared with corresponding cultures of *Csf1r*$^{fl/fl}$ crypts (**Figs. 5A-B**). These data suggest that CSF-1R deletion in steady state SI crypts has a direct impact on ISC function independently of other cell types that support the ISC niche.

Discussion

The role of growth factor receptor signaling in the GI is complex but with the use of conditional KO constructs, the development of ISC reporter mice and the advent of *in vitro* culture techniques greater clarity about specific and direct roles has emerged. Our observations that the classic hematopoietic growth factor receptor, CSF-1R in its *absentia* had a profound effect on the SI proliferation, differentiation and ISC gene expression were previously conducted in global KO mice (Huynh et al., 2009). The core observation of this study was the loss of PC in *Csf1r*$^{-/-}$ and *Csf1*$^{op/op}$ mice and the subsequent transgenic rescue of PC and ISC activity in *Csf1*$^{op/op}$ mice by locally restricted transgenic expression of CSF-1 (Huynh et al., 2009). We argued that this concurrence of PC maturation and ISC activity was further evidence that PCs play a role in the ISC niche. Studies using different mouse mutants in the Clevers lab have led to the same viewpoint (Sato et al., 2011). Subsequently, the role of PC in ISC biology has been challenged where *Atoh1/Math1* KO studies have resulted in deletion of PC without any apparent consequence to ISC function or progenitor cell proliferation (Durand et al., 2012; Kim et al., 2012). The implications of Atoh1 on PC and ISC will be addressed later.

To directly address the CSF-1R data it is apparent that global and intestine specific KO studies show a clear loss of PC in the SI as measured by histology and the loss of Lysozyme. Perhaps more compelling we show that CD24 is also lost. CD24 expression is both instructive and more encompassing as it marks both PC and ISC (Gracz et al., 2010; Sato et al., 2011; von Furstenberg et al., 2011). Although not exclusively a marker of ISC its location at the SI crypt base provides support to the view that it is employed within the ISC niche. Associated with CD24 loss is the reduced expression of non-PC genes that identify ISC, ie *Bmi-1*, *Olfm-4* and *Lgr-5*. To independently and functionally test where the loss of CSF-1R is important to ISC we asked whether primary organoid forming capacity was compromised. This allowed us to uncouple the ISC from the *Csf1r*$^{-/-}$ niche support and

in doing so it was evident that when CSF-1R was absent in ISC, and PC derived there from, their capacity to form organoids was markedly impaired.

The impact of deleting a gene globally and thus in the case of *Csf1r* during key point of development is genuinely different to tissue specific gene deletion in young fully developed mice. When we embarked upon deleting *Csf1r* using a Tamoxifen-inducible Cre restricted to the GI we were initially surprised that the phenotype was not similar to the global KO. However, with the proposition that the key role of CSF-1R in the SI is to support PC maturation, and further understanding that PC have relatively long life spans compared to other SI epithelial cells, the basis of our observations were predicable as follows. Firstly, survival of mature/established PC is not CSF-1R-dependent. Secondly, if PC have long life spans then it might be anticipated that several weeks may need to lapse before a phenotype is evident. The third prediction was that ISC gene expression signatures will be affected with the progressive PC loss due to the requirement of CSF-1R in PC replacement and maturation. The final realization based upon observations in other GI KO models is that when a gene is absolutely essential for crypt maintenance, escaper clones (Sato et al., 2011) that are not recombined will emerge to replace the void created by the deleted clones. Thus even with prolonged exposure of mice to Tamoxifen of 4 weeks plus a further chase of 4 weeks substantive but incomplete PC ablation was a predictable outcome. Indeed similar observations have been made when comparing *Sox9* GI-specific KO mice where deletion occurs early in development (Bastide et al., 2007; Mori-Akiyama et al., 2007) compared that seen in inducible KO in young mice (Sato et al., 2011).

Using the conditional deletion mice as sources of ISC we were able to show that just as deletion of *Csf1r* from the beginning of their propagation (germline deletion) led to defective organoid formation so too was the case when *Csf1r* loss was induced at the initiation of their culture (conditional inducible deletion). These data speak to the intimate relationship between PC and ISC and their dependence on CSF-1R. This observation is

interesting when comparison is made with the *Atoh1* KO mice where induced loss of PC is perhaps compensated for by allowing ISC activity to continue *in vivo*, but not when tested in organoid culture (Durand et al., 2012). Thus, mice with global CSF-1R loss seem to have an incomplete compensation of PC loss for ISC activity (Huynh et al., 2009), indicating that CSF-1 expressed by stromal cells may be required for support of the ISC niche.

Notch signaling is without doubt essential to ISC function in the SI crypt (VanDussen and Samuelson, 2010). Notch-1 deletion leads to expansion of all secretory lineages (Fre et al., 2005) however pharmacological inhibition of γ-secretase leads to disorganized Lysozyme granule deposition (VanDussen and Samuelson, 2010) suggesting that the PC are perhaps not normal in such treated mice. As Notch signaling favours ISC proliferation versus secretory cell differentiation via inhibition of Atoh1 (van Es et al., 2010) it is perhaps it instructive that Atoh1 expression is elevated at the crypt bottom and that PC may still need Atoh1 for survival. It is also noteworthy that when propagated *ex-vivo* that *Atoh1* null organoids did not grow which was argued may be due to the absence of some form of extra-epithelial stromal support (Durand et al., 2012). This implies that the loss of Atoh1 may lead to a change in the cytokine/growth factor milieu within the ISC that compensates for the loss of PC. Following on from this theme we showed that transgenic rescue of the PC and accompanied proliferative defects in the *Csf1*$^{op/op}$ mouse could be achieved by localized expression of a membrane-bound form or CSF-1 (Huynh et al., 2009).

As *Olfm4* expression is reduced when *Csf1r* is deleted and that *Olfm4* is a Notch target gene (VanDussen and Samuelson, 2010) implies that CSF-1R-dependent PC are directly or indirectly impacting on Notch signaling as well. Although *Olfm4* regulation is Notch-dependent, it is nevertheless Atoh1-independent (VanDussen and Samuelson, 2010) and this is evident when considering observations in *Csf1r* KO crypts that appear to

have elevated *Math1* and mucin production but reduced *Math-1* antagonist *Hes-1* as well as significantly reduced *Olfm4*. PC also serve as a source for Wnt3 among other factors (Sato et al., 2011) and in the absence of PC these might also be predicted to be in deficit leading to reduced Wnt signaling and targets there of, such as *Lgr5 and CyclinD1*. *Lgr5* expression is consistently down in SI crypts where *Csf1r* is deleted globally or when deleted solely in the SI epithelium. Therefore the removal of Atoh1 is unlikely to be functionally the same as activation of Notch signaling even though some morphological phenocopying may be apparent. It is thus reasonable to argue that in the absence of Atoh1 there may be redirected Notch and/or even Wnt signaling to allow the ISC compartment to operate or compensate. To this end in the case of the *Csf1r* KO SI it is apparent that the overall expression of *Hes-1* is substantially lower than in WT SI. As either Notch or Wnt signaling is capable of stimulating *Hes-1* expression in the SI (Peignon et al., 2011) these data imply that both pathways are compromised in the *Csf1r* KO mouse. Furthermore, as *Hes-1* expression is elevated to comparable levels in adenomas from mice with *Apc* mutant alone or *Apc* mutant plus activated Notch (Fre et al., 2009) it is however argued that Wnt and Notch activation are not additive in this setting with regard to this archetypical Notch target gene.

Additional studies will be required to investigate whether there is a relationship between CSF-1R and Notch signaling in the SI and why PC appear to be dispensable when Atoh1 is deleted. In contrast to Atoh1, CSF-1R is not essential for PC survival, but rather their *de novo* formation. Other gene KOs leading to loss of PC differentiation further differ from *Csfr1* KO and *Atoh1* KO with respect to proliferation and secretory cell defects (Bastide et al., 2007; Shroyer et al., 2005), indicating that these genes may act at different stages of PC differentiation. While *Atoh1* loss clearly abolishes terminally differentiated PC as demonstrated by the absence of granular structures CSF-1R loss also leads to proliferation defects, which might be explained by CSF-1R being necessary for the

maintenance of proliferating PC progenitors. Indeed, proliferating cells can be found within the epithelial cell population expressing the PC marker Lysozyme (von Furstenberg et al., 2011) and *Csf1r*$^{-/-}$ crypts retain some Lysozyme expression while CRS4C, another PC marker, is completely lost (Huynh et al., 2009).

In the context of the above studies and several others PC are not in all instances dispensable and here the data support an important role of PC in the integrity of the ISC niche. Specifically here the observations indicate a direct dependence of PC on CSF-1R signaling via locally presented CSF-1. Indeed defects in PC numeracy and morphology may serve as a sentinel for potential disruption of the ISC niche which may take time to manifest the ultimate consequences due to the long lived nature of PC. In line with this the onset of SI adenoma formation by *Apc* deletion in ISCs is characterized by an expansion of ISC-like and PC-like cells, which may represent expansion of a cancer stem cell niche (Schepers et al., 2012). Thus defects in the CSF-1R regulation of PC are likely to contribute to GI disease (Bevins and Salzman, 2011) that may reflect the important role PC play in innate immunity.

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Figure legends

Figure 1. Global CSF-1R loss affects on mature Paneth Cells and Intestinal Stem Cells (ISC). **(A)** The Paneth cell (PC) and ISC marker, CD24, is expressed at the base of the SI crypt (brown arrow). **(B)** Reduced CD24 expression and loss of PC and ISC in *Csf1r*$^{-/-}$ mouse SI crypts. **(C)** When crypts are liberated from the SI villi are usually sheared away (broken lines) allowing the production of enriched crypts separated from the ISC niche. A Cartoon depicting this is shown. **(D)** Confocal IF images reconstructed into a 3D format shows coincident expression of CSF-1R (green) and Lysozyme (red) at the crypt base. DAPI (blue) defines the crypt nuclei (*see Movies in supplementary data*). **(E)** 2D confocal image of *Csf1r*$^{-/-}$ crypts shows no CSF-1R (green) and minimal Lysozyme (red). Similarly prepared 2D confocal images of crypts show **(F)** DAPI-stained nuclei (blue) most **(G)** CSF-1R (green) associated with PC (green arrow). **(H)** Co-staining with β-catenin (red) as a merged image of **(F)** and **(G)** highlights the epithelial cells (red). Scale bar = 50 μm. Magnification x60.

Figure 2. Global CSF-1R loss affects on Intestinal Stem Cell (ISC) function and gene expression signatures *in vitro*. **(A)** Organoid cultures generated by WT and *Csf1r*$^{-/-}$ ISC shown at day 1, 3 and 5 indicate delayed growth of the KO organoids (Bar = 20 μM). **(B)** Quantitation of WT and *Csf1r*$^{-/-}$ organoid numbers after 7 days in culture (equivalent numbers of crypt nests were plated at day 0). (*p = 0.05; *** = 0.001; student t-test);. Organoid cultures initiated from WT and *Csf1r*$^{-/-}$ mice were harvested at day 7, RNA prepared and subjected to qRT-PCR using primers for ISC genes **(C)** *Bmi1*, **(D)** *Olfm4* and **(E)** *Lgr5* (* p<0.05; Mann Whitney test).

Figure 3. Conditional CSF-1R inactivation effects on Paneth cells and crypt proliferation. **(A)** CSF-1R (green) at the crypt base (green arrows) in association with Lysozyme (red) as

well as in the lamina propria and muscularis is shown. **(B)** PC loss in mice following Tamoxifen-induced inactivation (2 weeks) of the *Csf1r* gene and loss of Csf-1R expression (Green arrow). **(C)** 3D image of a SI crypt from a *Csf1r*$^{f//fl}$;*VillinCre*^{ERT2} mouse after 2 weeks of Tamoxifen exposure. **(D)** Lysozyme expression (brown arrow) determined by IHC in the ileum of *Csf1r*$^{f//fl}$;*VillinCre*^{ERT2} mice after 4 weeks of Tamoxifen exposure indicates substantive loss of mature PC. **(E)** ISC *Lgr5* expression is reduced in inducible, intestinal-specific *Csf1r* deleted mice. **(F)** Displacement of PC, or replacement by, PCNA+ve cells in the base of the SI crypts of *Csf1r*$^{f//fl}$;*VillinCre*^{ERT2} mice after 4 weeks of Tamoxifen exposure leads to significantly more PCNA+ve cells at the crypt base. (* p = 0.05, *** = 0.001; Student t-test).

Figure 4. Reduced ISC gene expression is durable for several weeks following loss of Paneth cells. Significant gene expression changes for *Csf1r, Lgr5* and *Olfm4* in the SI crypts of *Csf1r*$^{f//fl}$; *VillinCre*^{ERT2} mice after 4 weeks of Tamoxifen exposure, followed by a 4 week chase in its absence is shown. Cell cycle related genes c-*Myb*, *CyclinD1* and *c-Myc* expression was slightly but significantly reduced (* p = 0.05; Student t-test).

Figure 5. Effect of *in vitro* deletion of *Csf1r* on organoid formation. **(A)** Organoid cultures were initiated from WT and *Csf1r*$^{fl/fl}$;*VillinCre*^{ERT2} mice and 4-hydroxytamoxifen (4OHT) added on day 1 and assessed on day 7. **(B)** The number of organoids formed from the same number of starting crypt nests *Csf1r*$^{fl/fl}$:*VillinCre*^{ERT2} and wild type (WT) cultures are shown (* p<0.05; Student t-test).

Table 1. Antibodies

Antigen	Species (titre)	Source
CSF-1R	Rabbit anti-mouse (1:250)	Upstate Biotech, USA (06174)
PCNA	mouse PC-10 (1:100)	BD Transduction (610665)
Lysozyme	Rabbit anti-mouse (1:100)	Thermo Scientific (RB 372 A1)
CD24	Rat (1:100)	Santa Cruz (sc-52468)
β-Catenin	mouse (1:100)	BD Transduction Labs (610154)
IF Alexa Fluor 488 (green) secondary antibody	Mouse (1:500)	Molecular Probes (A11001)
IF Alexa Fluor 488 (green) secondary antibody	Rabbit (1:500)	Molecular Probes (A11034)
anti-mouse-HRP	Goat (1:250)	(Bio-Rad)
DAPI	(1:1000)	Molecular Probes (D1306)

Table 2. Oligonucleotide Sequences

Gene	Genbank Accession #	Forward Primer	Base number of Forward primer	Reverse Primer	Base number of reverse primer
Csf1rflox	*N/A*	***5' CAAGCTGGTGCGGATTCG***		***5' TCTTGGAAGTCACTGTTTAGGG***	
Csf1r	NM_001037859	***5' CCTCCTCTGGTCCTGCTG***	991-1008	***5' CATTCCACACTGCCATTGC***	4725-4744
c-Myc	NM_001177352.1	***5' AAGGCCCCCAAGGTAGTGA***	4963-4981	***5' TGCTCGTCTGCTTGAATGGA***	5011-5030
CyclinD1	NM_007631	***5' AGGCTACAGAAGAGTATTTATGGGAAA***	9129-9155	***5' TGCGTTTGAATCAAGGGAGAT***	9191-9211
Lgr5	NM_010195	***5' CAAGCCATGACCTTGGCCCTG***	109915-109935	***5' TTTCCCAGGGAGTGGATTCTATT***	113149-113171
Olfm4	NM_001030294.1	***5' GCCACTTTCCAATTTCAC***	6472-6489	***5' GAGCCTCTTCTCATACAC***	12104-12121
Bmi1	NM_007552	***5' AATTAGTCCCAGGGCTTTTCAA***	6454-6475	***5' TCTTCTCCTCATCTGCAACTTCTC***	6785-6808
c-Myb	NM_001198914.1	***5' AATTATCTGCCCAACCGG***	6891-8606	***5' AGACCAACGCTTCGGACC***	8953-8970
CD24a	NM_009846	***5' GGCAACCACAAGTCCAATG***	4252-4270	***5' AACTCCAGCAGATTCAATAGC***	4393-4413
Hes1	NM_008235	***5' CTCGCTCACTTCGGACTC***	3603-3620	***5' GTGGGCTAGGGACTTTACG***	3667-3685
Math1	NM_007500.4	***5' ATGCACGGGCTGAACCA***	1282-1298	***5' TCGTTGTTGAAGGACGGGATA***	1323-1343
Gapdh	NM_008084	***5' GTATGACTCCACTCACGG***	3746-3763	***5' GGTCTGGCTCCTGGAAGA***	3829-3846

Figure 1

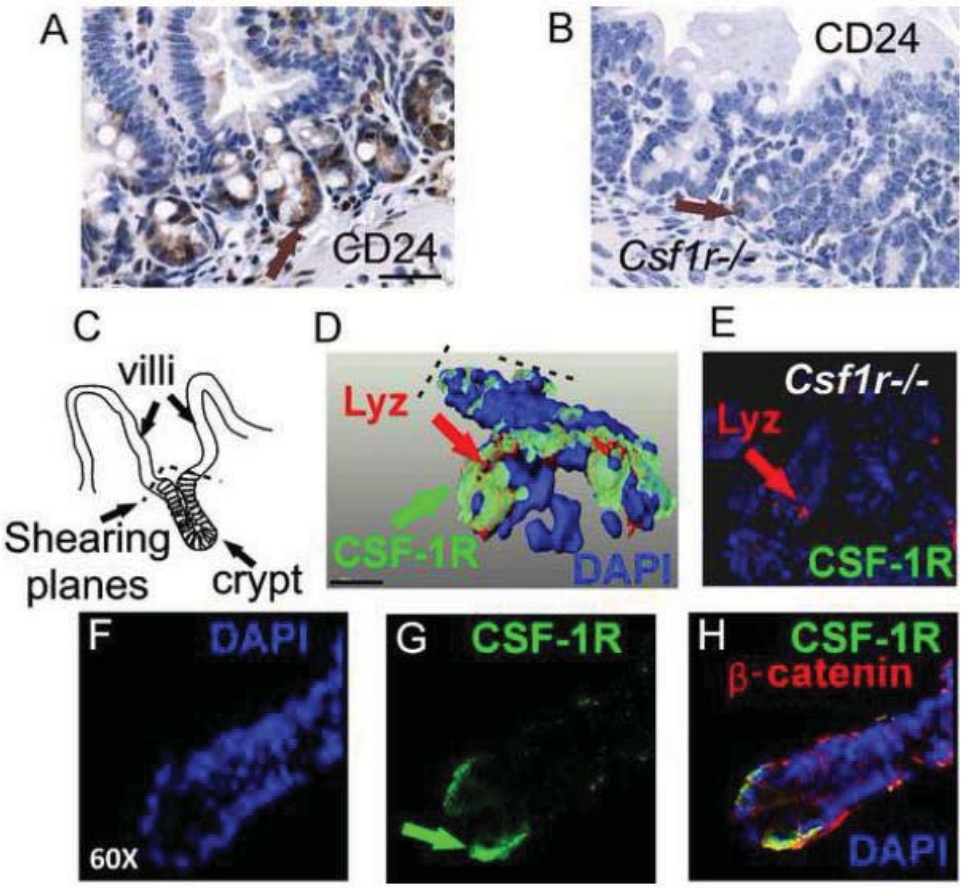


Figure 2

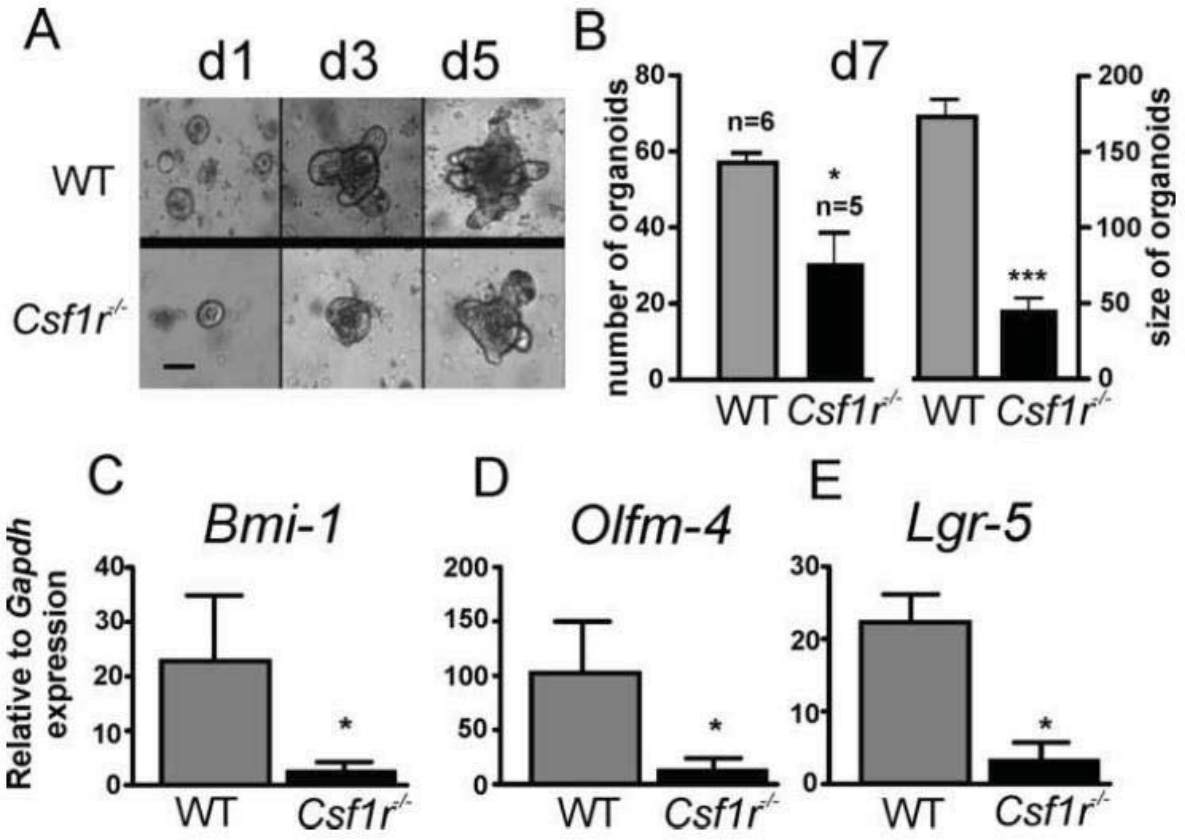


Figure 3

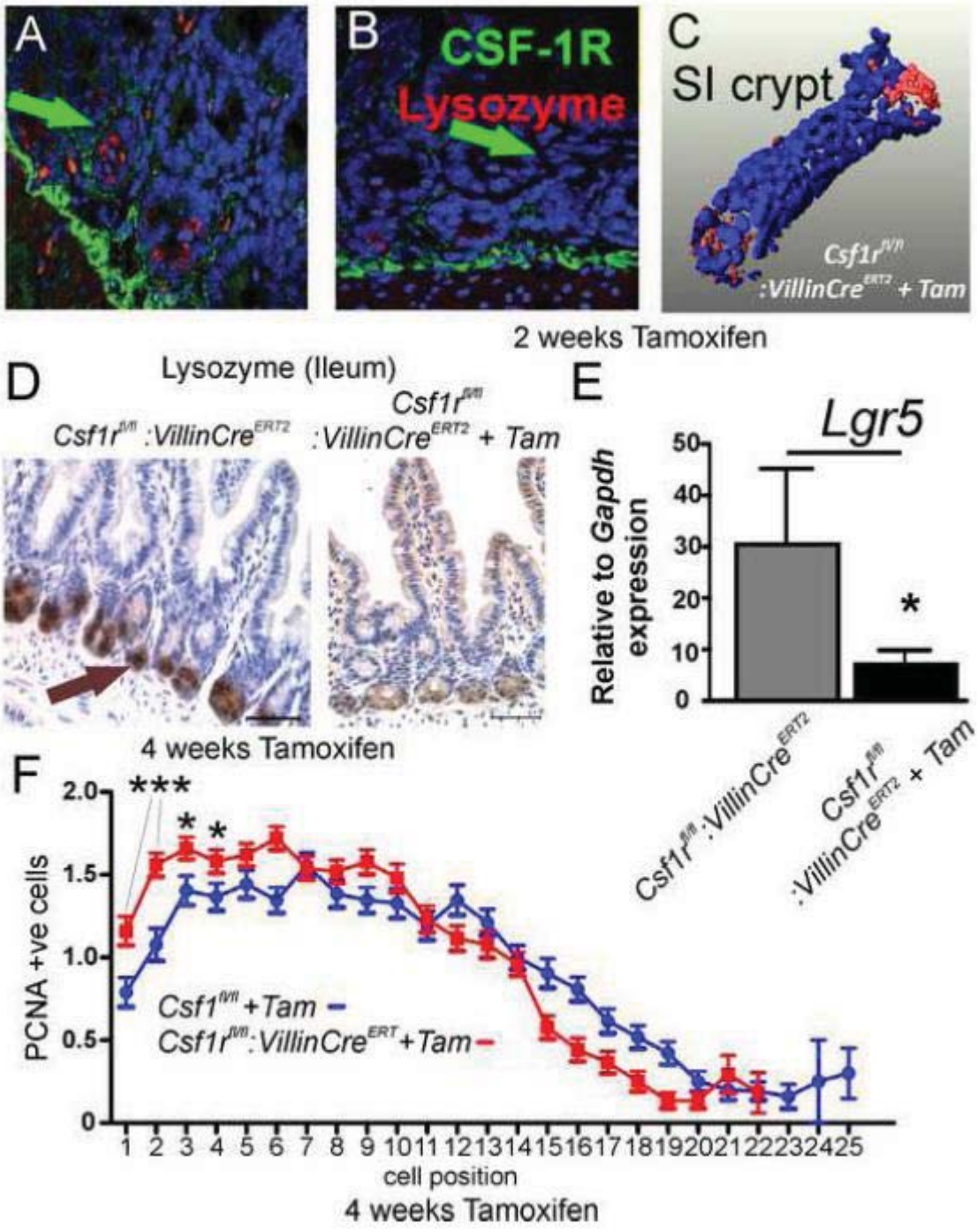
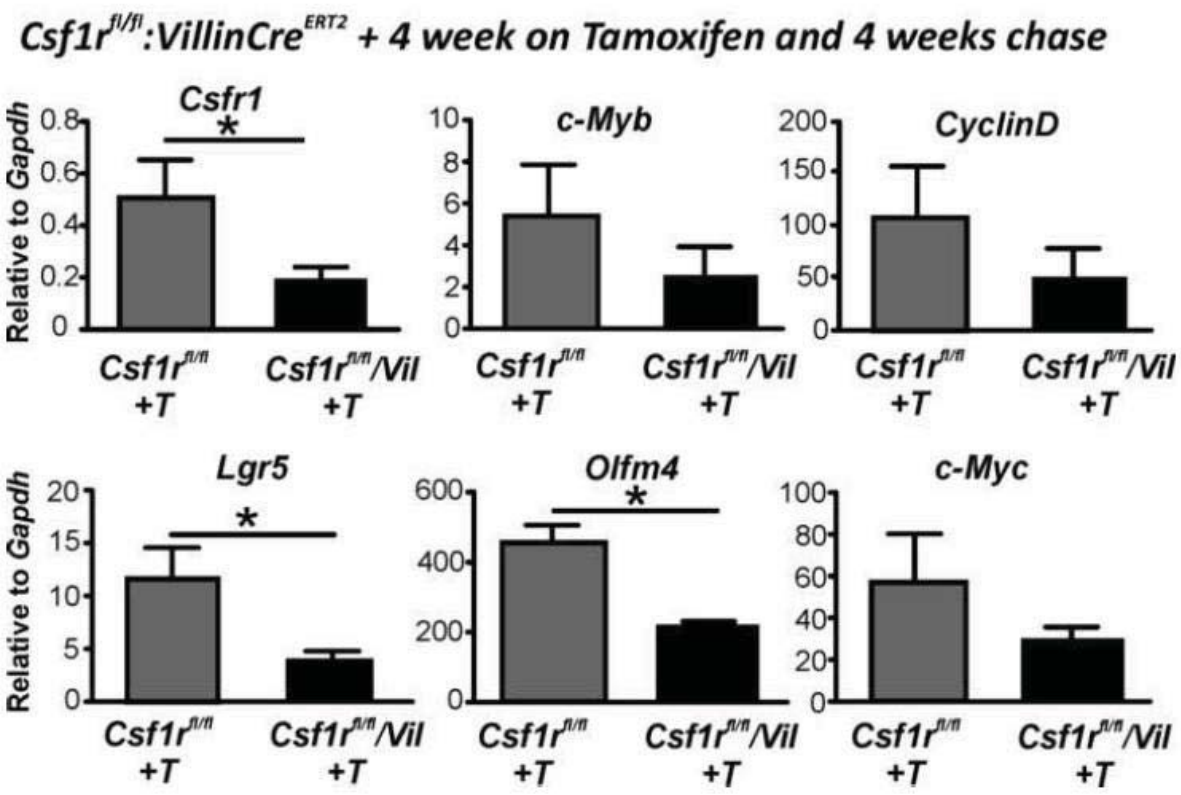


Figure 4



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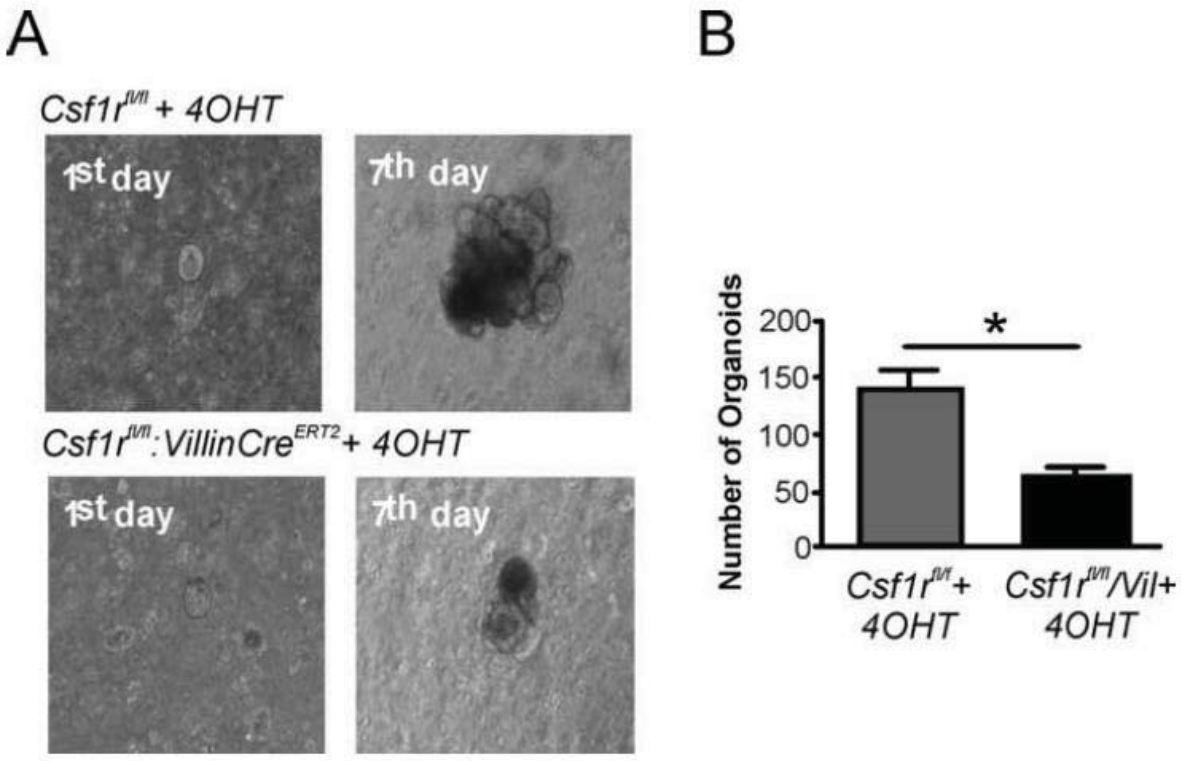
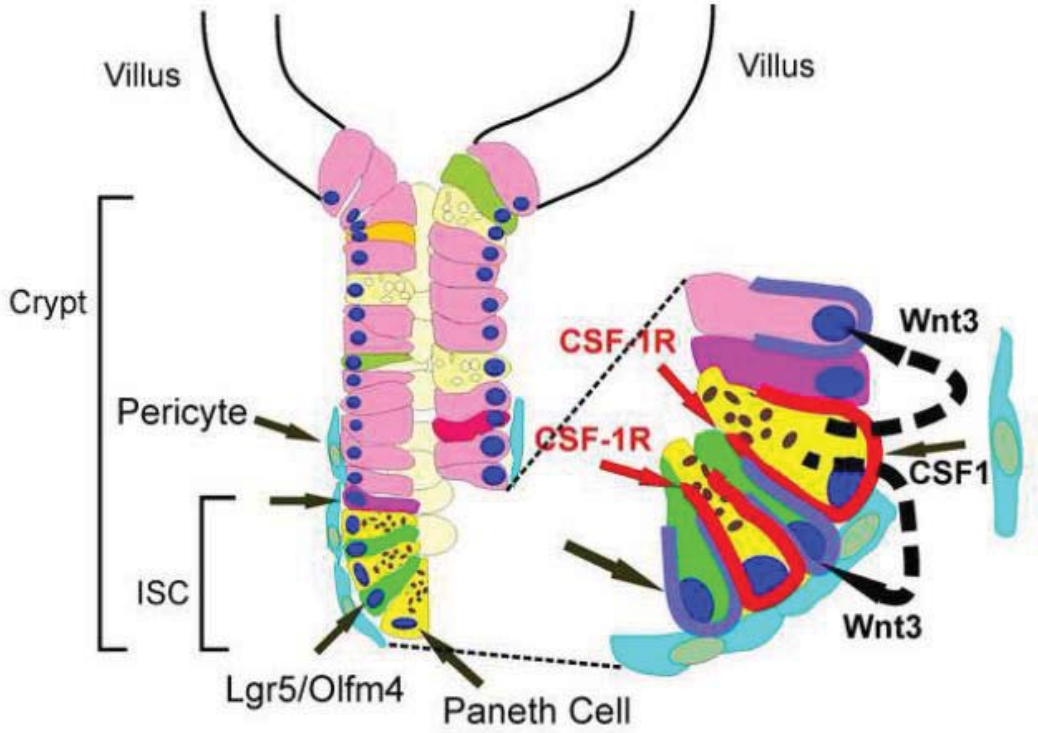


Graphical abstract



CSF-1 Receptor is required for Paneth cells which in turn support intermingled Intestinal stem cells (ISC) marked by Lgr5 and Olfm4. Pericytes provide local CSF-1 that engages CSF-1 R on PC. PC produce factors key to the ISC including Wnts.

Highlights:

- **CSF-1R KO eliminates stem and Paneth Cell marker CD24 in the intestinal stem cell niche**
- **The KO defect is cell intrinsic as shown by organoid cultures suggesting a pivotal role of Paneth cells**
- **Intestinal-specific KO of CSF-1R leads to a progressive, but delayed elimination of Paneth cells in the crypt**
- **This PC loss impacts intestinal stem cell gene expression**
- **CSF-1R signaling is central to the stem cell niche suggesting pathway defects may underpin GI disorders**

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