Cell and chromatin transitions in intestinal stem cell regeneration

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The progeny of intestinal stem cells (ISCs) dedifferentiate in response to ISC attrition. The precise cell sources, transitional states, and chromatin remodeling behind this activity remain unclear. In the skin, stem cell recovery after injury preserves an epigenetic memory of the damage response; whether similar memories arise and persist in regenerated ISCs is not known. We addressed these questions by examining gene activity and open chromatin at the resolution of single Neurog3-labeled mouse intestinal crypt cells, hence deconstructing forward and reverse differentiation of the intestinal secretory (Sec) lineage. We show that goblet, Paneth, and enteroendocrine cells arise by multilineage priming in common precursors, followed by selective access at thousands of cell-restricted cis-elements. Selective ablation of the ISC compartment elicits speedy reversal of chromatin and transcriptional features in large fractions of precursor and mature crypt Sec cells without obligate cell cycle re-entry. ISC programs decay and reappear along a cellular continuum lacking discernible discrete interim states. In the absence of gross tissue damage, Sec cells simply reverse their forward trajectories, without invoking developmental or other extrinsic programs, and starting chromatin identities are effectively erased. These findings identify strikingly plastic molecular frameworks in assembly and regeneration of a self-renewing tissue.

[Keywords: chromatin dynamics of dedifferentiation; intestinal secretory lineage; stem cell recovery after injury]

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Lgr5+ intestinal stem cells [ISCs] fuel lifelong epithelial self-renewal and their immediate daughters compete for limited niche space at the bottom of intestinal crypts [Lopez-Garcia et al. 2010; Snippert et al. 2010]. Exit from the crypt base initiates differentiation [Ritsma et al. 2014], first into two lineages, ATOH1+ enterocytes [Ents] and ATOH1+ secretory [Sec] cells, and then into Sec subtypes: goblet, Paneth, tuft, and enteroendocrine [EE] cells [Supplemental Fig. S1A; Shroyer et al. 2005; Beumer and Clevers 2021]. Paneth cells turn over slowly [Ireland et al. 2005] and remain in the crypt bottom, interspersed among ISCs [Barker et al. 2007], while goblet cells and EE cells [EECs] move into adjoining villi and mature at different rates [Cheng and Leblond 1974]; some EEC precursors linger near the ISC–Paneth zone at the crypt base [Bjerknes and Cheng 1981; Buczacki et al. 2013].

When native ISCs are injured, new Lgr5+ ISCs quickly restore homeostasis [Tian et al. 2011]. Efficient ISC regeneration, previously ascribed to Bmi1+ and other “reserve” stem cells [Li and Clevers 2010; Takeda et al. 2011; Yan et al. 2012], results principally from Sec and Ent cell dedifferentiation [van Es et al. 2012; Tetteh et al. 2016; Jadhav et al. 2017; Yan et al. 2017; Yu et al. 2018; Jones et al. 2019; Murata et al. 2020]. Dedifferentiation also drives tissue recovery in damaged lungs [Tata et al. 2013; Lynch et al. 2018], liver [Tarlow et al. 2014; Raven et al. 2017], prostate gland [Karthaus et al. 2020], and likely other epithelia. Therefore, cis-elements that underlie epithelial cell identities must be inherently plastic, helping to avoid organ failure.

Enhancers active in the dominant intestinal Ent population are insufficiently distinct from ISCs to assess chromatin transitions [Kim et al. 2014]. In contrast, the Sec lineage deploys thousands of enhancers that are inaccessible in Ents or ISCs and, at least in bulk cell isolates, those enhancers are decommissioned soon after ISC ablation [Jadhav et al. 2017]. However, it remains unclear whether dedifferentiation is limited to immature cells, even from studies on targeted Cre-driver mice [Yan et al. 2017; Schmitt et al. 2018], whether cell replication is a

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perequisite; whether reparative or developmental gene programs are deployed in the process; and whether restored ISCs retain cis-element memories of their starting identities. To address these questions, here we deconstruct forward and reverse differentiation in large numbers of single Sec cells.

The transcription factor [TF] NEUROG3 is necessary [Jenny et al. 2002] and sufficient [Gehart et al. 2019] to produce diverse EECs, and tamoxifen [TAM] treatment selectively marks the Sec lineage in Neurog3-CreERTK2 mice [Li et al. 2012]. Separately, Lgr5GFP mice [Tian et al. 2011] mark ISCs and enable ISC ablation by diphertheria toxin [DT]. We used these animal models to examine ISC regeneration critically in Neurog3-CreERTK2;R26RLoxstopLSL-TdTomato; Lgr5GFP mice [Neurog3-Cre;R26RTom;Lgr5GFP]. By profiling transcriptomes and open chromatin at single-cell resolution, we assessed forward Sec differentiation in animals treated with TAM alone and reverse differentiation after treatment with TAM and DT [Supplemental Fig. S1B]. Here we report the resulting insights into small intestine [SI] Sec cell diversity and dedifferentiation.

Results

Outline and validation of the experimental model

Neurog3-CreERTK2;R26RTom mice showed the expected distribution of fluorescent SI cells. One day after TAM injection, tdTom signals localized predominantly in crypt cells that lacked the mature EEC marker CHGA and were enriched just above the ISC zone, where EEC precursors are known to pause [Buczacki et al. 2013], and in a fraction of CHGA+ and CHGA− villus cells [Fig. 1A]. As expected, CHGA+ EECs that arose before Cre activation were unmarked and, without further TAM exposure, Tom+ cells traced the Neurog3-Cre lineage. Increasing numbers of Tom+ CHGA+ cells appeared along villi on days 2 and 3, in part reflecting maturation of labeled EECs into enterochromaffin [EC; 5-hydroxytryptamine [5-HT]] and non-EC cells [Fig. 1A; Supplemental Fig. S1C]. In line with low Neurog3 expression in Sec cells other than EECs [Schonhoff et al. 2004; Gehart et al. 2019; Li et al. 2021], Neurog3Cre also marked many goblet and fewer Paneth cells, recognized by morphology, anatomic positions, and cell-specific markers [Supplemental Fig. S1D]. Increasing numbers and upward migration of MUC2+ goblet cells [Supplemental Fig. S1D] indicate that Tom+ CHGA+ cells reflect Cre activity in multipotential Sec progenitors [Sec-Pros] and immature goblet cells, which proved to be an asset in our study. As expected, we detected no Tom+ FABP6+ Ent cells and rare Tom+ Lgr5+ ISCs at these early times [Supplemental Fig. S1D].

In Lgr5GFP mice, a single dose of DT ablated ISCs in some studies [Tian et al. 2011; Tetteh et al. 2016] but was inefficient in others [Tan et al. 2021]. In Neurog3-Cre; R26Tom;Lgr5GFP mice that received both TAM and DT, a typical 50 μg/kg dose eliminated Lgr5+ ISCs, as revealed by GFP microscopy, flow cytometry [Fig. 1B], and multiple subsequent single-cell isolations [see Figs. 3, 4, below]. GFP+ cells were undetectable in crypts for at least 2 d after DT exposure and did not reappear appreciably before day 3 [Fig. 1B]. Following ISC ablation, Tom+ cells were increased in crypts, especially at the base, and by day 4 GFP+ ISCs from both Neurog3-Cre [Tom+] and other [Tom−] sources were extensively restored [Fig. 1B,C; Supplemental Fig. S2A,B]. In the absence of ISC loss, tissue sections lacked clonal “ribbons” emanating from Neurog3Cre-labeled Tom+ ISCs, and intestine whole-mounts showed few instances [Fig. 1D]; sporadic clonal labeling, seen in all lineage-restricted CRE reporter mice [van Es et al. 2012; Yan et al. 2017; Yu et al. 2018], may reflect physiologic crypt cell plasticity or spurious Cre activity in rare ISCs. After ISC ablation, fully Tom+ crypts were readily detected in tissue sections by day 7, with a measurable increase in clonal ribbons; 1 mo later, following clonal fixation of such Tom+ crypts, SI ribbons were notably increased [Fig. 1D], indicating activity of long-lived ISCs recovered from Neurog3-labeled cells. Importantly, Tom+ crypt cells could be isolated by flow cytometry at any time during ISC recovery [Fig. 1D; Supplemental Fig. S2B]. Thus, although both Ent and Sec cells contribute to ISC recovery [van Es et al. 2012; Tetteh et al. 2016; Murata et al. 2020], Neurog3-Cre; R26Tom;Lgr5GFP mice allow critical examination of forward and reverse Sec differentiation; we focused on the crypt compartment, separated from villi.

Forward differentiation of Neurog3+ progenitors

To characterized forward Sec trajectories, we captured scRNA-seq profiles of Tom+ cells purified from the whole SI by flow cytometry, 24, 48, and 72 h after two daily injections of TAM [without DT]; i.e., between 48 and 96 h after initial Neurog3-Cre labeling and between 24 and 72 h after the second labeling [Supplemental Fig. S3A]. Uniform manifold approximation and projection [UMAP] [McInnes et al. 2018], coupled with known molecular markers, classified 5564 Tom+ cells by their scRNA profiles [Supplemental Fig. S3B–E]. Well-delineated clusters of hormone-expressing EECs and mature Paneth and goblet cells accounted for 49%–59% of Tom+ cells at any time, whereas cells isolated on days 1 and 2 were relatively enriched for ISCs and Sec progenitors [Sec-Pros]; replication was largely restricted to the latter populations [Supplemental Fig. S3F,G]. Cells clustered by type and not by regional SI markers, such as Gata4 and Gata6. For ease of presentation, we disregarded small clusters of tuft cells, Ents, and intraepithelial lymphocytes [Supplemental Fig. S3C,D] and focused on the remaining 5071 cells.

Slingshot, which imputes pseudotime relationships among cells [Street et al. 2018], traced the root of the lineage to a cluster that expresses ISC-specific genes, including Ascl2 and high Olfin4, and likely engendered the few Ents [Fig. 2A; Supplemental Fig. S3C–E]. A differentiation continuum extended from this ISC-like cluster to multipotential Sec-Pros, which produced a distant EEC branch and bipotential goblet–Paneth precursors [Gob/Pans], followed by resolution of each population into distinct daughters [Fig. 2A; Supplemental Fig. S3H]. Diverse EC subpopulations fell into three groups: Neurog3+ precursors [EEC-pres], which branch into serotonin/TPH1+...
enterochromaffin (EC) and peptide hormone-producing non-EC cells [Fig. 2A; Supplemental Fig. S3E,H]. For any active gene, recently generated cells carry more unspliced RNA than predecessor cells, where the mRNA pool is largely spliced (La Manno et al. 2018). Affirming the trajectories imputed by Slingshot, Sec-Pros expressed unspliced Chga, Neurod1 (EEC), Muc2, Tff3 (goblet), Lyz1, and Mecom (Paneth); Gob/Pans contained only non-EEC transcripts [Fig. 2B; Supplemental Fig. S3I]. Immature EEC precursors expressed Neurog3 [Fig. 2C, asterisk; Supplemental Fig. S3E] and all cell clusters expressed tdTom, confirming accurate cell purification. The number of Tom+ crypts present 30 d after Cre activation [Fig. 1D] suggests clonal fixation of one Neurog3-Cre-labeled cell.  

**Figure 1.** Neurog3-labeled cells contribute to the restoration of ablated Lgr5+ ISCs. **(A)** Representative images of Neurog3-labeled tdTom+ cells 1, 2, and 3 d after TAM injection. The tdTom label appears first in both immature crypt and mature villus Sec cells, including CHGA− EEC precursors, mature CHGA+ EECs, and diverse non-EEC populations. The signal later traces into larger fractions of CHGA+ EECs [also see Supplemental Fig. S1C]. (White arrowheads) Unlabeled CHGA− EECs, (yellow arrows) CHGA+ tdTom+ (double-positive) EECs. n = 3 mice per time point. Scale bars, 100 µm. Day 3 crypt images are magnified in the bottom row. **(B)** Schema to trace cell lineage in Neurog3-Cre;Lgr5Dtr;R26RTom mice during homeostasis (TAM only) and after ISC ablation (TAM + DT). Cells were labeled with tdTom by providing one dose of TAM 24 h before and a second dose concomitant with ISC ablation. Representative micrographs and flow cytometry plots show loss of GFP+ ISCs on days 1 and 2 after DT injection and their return by days 3−5 [also see Supplemental Fig. S2]. n ≥ 4 mice per condition. Scale bar, 100 µm. **(C)** Tom+ crypts were increased up to 30 d after ISC ablation [n = 3 mice per condition]. To ensure capture of the ISC zone in tissue sections, counts were limited to LYZ1+ crypts, as shown in a representative micrograph. (White arrows) Tom+ ISCs. **(D)** Representative whole-mount [collage of micrographs] and tissue sections [insets with DAPI costain] of Tom+ clonal ribbons [left], which were significantly increased in number throughout the SI from 7 d onward after ISC ablation [right]. Scale bars: micrograph collage, 1 mm; insets, 100 µm. n ≥ 4 mice per condition.
ISC per $\sim 10^4$ crypts, and because neutral drift (Lopez-Garcia et al. 2010; Snippert et al. 2010) likely eliminates most Tom+ ISCs over time, the yield of Neurog3-Cre-labeled ISCs is higher in the first few days.

From mRNA signatures for each cell cluster (Supplemental Table S1), we observed that labeling of ISCs and Sec-Pros mirrors low Neurog3 in those populations and that, although goblet and Paneth cells share hundreds of transcripts, >300 others (e.g., Plaur and Defensins) are sufficiently distinct to generate cell-specific profiles (Fig. 2C). Multipotent Sec-Pros express low levels of mRNAs specific to each daughter cell type, while Gob/Pan precursors

Figure 2. Cell states in normal (forward) differentiation of Neurog3-labeled cells. (A) UMAP plots from RNA analysis of 5071 single Tom+ cells showing each population, and Slingshot pseudotime trajectories (arrows) showing derivation of EE, goblet, and Paneth cells from ISCs and Sec-Pros. Only cells that contribute to any trajectory acquire scores (color) and dark-to-light color gradients depict pseudotime progression. (B) Unspliced unique transcript count residuals of Chga [EEC marker], Lyz1 [Paneth cells], and Muc2 [goblet cells] projected onto the UMAP plot. (Red) High relative levels of unspliced RNA, signifying ongoing transcription, (blue) low relative levels of unspliced mRNA, signifying residual transcript levels. Cells classified as Sec-Pros and Gob/Pans express low levels of gene markers specific to their respective descendants. (C, left) Differentially expressed genes ($n = 3168$) (Supplemental Table S1) condensed into signatures for each identified cell state. (Top right) Genes enriched in each cluster.
express transcripts from both cell types before adopting one or the other fate (Fig. 2C, blue box). Thus, multilineage priming [Hu et al. 1997], previously observed in putative bipotential Ent–Sec progenitors [Kim et al. 2016], is a theme in crypt differentiation. Our data affirm the existence and distinct mRNA signature of Gob/Pans, an elusive bipotential state previously inferred on morphologic grounds [Bjerknes and Cheng 2010]. Gob/Pan differentiation engenders less transcriptional diversity than the ECC branch, which is the most unique [Supplemental Fig. S3J]. ECC differentiation parallels the trajectory reported in Ngn3CreER mice [Gehart et al. 2019], with TPH1+ EC cells differing from those that make peptide hormones (Fig. 2C, yellow box; Supplemental Figs. S3H, J). Of note, peptide hormone genes express at >10-fold higher levels in non-EC cells but are not silent in other Sec derivatives (Fig. 2C). In summary, Neurog3-Cre labeled few ISCs and many progenitors that yield mature crypt Sec cells. Other than the distinction of EECs, gene profiles lie along a continuum with only two discernible intermediates: Sec-Pros and Gob/Pans. These data provide a path to investigate normal Sec diversification in vivo and robust cell-specific RNA reference standards to study cell fate reversal.

Early transcriptional response to ISC ablation

To examine dedifferentiation, we treated mice with both DT and TAM, and then captured Tom+ cells during crypt recovery 12, 24, 48, and 72 h after ISC ablation with DT (Supplemental Fig. S1B). Because any Tom+ cell could represent forward or reverse differentiation [Fig. 3A], we identified the latter cells by first integrating the scRNA-seq data with that from Tom+ cells in normal crypts [no DT treatment] (Fig. 2A). We matched groups of like cells across the two data sets, transferring labels from cells treated only with TAM to those isolated after TAM + DT [see the Materials and Methods]. After weighting by shared nearest neighbors (SNNs), 32.5% of the 10,074 cells captured after ISC ablation resembled their counterparts [Fig. 3B; Supplemental Fig. S4A]. To avoid uncertainty about the direction of differentiation, we set these 3272 “indeterminate” cells aside, including subtly perturbed cells whose cluster RNA signatures did not reliably differ from forward differentiation [sum of SNN score >3] [Supplemental Fig. S4B]; the 6802 remaining cells [67.5% of all Neurog3-labeled cells after TAM + DT] [Fig. 3B] differed materially. TAM exposure was the same before and after ISC loss [two daily doses to ensure Neurog3+ cell marking], so that Tom+ cells isolated the day after DT were labeled 24 and 48 h earlier, those isolated on day 2 were labeled 48 and 72 h earlier, and so on.

After DT treatment, Tom+ cells classified [based on forward differentiation profiles] as ISCs or progenitors exceeded the basal fraction of these immature cells, and mature cell fractions were proportionally reduced [Fig. 3C; also cf. Supplemental Fig. S3G]. Mean levels per cell of the seven most enriched ISC markers [scRNA] [Supplemental Table S1] or of six well-known ISC-enriched genes [Asc2, Axin2, Cdca7, Lgr5, Olfm4, and Smoc2] were substantially diminished 12 h after DT exposure [Fig. 3D], indicating that Tom+ cells classified as ISCs were not fortuitous survivors of DT exposure. Indeed, ISC-selective Olfm4 expression was undetectable by scRNA-seq or in situ hybridization at 12 h and increased on successive days; moreover, ISC-like cells 12 h after DT treatment were transcriptionally the least similar to resting or late-appearing ISCs [Fig. 3E,F]. On each day, an mRNA signature comprised of the top 10% ISC-enriched genes (minimally expressed in other cell types) [e.g., Supplemental Fig. S4B,C] was limited to dedifferentiating cells classified as ISCs or early Sec-Pros [Fig. 3B]. Thus, although regenerated ISCs lack Lgr5/GFP until at least day 3 [Fig. 1B], maturity was diminished in a large fraction of Sec cells soon after ISC ablation. Some ISC features reappeared within a day, but as we examine further in Figure 4, the complete mRNA profile returned gradually.

At any time, only 627 genes expressed aberrantly in any Sec cell cluster were not normally expressed in one or more sibling types [Fig. 3G], and a large fraction of even these genes represented marginal modulation of mature Sec markers [Supplemental Table S2]. Bona fide differential expression relative to the forward Sec trajectory was limited to 215 genes at 12 and 24 h. This 215-gene set was dominated by transcripts originally identified in fetal or newborn epithelial organoids, distinct from adult organoids [Fordham et al. 2013; Mustata et al. 2013]; overlapping genes were later reported in crypts responding to various forms of damage in vivo [Nusse et al. 2018; Yui et al. 2018; Ayyaz et al. 2019] and in organoids subjected to physical stretch [Tallapragada et al. 2021]. These mRNA signatures (for review, see Tallapragada et al. 2021) were absent from most normal Sec cell types but were enriched in normal crypt goblet cells, became stronger and more broadly distributed after ISC ablation, and abated by the next day [Fig. 3G, Supplemental Fig. S5A,B]. However, individual genes behaved differently; for example, S100a11, S100a6, Gsn, and Anxa2 were expressed in crypt goblet cells, whereas Clu, Sprt1a, Ly6a [also known as Sca1], Lactc2, and Anxa10 were largely silent in resting Sec and activated after ISC loss [Supplemental Fig. S5C–E]. All elements, whether silent or low-expressed at baseline, were most evident in cells reverting from the goblet–Paneth branch and classified at capture as Sec-Pros [Supplemental Fig. S5C–E].

Importantly, the module was absent in the 3272 indeterminate cells we set aside after DT treatment [Supplemental Fig. S5C], indicating that it reflects a response to ISC injury rather than to DT exposure per se. In situ hybridization for selected markers, Sprt1a and Clu, verified transient and spatially restricted activation, starting within 12 h of ISC ablation [Fig. 3H]; Clu was activated in both crypt cells and villus tips, where DT is known to eliminate underlying Lgr5+ mesenchymal cells [Bahar Halpern et al. 2020]. We detected few other aberrant mRNAs, such as Isg15, Acta1, Ddx60, R3hdml1, and Tap1, in various cell types [Supplemental Fig. S5E,F]. Thus, few genes not already expressed in Sec cells are activated upon ISC loss,
Figure 3. Reverse mRNA trajectories for ISC restoration. [A] Cells isolated from Ngn3-Cre;Lgr5<sup>Cre<sup>ERT</sup></sup>;<sup>R26<sup>R<sup>Tom</sup></sup></sup> mice after ISC ablation represent a mixture of cells in normal forward (left) and putative reverse (right) differentiation. [B] UMAP plots of nearest cell neighbors based on scRNA profiles of Tom<sup>+</sup> cells harvested 0.5, 1, 2, and 3 d after DT injection. Cells are distinguished by their resemblance to [left, n = 3272 indeterminate cells] or difference from [right, n = 6802 reversing cells] control (no DT). Reverse cells were captured at each time post-DT, and distinct populations carried an ISC signature (top 10% genes with minimal expression in other cell types) (Supplemental Table S1). [C] Cell proportions in indeterminate (TAM + DT-treated cells resembling normal TAM-only populations) and reverse (TAM + DT) cell collections on specified days after the second dose of TAM. Progenitor populations include Sec-Pros, bipotential Gob/Pans, and EEC-pres; mature cells include all other populations except ISCs. [D] ISC signatures (top seven ISC-enriched genes [shown in Supplemental Table S1] and classic ISC markers: Ascl2, Axin2, Cdc7a, Lgr5, Olfm4, and Smoc2) accumulate progressively. [E] Pearson correlations of global mRNA profiles of cells classified as ISC-like at each time point. Cells isolated at 12 h lack Olfm4 and differ most from control ISCs. [F] RNAscope in situ hybridization showing Olfm4 in control crypt cells (no DT), its absence 12 h after DT treatment, and its gradual restoration between 24 and 72 h. [Insets] Duodenum tissue rolls. (Graph) Olfm4<sup>+</sup> crypts in control (gray) and postablation SI. Scale bars: 100 µm; insets, 1 mm. n = 5 mice per time point. [G] Differentially expressed genes (ln fold difference >0.3, q <0.05, n = 627) (Supplemental Table S2) post-ISC ablation. By gene set enrichment analysis (GSEA), the 215 genes overexpressed at 12 and 24 h express signatures associated with fetal [Mustata et al. 2013; Nusse et al. 2018; Yui et al. 2018], revival stem cell [Ayyaz et al. 2019], Yap/TAZ [Gregorieff et al. 2015], and stretch-induced [Tallapragada et al. 2021] mRNA programs. GSEA enrichment scores at 24 h and expression at 24 and 48 h are quantified for one fetal signature (Mustata et al. 2013) in each defined cell cluster post-ISC ablation, compared with controls (TAM only, no DT). [H] RNAscope in situ hybridization revealing Spr1α and Chl in crypt and some villus cells, with peak expression 12 h after DT administration. Scale bars, 100 µm.
Figure 4. Sec cell states following ISC ablation. (A) During crypt recovery, ISC-like clusters express mature cell markers and low levels of ISC-specific markers. Coexpression of Paneth (e.g., Mmp7, Lyz1, and Defensins), goblet (e.g., Muc2 and Tff3), and low levels of ISC (e.g., Olfm4, Cps1, and Oat) genes implies that diverse Sec cells continuously replenish ISCs. (Gray dots) Genes expressed at similar levels before and after ISC ablation. (B) Counts (red = high) of unspliced Chga (EEC-specific), Plaur (goblet), Samd5 (Paneth), and Cdxca7 (ISC) transcripts projected on a UMAP plot of 2154 reverse Tom+ cells 48 h post-DT. Restored ISCs carry unspliced forms of mature cell RNAs, signifying recent synthesis and hence revealing diverse sources of ISC regeneration. (C) Proliferation mRNA index (G2/M score calculated in Seurat) projected onto a UMAP plot of Tom+ cells isolated 48 h post-DT. Cell replication is restricted to ISC-like and Sec-Pro-like cells. (Right) G2/M signatures in each cell cluster. (Bottom) Representative immunofluorescence images showing limited MKI67 in Tom+ cells before or after ISC loss and MKI67+ cell counts in 200 control (salmon) and postablation (green) crypts. n = 3 mice per time point. Scale bar, 100 μm. (D) Pearson correlations of global mRNA profiles among control (forward) and reversing cell clusters 48 h post-ISC ablation. Distinctions among all reversing cell types, including ISCs, were appreciably reduced compared with forward differentiation. Violin plots show enriched expression of goblet/Paneth markers (Ang4 and Itln1) in EECs and ISCs and enriched expression of Nhp2 (ISC marker) in non-ISCs. (Gray) Control, (green) post-ISC ablation. (E) During crypt recovery, ISC signatures from Muñoz et al. (2012) and from this scRNA study appear in ISC-like cells in distinct dynamic patterns. (F) RNAscope in situ hybridization showing Lgr5 in control crypt cells (no DT), its absence in DT-treated crypts at 12 h, and its subsequent gradual restoration [see also Supplemental Fig. S6E]. Graph displays counts of crypts with Lgr5+ basal cells in control [gray] and postablation SI. n = 2 animals per time point. Scale bar, 100 μm.
and those previously regarded as fetal, revival-related, or stretch-related do not represent a developmental module per se but an injury-responsive feature of normal crypt goblet cells.

**mRNA trajectories of reverse Sec differentiation**

Beyond this notable and transient transcriptional effect, discrete regenerating cell clusters that resembled ISCs (Fig. 3B) coexpressed Sec-specific genes, such as Lyz1, Spink4, Reg3g, and Defensins, and low levels of ISC genes; early mRNA profiles were dominated by goblet cell genes and later profiles were dominated by Paneth cell genes (Fig. 4A). Conversely, regenerating cells with defining features of each Sec cell type were unambiguously distinct from their control counterparts, showing reduced levels of canonical Sec markers; reversing goblet and Paneth cells outnumbered dedifferentiating EECs, but even immature and mature EECs expressed ISC genes such as Ifitm3 and Olfm4 at the expense of EEC-restricted genes (Supplemental Fig. S6A). These findings suggest direct and continuous conversion of crypt Sec cells that simultaneously adopt ISC and relinquish differentiated properties. Indeed, regenerating ISCs—distinct from forward-differentiating ISCs (Supplemental Fig. S6B)—showed unspliced forms of abundant mature Sec cell transcripts, such as EEC-specific Chga, goblet-specific Plaur, and Paneth-specific Samd5, 48 h post-DT (Fig. 4B). Furthermore, scRNA signatures of mitosis appeared only in ISCs and in cells at the brink of ISC restoration, and overall crypt cell replication was minimally perturbed in 48 h post-DT (Fig. 4C, Supplemental Fig. S6C); 24 and 48 h into ISC recovery, ~0.5% Mki67+ crypt cells were also Tom+. Thus, the provenance of regenerated ISCs traces to diverse Sec cells, including mature crypt cells, that initiate dedifferentiation without re-entering the cell cycle.

Ectopic signals were present in appreciable fractions of cells classified according to their dominant RNA signatures. Not only did cells from the Gob/Pan and EEC branches express each other’s canonical markers (Supplemental Fig. S6D), but 48 h after ISC loss, the normal distinction of various Sec cells from ISCs and from each other was substantially blurred, with all populations approaching the profiles of resting ISCs (Fig. 4D). This extensive transcriptional flux characterized the bulk of dedifferentiating Tom+ cells, yet on any day fewer than one-quarter of isolated cells classified as ISCs and most cells carried Sec-Pro features (Fig. 4C). The simplest interpretation of these findings is that ISCs regenerated from the Sec lineage arise through an intermediate state that closely resembles multipotential Sec progenitors. Before testing this idea by examining accessible chromatin in reverting cells (Figs. 6, 7, below), we considered a 510-gene mRNA signature reported for Lgr5+ ISCs (Muñoz et al. 2012); 273 of the 443 genes represented in our scRNA data were confidently ISC-enriched (Supplemental Table S3), and their low levels 12 h after DT exposure attest again to efficient ISC depletion. In regenerated ISCs, these transcripts appeared in distinct temporal patterns, and different sets of genes confidently enriched in ISCs in our scRNA data replicated these patterns (Fig. 4E, Supplemental Table S3). In line with these findings, and in contrast to early Olfm4 expression (Fig. 3E,F), Axin 2 (which is expressed in ISCs and other crypt cells) and Lgr5 (which is poorly represented in scRNA-seq) re-expressed at different speeds (Fig. 4F; Supplemental Fig. S6E). Thus, the final ISC state does not arise abruptly but over several days, with many ISC genes first reappearing 3 d after DT treatment [Supplemental Fig. S6F].

**Cis-element basis of forward Sec differentiation**

To examine the chromatin basis of Sec cell fate reversal, we first used scATAC-seq to map open chromatin during forward Sec differentiation at 12, 48, and 72 h after the second dose of TAM. We applied Signac (Stuart et al. 2021) to partition 9123 Tom+ cells based on similar chromatin access [Supplemental Fig. S7A]. Unlike cell clusters in scRNA-seq, which can be identified from specific transcripts (Fig. 2A), lineage attribution is challenging in scATAC-seq because cell identities reflect enhancers that may lie far from cell-restricted target genes (Heintzman and Ren 2009). However, dynamic access at a subset of promoters identified cell clusters based on differential gene expression in scRNA-seq and confirmed by known markers (Supplemental Fig. S7B). Pseudobulk analysis indicated that scATAC-seq data identified ~96% of peaks seen in bulk ATAC-seq from resting Lgr5+ ISCs (Supplemental Fig. S7C). After we excluded the small fractions of Ent, tuft cells, and lymphocytes (Fig. 5A), Slingshot analysis of aggregate scATAC profiles (Street et al. 2018) matched the pseudotime lineage relationships identified by scRNA-seq: ISCs differentiate first into multipotential Sec-Pros, followed by divergent Paneth/goblet and EEC tracks that resolve into terminal cell types (Fig. 5B).

More than 12,000 sites were differentially accessible (log fold difference >-0.1, FDR <0.005) between any two populations, with gains in accessibility outnumbering losses [Fig. 5C; Supplemental Fig. 7D]. Sec-Pros showed reduced access at 1945 ISC-specific enhancers, but few unique sites of increased access and, like their mRNA profile, their open chromatin resembled that of bipotential Gob/Pans. Although thousands of sites gave equally strong ATAC signals in mature goblet and Paneth cells, we could readily define groups of sites significantly enriched in one or the other (Fig. 5C). Overall, the EEC branch was the most distinctive [Supplemental Fig. S7D], accounting for approximately one-third of all ATAC changes (Fig. 5C). At some loci, chromatin open in multiple cell types was interspersed among sites that opened only in cells with high RNA expression (Fig. 5C, middle). Cis-coaccessible network analysis (Pliner et al. 2018) showed interactions of such “viewpoint” enhancers with the promoters of their respective target genes [Supplemental Fig. S7E]. Beyond the correlation of modulated chromatin with expression of linked genes (Fig. 5C, right), regions selectively open in individual cell types were enriched for sequence motifs that correspond to TFs expressed selectively in those cells (Fig. 5D). In sum, this scATAC-derived
Figure 5. Chromatin landscape of unperturbed Sec cell differentiation. (A) UMAP plot from scATAC-seq of 8274 Tom⁺ cells. Cell identities were imputed by integration with scRNA data (see Supplemental Fig. S7A,B). (B) Pseudotime differentiation trajectories, derived using Slingshot (Street et al. 2018), traced the origins of EE, goblet, and Paneth cell clusters to ISCs via Sec-Pros. (C) Differential regions \((n = 12,389)\) enriched in any cell cluster \((|L_\alpha| \text{ fold difference } >0.1, P_{\text{adj}} < 0.005\), minimum fraction of cells within an enriched cluster \([\text{pct.}1]\) \(>0.25\), minimum fraction of cells in other clusters \([\text{pct.}2]\) \(<0.15\)\), represented as aggregated pseudobulk data. Adjacent IGV tracks and violin plots [both pseudobulk] show representative differential regions [scATAC, left] and transcript levels of the corresponding genes [scRNA, right] in each cluster. (Bottom right) The graph shows overlap between scRNA data (columns) and the nearest scATAC peak (rows). Circle size represents the fractional mRNA:ATAC peak overlap computed at the gene level in each cell cluster and the color scale represents Bonferroni-corrected \(P\)-values from a hypergeometric test. (D) TF sequence motifs enriched at areas of differential chromatin access in each cell type affirm the veracity of scATAC data and cluster assignments.
Figure 6. Reversal and sublineage infidelity of cis-element trajectories during dedifferentiation. (A) UMAP plots from scATAC analysis of integrated forward (TAM-only control, left, n = 8274) and reverse (TAM + DT, right, n = 6990) cells resembling the control population were removed (see Supplemental Fig. S8A) Sec cell differentiation. (B) Graph of 131,216 ATAC peaks called in Neurog3-labeled cells (Y-axis) post-ISC ablation plotted against peaks called without DT treatment (X-axis). Each dot represents the signals at an ATAC site aggregated across all cells and gray signals were statistically increased post-ISC ablation (ln fold change >0.25, q < 0.05). (pie chart) Fractions of sites with increased post-DT signals (gray dots) associated with one (75.7%) or more (24.3%) forward-differentiating cell types. (C,D, top) Increased signals (scatter plots) and redistribution (UMAP plots) of goblet-specific (C) and Paneth-specific (D) sites (as defined in Fig. 5B) after ISC ablation. (Bottom) Chromatin sites differentially accessible in clusters classified as goblet (C) or Paneth (D) cells after ISC ablation. Pseudobulk aggregation of scATAC data (heat maps) and aggregate scATAC signatures (violin plots) reveal that cis-elements normally specific to ISCs or to one sublineage were accessible in dedifferentiating cells of other types. Similarities between control and post-DT cells at each time were quantified using the Kolmogorov–Smirnov test. The D statistics are noted within each violin; lower values reflect greater similarity, and control (TAM-only) cells serve as the reference. (IGV tracks) Representative examples of dynamic cell-specific enhancers. (E) EEC-restricted cis-elements from both EC and non-EC cells were less perturbed after ISC ablation than goblet- and Paneth-specific sites, reflecting predominant dedifferentiation of the latter cell types. EEC cis-element signatures nevertheless extended into other reversing Sec cells, consistent with dedifferentiation occurring via multipotential precursor states.
compendium of differentially accessible cis-elements outlines the sequential enhancer recruitment that underlies Sec cell diversity in vivo, explains multilineage priming in chromatin terms, and provides cell type-specific enhancer signatures as a tool to study reversal of cell identities.

**Enhancer trajectories during crypt Sec cell dedifferentiation**

To that end, we performed scATAC-seq in parallel on Tom+ cells collected after DT exposure for scRNA-seq. As with the scRNA data (Fig. 3B), we integrated control and post-DT cells in the computational analysis, and then separated informative reversing cells from indeterminate cells whose profiles of open chromatin were largely indistinguishable from those of controls (no DT); to be conservative, we regarded cells with subtle differences (sum of SNN score >3) in open chromatin as indeterminate (Fig. 6A; Supplemental Fig. S8A,B). We then plotted the 131,216 sites open in Neurog3-labeled cells (Fig. 6B, Y-axis) after ISC ablation against those that were open without DT treatment (Fig. 6B, X-axis). As each dot on this graph represents the signals at an ATAC site aggregated across all cells, a negative composite slope means that ATAC signals were generally higher after ISC ablation, even after rigorous normalization of the data. However, the graph lacked evidence for appreciable opening of previously inaccessible sites (Supplemental Fig. S8C) and only showed increased access at sites that were open in normal Sec cells of one (75.7%) or more (24.3%) types (Fig. 6B). The negative slope hence reflected expanded and ectopic distributions of open chromatin. Within 100 kb of TSSs, even loci that were silent in resting Sec cells but rapidly and briefly activated after ISC loss (Fig. 3) showed only small changes in constitutively accessible chromatin (Supplemental Fig. S8D), most pronounced in goblet and Gob/Pan precursors.

Global ATAC profiles of each Sec population most resembled those of its normal counterparts, indicating that cell clusters were correctly assigned (Supplemental Fig. S8A,B).
Fig. S9A). Nevertheless, enhancers otherwise selective for distinct Sec cell types, including 4%–24% of the 1945 enhancers otherwise restricted to ISCs (Supplemental Fig. S9B), became broadly and transiently accessible. These changes were most evident in cells classified as goblet [Fig. 6C] or Paneth [Fig. 6D], which carried chromatin features of each other and of ISCs within 12 h of ISC ablation. EEC-specific sites were less extensively redistributed [Fig. 6E], but even EC cells showed increased access [ln fold change >0.25 over normal EC cells] at 13% of goblet-enriched and 10.4% of Paneth-enriched enhancers [Supplemental Fig. S9C]. The appearance of ISC enhancers in Tom+ Sec cells indicates that the sum of these alterations was not spurious and reflects ISC regeneration. Indeed, across cell isolations from 12 to 72 h after DT treatment, enhancer dynamics mirrored, and preceded, the mRNA flux and blurred transcriptional identities and ISC revival [Fig. 6C,D]. Given that normal, forward differentiation occurs after sublineage-enriched enhancers first open in Sec-Pros, Gob/Pans, and EEC precursors [Fig. 5], these findings reinforce the idea that Sec cells reverse via bipotent and multipotent precursor states.

Within 12 h of DT exposure, cells classified as ISC-like showed chromatin access at 35.5% of goblet-specific and 15.4% of Paneth-specific regions (Fig. 7A), implying that these are the principal Sec sources. Lineage-specific epigenetic marks could in principle persist in regenerated ISCs and influence their properties; for example, Sec-derived ISCs may carry a bias to generate more Sec cells. Lineage-restricted ATAC signals, however, were strongest at 12–24 h, tapered thereafter, and then disappeared [Fig. 7B]. Consistent with that finding, Tom+ Sec-derived and Tom− cell-derived crypts were dominated by Ent cells and had similar fractions of MUC2+ goblet and 5-HT+ EC cells 30 d after ISC ablation [Fig. 7C]. Together, these observations reveal that Sec cells restore fully functional ISCs by reversing the course of forward differentiation and, in that process, erasing lineage identity or bias.

Discussion

Both Sec and Ent cells dedifferentiate to restore ablated ISCs. Prior studies showing ISC recovery from Sec cells [van Es et al. 2012; Yu et al. 2018; Castillo-Azofeifa et al. 2019; Jones et al. 2019] relied on lineage-restricted CRE expression, with inherent uncertainty about its onset and trajectory; one CRE-independent study relied on activation of Ascl2 at unknown and possibly variable times after ISC loss [Murata et al. 2020]. Using single Neurog3Cre+ labeled cells to define forward and reverse Sec cell composition as well as chromatin and transcriptional states, our study overcomes these limitations, and both scRNA and scATAC data pointed to dedifferentiation originating in all mature and immature Sec types. In the process, lineage-specific transcripts declined in these cells, which resembled each other far more than they do in normal crypts and recapitulated in reverse the transcriptional and chromatin states characteristic of multipotential Sec-Pros and Gob/Pans. Our findings reveal how dynamic chromatin transitions allow cells to readily acquire or relinquish Sec-specific properties and to enable tissue recovery when ISCs are depleted.

Four findings merit particular note. First, after ISC ablation, at least 60% of Neurog3Cre+-labeled cells had profiles distinct from their normal counterparts. Any crypt aims to restore only 12–15 Lgr5+ ISCs, many of which originate from the Ent lineage or by ISC replication. Therefore, many Neurog3Cre+-labeled cells may react only briefly to ISC attrition and then abort the process, or their daughters eventually succumb to neutral drift. Second, forward and reverse Sec differentiations occur along a continuum of precursor, immature, and mature cell states: Partially overlapping Sec-Pro, Gob/Pan, and EEC-pre signatures signify multilineage priming and no overt bottleneck. Third, cell replication was not evident at the onset of Sec fate reversal—only just before or after cells acquired ISC features. Fourth, although cell fate reversal was associated with extensive sublineage infidelity in RNA and chromatin states, few transcripts extrinsic to Sec cells and no new cis-elements were recruited. The few extrinsic transcripts overlapped with genes expressed in regenerating ISCs after helminth infection or chemical colitis [Nusse et al. 2018; Yui et al. 2018], in fetal mouse enteroids [Fordham et al. 2013; Mustata et al. 2013], in CLU+ "revival stem cells" [Ayaz et al. 2019], and in stretched adult enteroids [Tallapragada et al. 2021]. Some of these genes are expressed in normal goblet cells, while others are absent in normal Neurog3Cre+-labeled cells and appear transiently after ISC ablation. We suggest that the program reflects an injury response, possibly the physical void from crypt cell loss, and not a path to cell fate reversal per se. Taken together, our findings reveal self-contained Sec dedifferentiation occurring through bipotent and multipotent progenitor states that recapitulate multilineage priming in reverse.

Even at the few transiently activated loci, chromatin was previously accessible. Moreover, cis-elements specific to differentiated Sec persisted <48 h in regenerating ISCs. This absence of epigenetic memory is in contrast to stress responses in damaged epidermal [Naik et al. 2017] and hematopoietic [Kaufmann et al. 2018] stem cells, where new injury-responsive chromatin domains stay open indefinitely and respond rapidly to future challenge [de Laval et al. 2020; Larsen et al. 2021]. One notable difference is that DT treatment of Lgr5Dtr mice selectively ablates ISCs and partially depletes Paneth cells, in contrast to more extensive damage from skin wounds or pathogens that trigger lasting new memories in other self-renewing tissues. Other forms of intestinal damage or more severe epithelial attrition may elicit reactive chromatin memory and alternative pathways for ISC regeneration. Lgr5Dtr mice represent one of many possible models to study crypt dedifferentiation, each with its own caveats and limitations, such as reduced Wnt signaling strength in Lgr5Cre+ crypts [Tan et al. 2021]. Nevertheless, the facile dedifferentiation of Neurog3Cre cells in this model opens a window into normal crypt physiology, where ISCs and their immediate progeny coexist in dynamic equilibrium [Ritsma et al. 2014; Kim et al. 2016].
and the intestine may well respond differently to diverse injuries.

Materials and methods

Experimental mice and treatments

Lgr5<sup>Di-EGFP</sup> (MGE:5294798) [Tian et al. 2011], R26R<sup>Tom</sup> (MGE:3813512) [Madisen et al. 2010], Ngn3<sup>CreERT2</sup> (MGE:5449724) [Li et al. 2012, Li et al. 2019], and Lgr5-EGFP-IRE5-creERT2 (MGE:127123) [Barker et al. 2007] mice were maintained on a predominantly C57BL/6 background. Animals were housed under specific pathogen-free conditions in 12-h light/dark cycles at 23°C ± 1°C and 55% ± 15% humidity. Food and water were provided ad libitum. Animals were weaned 21–28 d after birth and handled and euthanized according to procedures approved by the Animal Care and Use Committee of the Dana-Farber Cancer Institute. Mice were at least 8 wk old at the time of experiments and cell isolations. Mice of both sexes were used in the experiments, with littermate controls. Ngn3-Cre<sub>Lgr5<sup>Di</sup></sub>, R26<sup>Tom</sup> mice were injected with two daily doses of intraperitoneal tamoxifen (Tam; 50 µg/kg, Sigma T5648), and whole SI was harvested 12, 24, 48, and 72 h after the second dose (Supplemental Fig. S1A). To ablate ISC’s, mice received one dose of intraperitoneal diphtheria toxin (DT; 50 µg/kg, Sigma D0564) at the same time as the second TAM injection.

Whole-mount imaging and immunohistochemistry

The whole SI was harvested from euthanized mice at the specified times, rinsed in PBS, and fixed overnight in 4% PFA (EMS 15714–30%) – 30% sucrose gradient and embedded in OCT compound (Tissue-Tek, VWR Scientific 4583), and 10-µm sections were prepared using a Leica cryostat. Incubation with CHGA (1:100, Abcam ab15160), LYZ1 (1:1000, Dako A0099), 5-hydroxy tryptamine (1:500; Immunostar 20080), MUC2 (1:1000, Santa Cruz Biotechnology sc15334), FABP6 (1:500; Abcam ab91184), and MKI67 (1:100, Abcam ab15580) antibodies was followed by washes and incubation with appropriate secondary Ab (Alexa fluor, Invitrogen). After counterstaining with DAPI, slides were imaged on a Leica SP8X laser scanning confocal microscope with 1-µm z-stem size and processed using ImageJ Fiji software [Schindelin et al. 2012].

mRNA in situ hybridization

mRNA in situ hybridization was carried out using the RNAscope method according to the manufacturer’s instructions [Advanced Cell Diagnostics (ACD), Bio-Techne]. In brief, fixed frozen tissue sections were subjected to heat-induced epitope retrieval at 98°C–102°C in antigen retrieval buffer and digestion in Protease III for 15 min. Probe sets designed by ACD for Olfm4 (Bio-Techne 311831-C2), Lgr5 (312171), Axin2 (400351-C3), Sprrla (426871), and Cldn (427891-C3) were hybridized with tissue sections for 2 h, and tyramide signal amplification with TSA fluorescein (Akoya Biosciences, NEL701AA01KT) was used to develop probe channels individually. Slides counterstained with DAPI were imaged with a Leica SP8X laser scanning confocal microscope with 1-µm z-stem size and processed using ImageJ Fiji software [Schindelin et al. 2012].

Single-cell preparation for scRNA-seq and scATAC-seq

To enrich for crypt cells, villi along the whole SI were scraped away using a glass slide. Intestine pieces were rinsed in PBS and then rotated in 5 mM EDTA (pH 8) in PBS for 45 min at 4°C, with manual shaking every 10 min and a change of solution after 30 min. Released crypts were passed through a 70-µm strainer to eliminate any remaining villi and dissociated into single cells by rotating in 4% TrypLE solution [ThermoFisher A2121702] for 45 min at 37°C. Single cells were diluted in Dulbecco’s modified Eagle medium [DMEM, Corning 17-025-05], containing 2% fetal bovine serum (FBS). Tom+ cells were isolated on a FACSAria II SORP flow cytometer, with elimination of dead cells using DAPI, pelleted by centrifugation, and counted manually by trypan blue exclusion. For scRNA-seq, cell pellets were resuspended in PBS according to recommendations provided by 10X Genomics, and the suspension was loaded onto the 10X Chromium Chip G (10X Genomics PN-1000127). Libraries were prepared according to the manufacturer’s protocol using 10X Single-Cell 3′ v3.1 chemistry [10X Genomics PN-1000128]. For scATAC-seq, nuclei were isolated from sorted cells, counted, and processed as described in the manufacturer’s protocol [10X Genomics, Chromium NexGEM Single-Cell ATAC reagent kits v1.1 PN-1000176] using 10X Chromium Chip H (10X Genomics PN-1000162). Libraries were sequenced on Illumina HiSeqX instruments to generate 150-bp paired-end reads using Novogene services [https://en.novogene.com]. Computational analysis of scRNA-seq and scATAC-seq is described in the Supplemental Material.

Data availability

All scRNA-seq and scATAC-seq data have been deposited in the Gene Expression Omnibus (GEO), accession number GSE183299.

Competing interest statement

The authors declare no competing interests.

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