

Sexual dimorphism in epidermal stem cell dynamics

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)

This manuscript by Engelman et al. presents an elegant and technically sophisticated analysis of epidermal homeostasis in the mouse ear interfollicular epidermis. By combining long-term intermittent intravital time-lapse imaging and clonal lineage tracing, the authors directly track the dynamics of individual labelled clones over extended periods. This approach enables the detection of transient, short-lived clones that were previously undetectable in fixed sample lineage analyses based on pooled data, thereby providing critical temporal resolution. The major strengths and advancements of this study are; 1) the direct observation of clonal extinction represents a significant technical and conceptual advance, allowing access to temporal dynamics beyond the reach of static lineage tracing; 2) the integration of short-lived clones into a hierarchical SC–TA model provides a compelling framework to reconcile both long-term clonal persistence and transient dynamics; 3) the study connects clonal fate patterns with sex hormones, providing insight into how systemic factors influence epidermal homeostasis. This is a high-quality and timely study that addresses an important question in epithelial stem cell biology using sophisticated tools. While the main conclusions are convincing, some key mechanistic claims, especially those regarding coordination between sister cell fates, rely more on inference than direct experimental evidence. Strengthening the logical and experimental support of these aspects would further improve the clarity and overall impact of the work.

Major concerns:

1. The model proposed by the authors relies heavily on the assumption of coordinated cell fate behavior—specifically, the temporal coupling of sister cell division and delamination within each clone for long period of time—to account for the observed enrichment of even-numbered clone sizes. However, the manuscript does not clearly specify what type of coordination is being assumed at the mechanistic or dynamical level (e.g., whether divisions and delaminations are synchronised in time, spatially coupled, or triggered by local feedback). Without such clarification, it is difficult to evaluate the plausibility or testability of the coordination-based explanation.

Moreover, while the authors cite previous work by Rempel et al. (Science 2016) and Mesa et al. (Cell Stem Cell 2018), they do not clearly describe how the types of coordination observed in those studies relate to the mechanisms proposed in their own model. For example, they showed that delamination events can trigger division in neighboring cells, often with planar (horizontal) division orientations that serve to maintain tissue integrity. This raises the possibility that division and delamination may occur in an alternating or compensatory manner, rather than in a synchronously coordinated way. The current manuscript does not provide direct experimental evidence—such as long-term single-cell/clone tracking or quantitative fate mapping—that sister cells divide or delaminate in a coordinated manner over time. If coordination is indeed central to the mechanistic interpretation, then empirical demonstration within the current system, following the standards of these foundational studies, is essential.

2. Key terms such as “basal clone”, “suprabasal clone”, and “TA-like clone” are used throughout the manuscript, but their definitions and quantification methods are not clearly described in this paper. For instance, it is unclear whether suprabasal cells are considered part of the same clone as their basal counterparts or counted separately. Likewise, the use of clone size (≤ 3 cells) to define TA-like clones is operationally convenient, but it may not accurately reflect lineage origin or fate potential. Some stem cell–derived clones may remain small, while TA cells could produce more than three progeny. The authors should discuss the biological limitations of this size-based classification and how it might influence the interpretation of modelling results.

3. The manuscript integrates clonal analysis, scRNA-seq, and mathematical modelling to support a hierarchical SC→TA→D framework. However, it remains unclear how consistently the terms “SC”, “TA”, and “D” are defined and interpreted across these platforms. For example, do SC-like clones identified via fate-mapping correspond to transcriptionally defined stem cell states in scRNA-seq? Are TA-like clusters associated with short-lived or extinct clones? Clarifying whether these definitions are intended to align or diverge is intentional would improve the conceptual coherence of the model.

4. In Fig. S6e, RNA velocity vectors appear to point from TA-like to SC-like cells, which contradicts the proposed irreversible SC→TA hierarchy. This apparent inconsistency must be directly addressed. Is this a technical artifact or a biological suggestion of reversibility? Leaving this unexamined undermines confidence in the model's directionality.

5. If SC clones generate TA cells as a key feature, the rate or proportion of such transitions should be quantified. The manuscript does not provide estimates of how often SCs generate TA progeny or how many TA cells arise per SC division. Without these data, it is difficult to assess the quantitative validity of the two-compartment model.

6. The observed sex-specific differences in epidermal turnover are interpreted primarily as a consequence of hormonal influences directly to epidermal gene expression. However, other factors, such as differences in dermal thickness, hair follicle cycling, could also contribute to these patterns. A consideration of these potential confounders would help strengthen the causal interpretation of the sex-related findings. For example, do you see dermal thickness reduction, gene expression changes and hair cycle progression differences in castration mice?

Minor comment:

7. The authors propose a two-compartment SC→TA model as a biologically more accurate alternative to the one SC pool model. While this framework is compelling, the manuscript would benefit from a more explicit discussion of which specific observed phenomena cannot be adequately explained by the one SC pool model and why. For instance, are the biphasic clone persistence patterns, high frequency of clone extinction, or even-number clone bias incompatible with predictions of the SC pool model under reasonable parameter settings? Clarifying this point would help to justify the necessity of introducing a distinct TA compartment rather than relying on continuous fate transitions within a single stem cell population.

Referee #2

(Remarks to the Author)

Comments:

In this work, Engelman and colleagues show for the first time that epidermal stem cell dynamics that ensure homeostasis are under hormonal control thus showing sexual dimorphism. Sex-dependent differences in physiology might underlie differences in the incidence and level of many pathologies. However, the mechanistic reasons that explain these differences in homeostasis and disease are almost completely unknown. In this manuscript, the authors study this issue in the epidermis, where clear sexual dimorphism is known to occur regarding essential aspect of the function of the human skin, such as skin immunity, skin aging, and the prevalence and progression of tumors, mainly melanoma and basal cell carcinoma.

The two main labs conducting this elegant work were at the forefront of their fields in understanding the importance of stem cell dynamics regarding skin physiology. Both groups developed the in vivo models and mathematical tools which were used later by many others to almost open a new field within the stem cell research field. They now also include beautiful intravital microscopy to obtain very robust data. As such, I do not have any comments regarding the data presented.

However, I think the authors have a wonderful opportunity to expand this current work to try to understand whether the sex-dependent differences they have observed regarding tissue-level stem cell dynamics, underlie some of the physiological and disease differences seen in human skin at the clinical level. Recently, Chi L et al (2024) showed sexual dimorphisms in skin innate immunity. In addition, the group of Ashani T. Weeraratna has shown that male dermal fibroblasts age faster than female dermal fibroblasts. Very interestingly, age-dependent sex difference patterns are observed in melanoma and BCC in humans. Specifically, melanoma and BCC show higher incidence in young women and old men, with a switching age around menopause. This switching age suggests involvement of sex hormones, but other potential contributors to this sexual dimorphism could be a direct consequence of the well-characterized sexual dimorphism in skin color in humans (males showing a darker and redder skin than females). However, in none of these previous works they took the extra step to relate these sex-dependent differences to specific physiological aspects with clinical importance. Thus, the results in this ms could constitute a beautiful working frame to test whether the sexual dimorphism they have identified in epidermal stem cell behavior may be a contributing factor to the differences in BCC incidence between males and females. Another physiological example of sex dimorphism relates to wound-healing in that human male skin heals slower than female skin upon acute wound healing.

I think if the authors could show that these hormone-dependent sexual dimorphism are at the root, or contribute in any way, to the differences in BCC development, or rate of wound healing, then this would be a hallmark paper in the field that underscores the relevance of sexual differences regarding health and disease.

(Remarks to the Author)

In this work, the authors performed intravital timelapse imaging to track the long-term fate of individual lineage-labelled cells in the ear skin interfollicular epidermis, and observed enhancement of even basal clone sizes, especially in female mice. Then, the authors proposed a mathematical model with two-proliferative compartment to explain and reproduce the observations. A key discovery is a sex-specific difference in this process: female mice exhibit more synchronized patterns of cell division, differentiation and delamination. Single-cell and bulk RNA-sequencing and clonal analysis following castration revealed that male sex hormones appear to promote stem cell differentiation and delamination.

Overall, the study presented an interesting observation of even basal clone size enhancement and the sex-specific differences, proposing a stem cell lineage model to predict potential mechanisms. However, several key weaknesses limit its current impact. Most critically, the study lacks experimental validation of core model predictions, such as synchronized behaviors in female mice. Furthermore, the connection between molecular mechanisms and the kinetic parameters used in modeling remains insufficiently developed. Additionally, contradictory transcriptomic interpretations and limited exploration of immune-related signaling raise concerns about consistency and depth. Taken together, these issues must be addressed to solidify the conclusions and improve the overall rigor.

My comments are as follows:

Major comments:

1. A key prediction by the two-proliferative compartment model is that female mice exhibit more synchronized patterns of cell division, differentiation and delamination. Is there any experimental evidence for the prediction? Is it possible to observe/measure the synchronizations in experiment? If so, it would validate the prediction and enhance the conclusion of this study.
2. Similarly, experimentally validating the prediction that “male sex hormones had an impact on the basal cell delamination rate and on the relative frequency of asymmetric TA cell divisions” would strengthen this study.
3. While the authors show that one proposed mathematical model, the two-compartment model, generates dynamics that are more consistent with the observed experimental data, there is no reason that there could not be other mechanisms that could give rise to the same dynamics. It would be worth proposing alternative models in the Discussion and how one may “discriminate” between these models.
4. While the authors proposed a mathematical model to explain the sex-specific difference and performed scRNA-seq and bulk RNA-seq to characterize the transcriptomic difference, there is a gap between the differences in molecule expressions and different kinetic parameters defined in the proposed mathematical model. The rigor of this study would be improved by explicitly establishing the link from molecular characterizations to kinetic parameters, and ultimately to clonal dynamics.
5. How is the parameter scan performed for the one- and two-proliferative compartment model? For the scan space, what's the percentage of parameter sets that yield the desired behavior? Which parameters are critical to the model? How robust/sensitive is the model to parameters?
6. Line 296: the justification of the 3D manifold is a little speak, considering that either 2D or 3D visualizations could lead to misinterpretation of the data, as is well known about visualizations such as UMAP, diffusion maps, etc.
7. Considering that replicates for male and female were sequenced for scRNA-seq analysis, it would be better to perform pseudobulked differential expression analysis per cell type to identify more cell-type-specific differences, using statistical models that can be specified using, e.g., DESeq2 or edgeR. This is the more “consensus” way of performing differential analysis using scRNA-seq analysis these days.
8. Two contradictory pieces of information appeared in the results section “Molecular characterization of the IFE reveals sexual dimorphism at the transcriptomic level”. At lines 315-316, “Male IFE SC cluster showed increased levels of Skint1 and differentiation-related genes such as Ehf and Krt14 (Fig. 5n)...” However, at lines 342-343, “In contrast, many differentiation markers were strongly upregulated in female basal cells compared to male...” It would be necessary to interpret the two contradictory pieces, in order to avoid confusion and improve the rigors.
9. Given that bulk RNA-seq was generated a statistical model built using, e.g., DESeq2 or edgeR, should be used over mere log-fold-change calculation to test for differential expression.
10. It would be interesting to investigate how cell-cell communications may shape the differentiation landscape in male and female mice.
11. Something that would help the study is addressing in the Discussion why no previous study of stem cell lineages in IFE has identified these sex-specific differences, i.e., what was missing from previous work, or what was “done correctly” in this study.

12. Lines 820–823: considering that the samples were integrated using Harmony to generate the diffusion map (lines 860–861), why weren't the samples integrated for the "original" analysis?

Minor comments:

1. At line 41, change "comprises" to "is".

2. Line 49, '...in the 1960s' should have citations to the original works.

3. At line 74, the term "CreER" is used without explanation. Consider briefly defining this term for readers who may not be familiar with genetic engineering tools, even if it is common jargon in the field.

4. In the paragraph beginning with (line 73), "However, several observations challenged the one-compartment model. First, lineage tracing using different CreER mice results in differences in the long-term survival and growth potential of clones.^{13–18} Second, fate mapping studies of back skin epidermis under perturbed conditions showed evidence of an enrichment of clones with even cell numbers, a feature difficult to explain within the framework of a one-compartment model. Finally, fate mapping of the ear skin epidermis based on a Keratin 10 (Krt10) reporter showed evidence of proliferative cells with strictly limited proliferative potential," the relation of the first and final points to one-compartment model is not clear.

5. Typos and grammar errors: "beside" at line 86 should be "besides"; "recording of" to "recording" at line 118; "some 1171 clones" at line 127; "5-months" to "5-month" at line 134; "Further, we the model could not capture temporal features..." at line 199.

6. The paragraph "Finally, beside reproductive organs, sex has been associated with differences in organ morphology, tissue composition, and brain specific circuits.^{31–33} However, little is known about the role of sex hormones in adult SC fate decisions. In the skin, morphological difference between male and female have been described.^{34,35} However, whether sex affects stem and progenitor cell dynamics during epidermal homeostasis is not known" should be reorganized.

7. All figure panels should be cited in the main article text and numbered in the order of their first appearance. For example, Figure 1a and 1b are cited in reverse order.

8. It's not clear how the clonal persistence is calculated from the data (Fig. 1f). Given a time point, does it mean the fraction of clones that retains at least one basal cell?

9. Lines 150-151: was any statistical test performed to confirm this difference was significant?

10. It seems that the enrichment of even cell numbers in female mice is not obvious at D6 (Fig. 2l). How to interpret this?

11. Lines 195-196: "Formally, to capture the abrupt increase followed by the shallow linear-like growth in the average basal clone size (Fig. 2e-h)..." . It's unclear how Fig. 2e-h is related to the sentence. Maybe Fig. 2a?

12. Lines 286-287: "As previously described, we found three G0/G1 clusters." The sentence is confusing because the "three G0/G1 clusters" seems not mentioned before.

13. Lines 298-299: "Leiden clustering with a resolution of 0.5 identified three clusters not related to cell cycle..." It's not clear whether the clustering is based on G0/G1 cells or all cells. In addition, justifications are needed why these three clusters are not related to cell cycle.

14. It's not clear how the conclusion "Cells from both the SC and TA cluster were able to enter the cell cycle loop, while most of the differentiated cells were post-mitotic (Fig. 5i)" is made from Fig. 5i, as there is no trajectory direction or pseudotime information. It would be better to incorporate Extended Data Figure 6e (i.e, the RNA velocity analysis) to support this conclusion.

15. How to interpret the increase of the differentiation markers in male SC and TA clusters, such as Krt14?

16. Lines 832–836: what was the p.adjust threshold used to select for marker genes?

Referee #4

(Remarks to the Author)

This paper employs an elegant long-term in vivo imaging approach to revisit a longstanding question regarding clonal stem cell dynamics in the mouse epidermis. The findings suggest sex-specific differences in the hierarchy of stem and transit-amplifying (TA) cells in the interfollicular epidermis of mouse ears, with male and female mice displaying distinct patterns. Analysis of castrated males supports the argument that these differences are driven by androgen signaling. As it is, the study is highly descriptive and of questionable general significance. There are several important limitations:

1. While the imaging technique is technically impressive, it provides only incremental insights relative to an extensive body of prior work, without introducing a conceptually novel framework.
2. The conclusion that androgens directly influence epidermal stem and TA cell dynamics is rather questionable, as the observed effects could stem from indirect influences. Androgens are known to affect immune cell populations in the skin as well as dermal fibroblasts, which in turn can impinge on epidermal cells.
3. The potential contribution of sex chromosome-linked genes has not been explored. This could be addressed using the "four core genotypes" (FCG) model, which has previously been applied to studies of mouse skin, including the interplay between androgens and sex chromosomes in control of ILC2 and dendritic cells.
4. The observed differences may be limited to the mouse ear epidermis, and the broader relevance of the findings to other anatomical sites and to human biology remains unexplored.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)

The revised manuscript is substantially strengthened by the extensive new experiments and analyses. The additional live-imaging experiments provide direct evidence for temporally coordinated sister-cell fate events, and the new *Lgr6CreER* and *Notch1CreER* lineage-tracing studies establish an important connection between transcriptionally defined populations, clonal behaviour, and the proposed mathematical framework. Together, these additions address several of the major concerns raised in the original review and significantly improve the manuscript. I therefore find the central conclusion that epidermal homeostasis is regulated in a sexually dimorphic manner to be well supported. The manuscript makes an important contribution to our understanding of epidermal dynamics and stem-cell regulation.

After reading the revised version, however, I find one remaining gap between the experimental observations and the model-based explanation of the reported sexual dimorphism. I am not requesting additional experiments, but I think further discussion would help readers better understand how the proposed mechanism is supported by the available data.

The revised live-imaging experiments demonstrate that temporally coordinated sister-cell behaviour occurs within the experimental observation window (12-h imaging intervals over 5 days). At the same time, the modelling framework, particularly the analyses presented in Extended Data Fig. 7, suggests that coordinated division and delamination events play a central role in generating and maintaining the characteristic even-odd clonal structure. However, it remains less clear how the experimentally measured coordination relates quantitatively to the coordination parameters inferred by the model, and how observations made over a relatively short temporal window are linked to the long-term clonal statistics analysed over several months. It would be valuable for the authors to discuss more clearly and directly how the experimentally observed coordination events map onto the corresponding model parameters, and how these measured and inferred quantities together account for the persistent enrichment of even-numbered clones and the reported sexual dimorphism. Such discussion would help readers distinguish more clearly between features that are directly supported by experimental observation and those that rely more heavily on model-based inference.

This point does not diminish the importance of the study or the substantial advances achieved through the revision. Rather, a more unambiguous discussion of this issue would help readers better appreciate both the strengths of the experimental findings and the role of the analytical framework in interpreting their biological significance.

Referee #2

(Remarks to the Author)

The authors have addressed all the issues raised from the first revision. The new results added on the wound healing and new mouse models of sexual dimorphism have greatly strengthened the work. I think this is an outstanding study highlighting a previously unknown sex dimorphic adult stem cell behavior with clear consequences over tissue homeostasis and overall physiology. As such, I strongly recommend its publication in *Nature*.

Referee #3

(Remarks to the Author)

The authors have done commendably well to address reviewer comments and revise the initial submission substantially. However, there are still some areas that remain unresolved.

Response to previous comments:

Major comment #1:

Not fully addressed. I thank the authors for the additional experiments and analyses quantifying synchronization between sister cell events. The lineage tracking and the correlation analysis provide useful evidence for strong temporal coordination of sister cell fates. However, it remains somewhat unclear whether the synchronization metrics support the model's predicted sex difference. Among the three correlation measures reported, only the correlation between dividing sisters appears slightly higher in females, without reaching statistical significance, whereas the correlations for delaminating sisters do not show a

consistent trend favoring females, and the confidence intervals overlap substantially. It would therefore be helpful if the authors could further clarify how these measurements support the predicted sex-specific difference in synchronization of cell division, differentiation and delamination, or explicitly acknowledge that the current data primarily demonstrate strong synchronization overall rather than a clear sex difference.

Otherwise, the phrase “female mice showing a highly synchronized pattern of cell division, differentiation and delamination” may overstate the strength of the evidence, given the lack of consistent, statistically supported differences between sexes.

Major comment #3:

Not fully addressed. The additional lineage-tracing experiments strengthen the evidence for distinct SC and TA compartments and support the two-proliferative-compartment model. However, the original concern regarding the link between molecular characterization and the kinetic parameters of the model remains unaddressed. It would be helpful if the authors could further clarify how the sex-specific transcriptomic differences relate to the sex-specific model parameters, e.g., correlations between cell sisters, proliferation, differentiation, and delamination rate.

Major comment #7:

Mostly addressed. The authors are correct in their response that 2 biological replicates per sex is not a lot to use methods like edgeR or DESeq. Given the amount of extra (and arguably, much harder) work the authors did to address other reviewer points, these underpowered experimental designs do detract from the credence of the results, even if multiple bioinformatic methods were applied.

Major comment #8:

Not fully addressed. First, the authors state that the “Male IFE SC cluster showed increased levels of Skint1 and differentiation-related genes...”, whereas the response appears to attribute the differentiation marker signal primarily to differentiated cells.

Major comment #12:

Why wasn't only CCA or only Harmony used for integration? Both methods have different underlying assumptions and potentially different effects on downstream analyses. It is fair that there is no single “best” integration method, but at least ensuring consistency with bioinformatic analyses would help strengthen the validity of the results.

Other major comments:

- How much is clone size affected by the size of the mouse? Male and female mice differ significantly in weight, bone structure, and so forth. Does that confound the clone sizes estimated? Is there a way to look at “relative” clone size or clone density (per area)?
- In these types of Voronoi models, usually the energy to be minimized includes a term describing interfacial tension (based on adjacent center-to-center lengths). Is there a reason it was neglected in this model, other than the fact that it wasn't included in the original proposed framework? That would also help reduce numerical instabilities when modelling cell division, as one could introduce a rule that the reference length between newly divided cells slowly increases to the mature reference length.
- Considering the epidermis consists of several confluent layers, I am not fully convinced that modeling a top-down view of a monolayer interspersed with basal and suprabasal cells is a suitable abstraction of the skin epidermis, as the cellular neighborhoods would be significantly different. As an alternative, the authors may want to consider a model of 2D cross-sectional view of the epidermis and model the different layers or implement a 3D Voronoi model. That way, detachment and subsequent removal of suprabasal cells would be clearer.
- Considering the authors already modelled mechanical feedback on cell division, did the authors consider density-dependent cellular extrusion?
- “3D visualization allowed to remove the confounding effect of cell cycle on G0/G1 cells clustering, while leaving the opportunity to study the ability of G0/G1 cells to divide.” I am not sure how 3D visualization removes a confounding effect rather than, say, additional statistical analysis (like the regression step performed by the authors prior to clustering) or removing the non-G0/G1 clusters from further analysis.
- Throughout the paper, the adjusted p-value threshold to determine significance or differential expression changes between analyses. What is the main reasoning behind these choices?
- The previous comment applies to assessment of “differential regulon activity”.
- Considering that other organs can produce sex steroids, like adrenal glands, adipose tissue, was it confirmed that castrated males and ovariectomized females do indeed have reduced sex steroid levels?

Minor comments:

- Some of the manuscript text was difficult to follow. Granted, I understand that adding all of these revisions has significantly expanded the scope of the manuscript, but I think taking some time to make the results text session as clear as the Introduction (which is very well written) would help improve clarity a lot.
- Fig. 2f-k: It would may make the results easier to interpret if the clone size fractions of males and females (basal vs. basal, suprabasal vs. suprabasal) were overlaid on top of each other, like what was done for Fig. 2a-d. This comment also applies to Fig. 3i, and Extended Data Figures 3, 4, 5, 6, and 14.
- Fig. 4a and Fig. 4c have typos: ‘Casrtated’
- Is the Voronoi model used in this work a cell-center (one point per cell) or a cell-vertex model (multiple points per cell)? The

mathematical descriptions imply that it's the former, but the text description make it sound like it's the latter.

- Fig. 5: Does the G2/M population more resemble the stem or TA population? This can be resolved by examining the expression of the marker genes shown in Fig. 5d in this cluster.
- Lines 1234–1239: technically differentially.
- The phrase 'RNA velocity' is more widely used than 'cell velocity' in the field.
- It would be nice to see the PCA plot of the bulk RNA-seq samples as described in the Methods.
- What are the cluster markers in Extended Fig. 11a?

Referee #4

(Remarks to the Author)

The authors have addressed my concerns and should be congratulated for their comprehensive study.

Version 2:

Reviewer comments:

Referee #3

(Remarks to the Author)

The authors have answered all comments thoroughly and carefully, clarifying several points of confusion, and have improved the work significantly from its initial submission. I am happy for the manuscript to be accepted for publication.

There are some final, minor comments:

- The axes of some plots across figures, e.g. like the ones shown in Fig. 1m, look strange with the '0, 5, 0', and '0.0, 0.3, 0.0' tiling. Maybe stagger 10/0 and 0.6/0.0, respectively, or add small double ticks.
- As it is hard to draw meaningful conclusions about tissue composition from scRNA-seq data, which is biased in cell capture ability across cell type, I would tone down the conclusions in lines 549-552 in the Discussion section.

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We thank the referees for their positive assessment of our paper as well as their comments and suggestions, which have helped us to improve our manuscript. In the light of these comments, we have performed multiple additional experiments that, together with the original findings, provide new mechanistic insights and important conceptual advances in the field of stem cell biology, and sexual dimorphism in epithelial tissues.

In our revised manuscript, we have addressed all the questions and suggestions made by the reviewers by performing many new experiments, highlighting implications of our findings during wound healing, as well as better explaining the novelty of the data and conceptual advance that our paper provides. We have performed many important experiments during the revision that have been included in the revised manuscript to address the following topics:

1. Direct validation of the proposed coordinated cell division and delamination using continuous live imaging.
 - We performed new in vivo lineage tracing at single cell resolution by imaging the same clone every 12 hours. This allowed us to trace the dynamics of individual cells and their interaction with other cells within the same clone.
2. Validation of cell states (SC-TA-Diff) uncovered by scRNA-seq using novel in vivo lineage tracing experiments.
 - To validate scRNA-seq predictions, we performed in vivo lineage tracing experiments using new genetically modified mouse models (Lgr6CreER-ControlMosaic and Notch1CreER-ControlMosaic mice).
 - We performed new bioinformatic analysis including cell velocity, regulon activity, GO term expression and cell-cell communication.
3. Genetic analysis of the role of sexual chromosome and specific sex hormones on epidermal cells.
 - We used a new mouse model, the Four Core genotype (FCG) mice, to study the role of chromosomal sex over gonadal sex on basal cell fate.
 - We performed hormonal replacement studies (dihydrotestosterone and estradiol) using hormonal implants to study the role of sex hormones on basal cell fate.
4. Assessing the human relevance of the role of sex hormones on keratinocyte differentiation and delamination.
 - We used 3D cell culture of primary human cells to study the impact of DHT on human basal cell proliferation, differentiation and delamination.
 - We used human epithelioids to study the transcriptional impact of DHT on human keratinocyte differentiation.
5. Assessing the impact of sexual dimorphism on wound healing
 - We assessed potential sex related differences in wound healing using macroscopic closure measurements, in vivo proliferation assays, and clonal dynamic analysis during wound healing in female and male mice.
6. Assessing the sexual dimorphism in epidermal stem cell dynamic in other skin regions
 - We performed lineage tracing and clonal analysis of IFE clones in mice back skin and tail skin and identified the same pattern as found in the ear skin.
7. Assessing the impact of sex hormones on dermal cells and hair follicle cycle

- We performed dermis measurements and hair follicle cycle assessment on ear skin following different hormonal treatments.
8. We clarified some wording and definitions, as we improved the model description and our analysis of other alternative models.
 9. We have corrected spelling mistakes and typos reported by reviewers.

Referee #1 (Remarks to the Author):

This manuscript by Engelman et al. presents an elegant and technically sophisticated analysis of epidermal homeostasis in the mouse ear interfollicular epidermis. By combining long-term intermittent intravital time-lapse imaging and clonal lineage tracing, the authors directly track the dynamics of individual labelled clones over extended periods. This approach enables the detection of transient, short-lived clones that were previously undetectable in fixed sample lineage analyses based on pooled data, thereby providing critical temporal resolution. The major strengths and advancements of this study are; 1) the direct observation of clonal extinction represents a significant technical and conceptual advance, allowing access to temporal dynamics beyond the reach of static lineage tracing; 2) the integration of short-lived clones into a hierarchical SC-TA model provides a compelling framework to reconcile both long-term clonal persistence and transient dynamics; 3) the study connects clonal fate patterns with sex hormones, providing insight into how systemic factors influence epidermal homeostasis. This is a high-quality and timely study that addresses an important question in epithelial stem cell biology using sophisticated tools. While the main conclusions are convincing, some key mechanistic claims, especially those regarding coordination between sister cell fates, rely more on inference than direct experimental evidence. Strengthening the logical and experimental support of these aspects would further improve the clarity and overall impact of the work.

We thank the reviewer for highlighting the significant technical and conceptual advances brought about by our work. In particular, the reviewer's statement on access to temporal dynamics and interrogation of short-lived clones provides a compelling framework to reconcile conflicting theories. We have now strengthened our proposed model by using repeated short-term imaging of single cell lineage tracing, to provide direct experimental evidence rather than inference of the coordination between sister cell fates, as requested. We have also used novel mouse lineage tracing experiments to link the cell state heterogeneity identified by single-cell sequencing with the clonal dynamic of these distinct cell populations in vivo.

Major concerns:

1. The model proposed by the authors relies heavily on the assumption of coordinated cell fate behavior—specifically, the temporal coupling of sister cell division and delamination within each clone for long period of time—to account for the observed enrichment of even-numbered clone sizes. However, the manuscript does not clearly specify what type of coordination is being assumed at the mechanistic or dynamical level (e.g., whether divisions and delaminations are synchronised in time, spatially coupled, or triggered by local feedback). Without such clarification, it is difficult to evaluate the plausibility or testability of the coordination-based explanation.

Moreover, while the authors cite previous work by Rompolas et al. (Science 2016) and Mesa et al. (Cell Stem Cell 2018), they do not clearly describe how the types of coordination observed in those studies relate to the mechanisms proposed in their own model. For example, they showed that delamination events can trigger division in neighboring cells, often with planar (horizontal) division orientations that serve to maintain tissue integrity. This raises the possibility that division and delamination may

occur in an alternating or compensatory manner, rather than in a synchronously coordinated way. The current manuscript does not provide direct experimental evidence—such as long-term single-cell/clone tracking or quantitative fate mapping—that sister cells divide or delaminate in a coordinated manner over time. If coordination is indeed central to the mechanistic interpretation, then empirical demonstration within the current system, following the standards of these foundational studies, is essential.

We thank the reviewer for raising these important questions. Taking their concerns in order, to account for the observed statistical features in the clonal data, the model demands the existence of temporal coordination between dividing and delaminating sister cells, a behaviour consistent with that reported in the study of Rompolas et al. (Science 2016). More precisely, within the modelling framework, sister SC and TA cell divisions were assumed to be fully synchronized, while sister differentiated cell delamination is considered as correlated with the degree of coordination treated as a fit parameter. A further fitting parameter is introduced for the synchronization of sisters produced by symmetric stem cell divisions ($SC \rightarrow 2SC$, $SC \rightarrow 2TA$). However, our model does not assume any coupling between the timing of cell division and delamination; but this cannot be ruled out from the present data, and may indeed prevail in some skin types, as reported in the study of Mesa et al. (Cell Stem Cell 2018). Moreover, the clonal data does not provide the resolution to allow us to determine whether there is local spatial coupling in the timing of loss and replacement events, as has been reported by Mesa et al. If such a coordination was imposed on the model dynamics, the quality of the fits to the clonal data would be unchanged.

To directly assess the coordination of sister cell division and delamination, as requested by the reviewer, we performed single cell tracking in mice ear skin and followed the fate of the sister cells by repeat imaging of the same area every 12h. This allowed us to track individual cells within a given clone for up to 5 days (Extended Data Fig. 7a,b). This temporal resolution allowed us to resolve lineage trees alongside clone sizes, from which we could determine the correlation between sister cell events. We assessed 69 clones (188 pairs of unique events where both sister fates were observed) in female mice and 60 clones (151 pairs of unique events) in male mice.

The results revealed a strong temporal correlation for all types of sister cell events, as measured by correlation coefficients between sister cell lifetimes. Here, a coefficient of 0 indicates no correlation and 1 indicates that all sister cell events happen during the same 12h window. For male mice, the sister lifetime correlation coefficient was 0.89 ± 0.03 (78 pairs of events) between delaminating sisters, 0.7 ± 0.15 (86 pairs of events) between dividing sisters and 0.75 ± 0.19 (24 pairs of events) between one sister dividing and one sister delaminating. For female mice, the sister lifetime correlation coefficient was 0.82 ± 0.14 (62 pairs of events) between delaminating sisters, 0.8 ± 0.12 (74 pairs of events) between dividing sisters and 0.3 ± 0.3 (15 pairs of events) between one sister dividing and one sister delaminating. Further, we frequently observed two delamination events within the 12hr time window within a clone. For male mice 84% (42/50) of these events were sisters, whereas for female mice 98% (45/46) of these double delamination events were sisters. Together, these findings provide evidence for the temporal correlation of sister cell events.

As further evidence for the tight temporal correlation between sister cell events (Extended Data Fig. 7c), we considered the frequency of events, starting from basal clone sizes of 2, 3 and 4 cells, aggregating clones across all time-points. From a basal clone size of 2 cells, we observed that the most common event was that both cells divide (~40% of clones experiencing such events). From a clone size of 3 cells, the most common event was a single cell division (~35% for M and ~50% for F). Finally, for a basal size of 4 cells, the most common event was that two cells delaminate (~50% of clones with events). This highlights once again that clones with an even number of basal cells stay coordinated, while clones with an odd number of basal cells tend to go back to evenness, reflecting both the coordination and symmetric (paired) nature of events.

These strong correlations support the predictions of our model concerning the tight coordination between dividing sisters and the coordination between delaminating sisters (and are discussed in full in Supplementary Theory). These new data are now included and discussed in the revised manuscript in lines 265-283.

2. Key terms such as “basal clone”, “suprabasal clone”, and “TA-like clone” are used throughout the manuscript, but their definitions and quantification methods are not clearly described in this paper. For instance, it is unclear whether suprabasal cells are considered part of the same clone as their basal counterparts or counted separately. Likewise, the use of clone size (≤ 3 cells) to define TA-like clones is operationally convenient, but it may not accurately reflect lineage origin or fate potential. Some stem cell-derived clones may remain small, while TA cells could produce more than three progeny. The authors should discuss the biological limitations of this size-based classification and how it might influence the interpretation of modelling results.

We acknowledge the importance of clarity and consistency in the terminology. In revision, we have sought to ensure that all definitions are made clear. Specifically, the term “basal clone size” and “suprabasal clone size” refer, respectively, to the number of basal and suprabasal cells within a clone of a given category. In each case they refer to only to the ensemble of “surviving” clones, which are defined as clones containing at least one basal cell (unless otherwise explicitly stated in the caption). We note that suprabasal cells were scored only when they were part of a clone containing at least one basal cell. The only exception was at the first time point, where many labelled cells were only present in the suprabasal layer (termed suprabasal-only clones). These events did not impact on the modeling analysis. We note that clone persistence was scored based on the abundance of surviving clones.

To assess the validity of the model, it was convenient to group clones according to the maximum size they achieve over the time course. For this purpose, we assigned clones that achieve a maximum size of 4 cells or more as “SC-like”, clones that expand to a maximum of 2 or 3 cells were termed “TA-like”, and the remaining single-cell clones as “D-like” clones. This denotation does not *impose* an identity per se; but enables us to assess finer-grained features of the data more directly than pooling all clones together. Although D-like clones will be associated predominantly with the induction of differentiated (post-mitotic cells) basal cells, it is possible that “TA-like” clones could be derived from SCs that have a lower proliferative capacity than the model would predict, or that “SC-like” clones could be derived from TA-cells that showed higher proliferative capacity. Operationally, to compare the model and data, we treat both data sets in the

same manner (i.e., we classify based on observed cell numbers at the experimental time-points, without imposing a particular identity on the induced cell). In this way, the comparison between model and data is consistent, irrespective of the choice of model, and the fate potential of the cell type in question. For the model fit, we find that these categories map well onto the cell types of the model.

The key biological limitation of the classification is that, because of the limited temporal resolution, some clone types may be misassigned (where, e.g., a clone is able to both expand and contract between two consecutive time-points).

These considerations have now been more carefully discussed in the revised manuscript in the mathematical modeling section.

3. The manuscript integrates clonal analysis, scRNA-seq, and mathematical modelling to support a hierarchical SC-TA-D framework. However, it remains unclear how consistently the terms “SC”, “TA”, and “D” are defined and interpreted across these platforms. For example, do SC-like clones identified via fate-mapping correspond to transcriptionally defined stem cell states in scRNA-seq? Are TA-like clusters associated with short-lived or extinct clones? Clarifying whether these definitions are intended to align or diverge is intentional would improve the conceptual coherence of the model.

We thank the reviewer for raising this important question. To address the reviewer’s comment and link clonal fate mapping, modelling and scRNA-seq, we performed lineage tracing experiments using different CREER mice models based on putative marker genes enriched in SC and TA clusters (Fig. 5d). We used *Lgr6*CreER (Snippert et al., 2010) to target preferentially the SC cluster and *Notch1*CreER (Fre et al., 2011) to target the TA and Diff clusters. As fate decisions and clonal loss in TA and Differentiated cells can arise early, we performed the first imaging time point at D3 instead of D7, as some early events in the *Notch1*CreER lineage would already have been lost at D7. Specifically, to test the prediction that the basal cell layer hosts two distinct, proliferative populations, SCs and TA cells, we imaged from D3 to D28 in mice ear skin using *Notch1*CreER-ControlMosaic and *Lgr6*CreER-ControlMosaic.

By first combining clonal data from all animals, irrespective of sex, we found that the clonal persistence – the probability that a clone retains at least one basal cell – was dramatically different between models, with $44.6 \pm 4.0\%$ of clones persisting for *Lgr6*CreER at D28 (Fig. 5j) compared to $10.0 \pm 0.3\%$ for *Notch1*CreER (Fig. 5k). This major difference already highlighted that using different Cre, targeting the cells-enriched SC marker gene *Lgr6* and the TA (and Diff)-cell enriched marker gene *Notch1*, label functionally discrete basal populations. To further assess whether these targeted cells were rooted in self-renewing populations, we looked at the change in average labelled cell number – equivalent to the mean number of cells per clone including clones of size “zero”, the latter representing those that have become lost through differentiation (Fig. 5l,m). Notably, an early drop in labelled cell number in the *Notch1*-targeted population indicated that initially labelled proliferative cells is not enriched in cells with self-renewal potential, in line with a putative TA cell population (Fig. 5m). In the longer term, the labelled cell density then stabilized, most likely due to a residual labelling of SCs by the *Notch1*CreER. (However, although not included explicitly in the model, we cannot rule out the possibility that TA cells may revert back to a SC identity, albeit at some low frequency.) By contrast, the *Lgr6* population showed an early increase in average labelled cell

number, followed by rapid convergence to a constant value, highlighting the self-renewal potential of this population, in line with a putative SC identity (Fig. 5l).

The mean clone size and clonal persistence of Lgr6CreER targeted cells were similar between male and female (Extended Data Fig. 13e). However, clones from Notch1CreER female animals expanded more than male animals (5.1 ± 0.4 vs 3.1 ± 0.3 cells at D28) (Extended Data Fig. 13f) and the labelled cell density was higher in female mice compared to male (Extended Data Fig. 13j).

In Notch1CreER tracing, $75.5 \pm 1.0\%$ of clones showed no cell division over the time course, while in Lgr6CreER tracing non-dividing clones represented just $18.7 \pm 5.1\%$ of cases (Fig. 5n). This highlights that Notch1CreER labelled cells comprising both terminally differentiated cells (Diff) and cells able to divide (potentially TA and some SC). By focusing on clones that did divide, the frequency of clones that stayed within the maximum 3-cell threshold (putative TA cells) was around $62.1 \pm 3.7\%$ in Notch1CreER and $38.6 \pm 5.7\%$ in Lgr6CreER (Fig. 5o), consistent with the preferential labeling of TA cells by Notch1CreER compared to Lgr6CreER. The fraction of dividing clones that was lost through differentiation was similar with $6.8 \pm 2.0\%$ for Notch1CreER and $8.9 \pm 1.6\%$ for Lgr6CreER (Fig. 5o). The proportion of clones that kept expanding at D28 was $52.5 \pm 4.7\%$ in Lgr6CreER versus only $32.2 \pm 4.4\%$ in Notch1CreER (Fig. 5o), providing further evidence for the preferential labeling of SC by Lgr6CreER compared to Notch1CreER.

To assess the degree of differentiation of targeted cells and their immediate progeny, we assessed Krt10 expression in basal targeted cells at D3 following TAM administration. The fraction of basal labeled cells expressing Krt10 was $7.7 \pm 2.9\%$ in male Lgr6CreER targeted cells and $9.24 \pm 1.0\%$ in female Lgr6CreER targeted cells (Extended Data Fig. 13k-m). For Notch1CreER, the proportion was $38.2 \pm 4.5\%$ in male and $55.3 \pm 3.8\%$ in female animals (Extended Data Fig. 13m). Regarding the composition of clones at D3, the fraction of suprabasal cells over total labelled cells was 0% in male Lgr6CreER and $1.4 \pm 1.4\%$ in female Lgr6CreER animals, compared to $27.4 \pm 4.4\%$ in male Notch1CreER and $21.3 \pm 1.4\%$ in female Notch1CreER animals (Extended Data Fig. 13n). These results were consistent with the faster delamination of terminally differentiated cells in males, and a higher content of cells committed to differentiation (basal cells expressing Krt10+) on the basal layer of female mice.

Computational modelling of the Notch1CreER and Lgr6CreER lineage tracing showed that the two proliferative-compartment model captures well the dynamics of each population (Extended Data Fig. 14). For the Lgr6 population, we found that 50% of clones from males and 52% of clones from females were SC-like, relative to 41% for K14 Male, 50% K14 Female, 10% for Notch1 Male and 12% for Notch1 female. This indicates that the Lgr6 promoter captures SCs in similar to proportion to K14, but the Notch1 captures only a small number of SCs. For both Notch1 and Lgr6, we found that the model could accurately capture the average size and distribution of SC-like clones (Extended Data Fig. 14a-d). Further, the model captured reasonably the dynamics of TA-like and D-like clones with the exception of the persistence of TA-like clones (Extended Data Fig. 14e-l). In terms of parameters, the homeostatic composition of SC/TA/D is comparable by sex to that found for the K14 promoter for both Lgr6 and Notch1 (Extended Data Fig. 15).

Altogether, these results support the existence of two proliferative basal cell compartments, with SCs preferentially targeted by the Lgr6CreER and TA and differentiated cells preferentially targeted by the Notch1CreER.

These results have been added to the revised manuscript in the molecular characterization section.

4. In Fig. S6e, RNA velocity vectors appear to point from TA-like to SC-like cells, which contradicts the proposed irreversible SC→TA hierarchy. This apparent inconsistency must be directly addressed. Is this a technical artifact or a biological suggestion of reversibility? Leaving this unexamined undermines confidence in the model's directionality.

We thank the reviewer for raising this important point. We carefully re-examined the RNA velocity results and performed complementary analyses to clarify the apparent vector orientation.

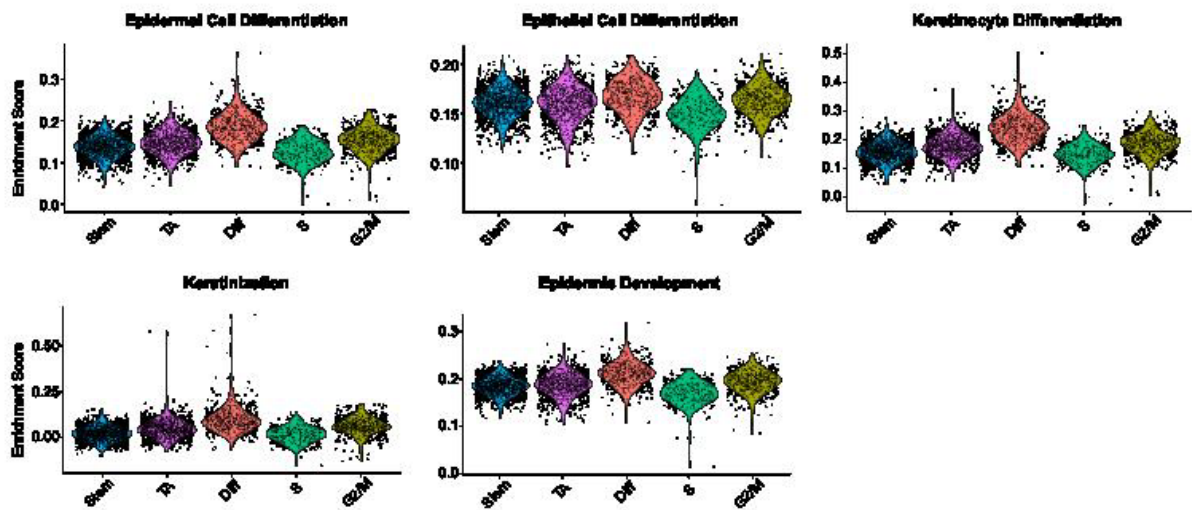
RNA velocity is known to be sensitive to cell cycle-related transcriptional dynamics, which can sometimes produce “backward-pointing” vectors in rapidly cycling populations, even in the absence of biological reversibility (Bergen et al., 2020). Indeed, our TA cell compartment is highly proliferative, and this likely contributes to apparent vector noise toward SCs.

Moreover, the stream plot of velocity (old Extended Fig. 6e now Extended Fig. 12a-c) tries to integrate cell-wise velocities over long distances for simplification. If the velocity vector length is minimal for a given cell state, the streamlines passing through that region may not reliably reflect the true dynamics. We checked the original velocity embeddings before streamlining, as well as the quantitative speed (velocity length) and acceleration values for better interpretation (Extended Fig. 12a-c). The zoomed-in view of the cell-wise velocity embedding showed that both stem and TA cells could enter cell cycle and subsequently exit cycle. Upon exiting cycle, TA cells may either re-enter the cycle or commit to differentiation. The violin plots revealed that transcriptomic speed and acceleration are the smallest in SCs. They are significantly increased in TA cells and dramatically increased in Diff cells. These observations suggest that SCs have the most conservative expression pattern and tightly preserve their identity, whereas TA cells undergo rapid remodeling of their transcriptomic profiles to shift identity. Diff cells, in contrast, exhibit the fastest changes in expression and establish a stable commitment. Together, the cell-wise velocity embedding and the changes in speed and acceleration further support a stepwise differentiation trajectory from SC to TA to Diff.

We also performed TF analysis to compare the regulatory programmes across cell states. SCENIC detected a total of 171 regulons – TFs together with their direct target genes – from the scRNA-seq data and scored each regulon's activity (using AUCell) in individual cells. We used t.test to compare the activity of regulons between cell states (Fig. 5e-g and Extended Dat Fig. 12d-i). Notably, activities of the “stemness-associated” Trp73 regulon and the “differentiation-associated” Klf4 regulon exhibited opposite trends. Trp73 regulon activity was enriched in SCs and progressively declined along the differentiation trajectory. Meanwhile, Klf4 regulon activity was minimal in SCs, significantly elevated in TA cells, and peaked in differentiated cells. The observation that the “stemness-associated” regulatory programme declines while the “differentiation-associated”

programme markedly increases in TA cells is consistent with a stepwise SC→TA→Diff differentiation trajectory.

To assess whether TA cells might have SC-like characteristics consistent with de-differentiation, we additionally performed GO term enrichment analysis across SC, TA and differentiated (Diff) populations using per-cell module scoring. Across epidermal/keratinocyte differentiation terms (epidermal cell differentiation, epithelial cell differentiation, keratinocyte differentiation, keratinization, epidermis development and epithelium development), enrichment scores increased from SC → TA → Diff, with the highest scores observed in Diff cells. TA cell populations already showed elevated enrichment for fate-specification and differentiation-regulatory programmes, whereas SCs consistently displayed low scores, indicating absence of differentiation signatures. These enrichment score distributions provide orthogonal, cell-level evidence for a unidirectional SC → TA → Diff trajectory and argue against a strong contribution from TA → SC dedifferentiation.



It is therefore likely that the occasional TA → SC arrows observed in RNA velocity analyses (presented in the original Extended Data Fig. 6e) represent technical artifacts of velocity inference in the proliferative population introduced during streamline integration and long-distance smoothing, rather than evidence of true biological reversibility. In the revised manuscript, we have replaced the stream plot with cell-wise velocity vectors together with speed/acceleration plots (Extended Data Fig. 12a-c).

5. If SC clones generate TA cells as a key feature, the rate or proportion of such transitions should be quantified. The manuscript does not provide estimates of how often SCs generate TA progeny or how many TA cells arise per SC division. Without these data, it is difficult to assess the quantitative validity of the two-compartment model.

We thank the reviewer for this question. These details are presented in the main text figures, with the rates and fate outcomes presented in the original *Table 1* (now in Extended Data Fig. 6g) alongside the other parameters of the model. The rate of TA cell divisions is the same as SC divisions, which is now emphasized in the caption of *Figure 2* and in *Extended Data Fig. 6g*. The remaining technical details of the model and its implementation are provided in depth in the *Supplemental Theory*.

6. The observed sex-specific differences in epidermal turnover are interpreted primarily as a consequence of hormonal influences directly to epidermal gene expression. However, other factors, such as differences in dermal thickness, hair follicle cycling, could also contribute to these patterns. A consideration of these potential confounders would help strengthen the causal interpretation of the sex-related findings. For example, do you see dermal thickness reduction, gene expression changes and hair cycle progression differences in castration mice?

We thank the reviewer for this important question. Previous papers have reported differences of dermal thickness between male and female animals (Azzi et al., 2005). In dorsal skin of adult mice, males have thicker dermis than females. In the same study, castration (with or without hormonal replacement) did not significantly modify male dermal thickness. To address the reviewer's question, we measured dermal thickness in the ear skin of control male and female mice, together with castrated mice with or without hormone replacement (DHT for male, E2 for female). In line with back skin data, male ear dermis was significantly thicker than female ($53.9 \pm 3.5 \mu\text{m}$ vs. $28.2 \pm 4.0 \mu\text{m}$) (Extended Data Fig. 16a,b). However, the castration of male mice did not significantly modify dermal thickness ($53.9 \pm 3.5 \mu\text{m}$ vs. $49.9 \pm 3.9 \mu\text{m}$) (Extended Data Fig. 16b). Only exogenous DHT replacement in castrated males significantly increased dermal thickness compared to castrated males ($65.7 \pm 2.8 \mu\text{m}$ vs. $49.9 \pm 3.9 \mu\text{m}$) but not compared to control males ($65.7 \pm 2.8 \mu\text{m}$ vs. $53.9 \pm 3.5 \mu\text{m}$) (Extended Data Fig. 16b). Therefore, as castrated males did not present significant difference compared to control males, it is unlikely that the differences observed in the epidermal dynamic arise from modification of dermal thickness. We have added and discussed these data in our revised manuscript.

Concerning the hair follicle (HF) cycle, it should be noted that HF have a very specific behaviour in the ear skin compared to other regions of the mouse skin. Indeed, as highlighted below, in contrast to HFs in mouse back skin that undergo hair cycle, the HF in the ear skin only performs one initial cycle (known as morphogenesis: anagen-catagen-telogen) and stay in telogen for extend period and are then considered afterwards as refractory to cycling (Wang et al., 2017). Few solitary anagen HFs could be observed, but the vast majority of HFs stay in the resting, telogen phase. To verify that castration did not interfere with this established, global telogen, we performed HF cycle assessment in both control and castrated males. Two weeks after castration, males had $96.7 \pm 3.3\%$ of HF in telogen phase, while control males presented $96.0 \pm 2.0\%$ (Extended Data Fig. 16d). Therefore, as castrated males did not present significant differences compared to control males, it is unlikely that the differences observed in the presented data arise from modification of the HF cycle. We have added and discussed this data in our revised manuscript.

[REDACTED]

Source: Figure 5 in Wang et al., 2017, eLife

Minor comment:

7. The authors propose a two-compartment SC–TA model as a biologically more accurate alternative to the one SC pool model. While this framework is compelling, the manuscript would benefit from a more explicit discussion of which specific observed phenomena cannot be adequately explained by the one SC pool model and why. For instance, are the biphasic clone persistence patterns, high frequency of clone extinction, or even-number clone bias incompatible with predictions of the SC pool model under reasonable parameter settings? Clarifying this point would help to justify the necessity of introducing a distinct TA compartment rather than relying on continuous fate transitions within a single stem cell population.

We thank the reviewer for this question. The failure of a one progenitor model to account for the clonal dynamic rests on two key pieces of experimental evidence linked especially to female animals, which cannot be captured simultaneously. Specifically, in common with other clonal fate studies of squamous tissues, the average surviving basal clone size shows a fast initial increase followed by slowed linear-like growth (Extended Data Figure 3c,g). For models based on population asymmetry of the SC compartment, such behaviour can only be explained by imposing a high propensity for SC divisions leading to asymmetric fate outcome. Such dynamics ensures that the fast initial increase in clone size, which correlates with the cell division rate, crosses over to a reduced effective SC loss/replacement rate, with asymmetric divisions leaving the SC clone size unchanged. By contrast, to recapitulate the observed strong enhancement in even clone sizes in the basal and suprabasal layer with the framework of a single progenitor model, we require

that SC divisions lead predominantly to symmetric fate outcome (duplication or terminal division) with strong sister cell coordination, enabling clone sizes to jump in units of 2 in the basal and suprabasal layer (Extended Data Figure 5j-l).

Moreover, additional statistical measures based on the classification of SC-like (clones which grow to 2 or more cells), and D-like (clones which are only ever one cell) present incompatibilities between the one progenitor model and experimental data. For both male and female mice, the average basal clone size from the best fit values is overestimated at the longest time-points (Extended Data Figure 4c,g) for both male and female mice, and the clonal persistence for male animals is underestimated. These discrepancies reflect the tension between the fine-grained features of clone-size distributions, which require a larger proportion of symmetric divisions, and the average basal clone size, which requires a large proportion of asymmetric divisions. A detailed explanation and examples of each situation is provided in the *Supplemental Theory* (Section: Fits of the SD-model to the time-lapse clonal data).

In revision, we have incorporated a few clarifying statements in the main text in lines 213-219, while a full description is presented in the *Supplemental Theory*.

Referee #2 (Remarks to the Author):

In this work, Engelman and colleagues show for the first time that epidermal stem cell dynamics that ensure homeostasis are under hormonal control thus showing sexual dimorphism. Sex-dependent differences in physiology might underlie differences in the incidence and level of many pathologies. However, the mechanistic reasons that explain these differences in homeostasis and disease are almost completely unknown. In this manuscript, the authors study this issue in the epidermis, where clear sexual dimorphism is known to occur regarding essential aspect of the function of the human skin, such as skin immunity, skin aging, and the prevalence and progression of tumors, mainly melanoma and basal cell carcinoma.

The two main labs conducting this elegant work were at the forefront of their fields in understanding the importance of stem cell dynamics regarding skin physiology. Both groups developed the in vivo models and mathematical tools which were used later by many others to almost open a new field within the stem cell research field. They now also include beautiful intravital microscopy to obtain very robust data. As such, I do not have any comments regarding the data presented.

However, I think the authors have a wonderful opportunity to expand this current work to try to understand whether the sex-dependent differences they have observed regarding tissue-level stem cell dynamics, underlie some of the physiological and disease differences seen in human skin at the clinical level. Recently, Chi L et al (2024) showed sexual dimorphisms in skin innate immunity. In addition, the group of Ashani T. Weeraratna has shown that male dermal fibroblasts age faster than female dermal fibroblasts. Very interestingly, age-dependent sex difference patterns are observed in melanoma and BCC in humans. Specifically, melanoma and BCC show higher incidence in young women and old men, with a switching age around menopause. This switching age suggests involvement of sex hormones, but other potential contributors to this sexual dimorphism could be a direct consequence of the well-characterized sexual dimorphism in skin color in humans (males showing a darker and redder skin than females). However, in none of these previous works they took the extra step to relate these sex-dependent differences to specific physiological aspects with clinical importance. Thus, the results in this ms could constitute a beautiful working frame to test whether the sexual dimorphism they have identified in epidermal stem cell behavior may be a contributing factor to the differences in BCC incidence between males and females. Another physiological example of sex dimorphism relates to wound-healing in that human male skin heals slower than female skin upon acute wound healing.

I think if the authors could show that these hormone-dependent sexual dimorphism are at the root, or contribute in any way, to the differences in BCC development, or rate of wound healing, then this would be a hallmark paper in the field that underscores the relevance of sexual differences regarding health and disease.

We thank the reviewer for the positive assessment of our work. We agree with the reviewer that expanding our work to wound healing could enhance its impact.

As suggested by reviewer, we have investigated the potential sexual dimorphism of mouse skin epidermal SC dynamic during wound healing.

Evidence for sex related differences in wound healing has been reported previously. Topical estrogen improves skin wound healing (Ashcroft et al., 1999) whereas androgen slows wound healing (Ashcroft and Mills, 2002). Most of these effects have been attributed to the ECM and inflammatory response. However, some data indicated that keratinocytes could be involved in this dimorphism, as the deletion of ER-beta in the epidermis phenocopy the delay in wound healing observed in full ER-beta KO mice (Campbell et al., 2010). However, little is known about the cellular mechanisms underlying the sexual dimorphism in wound healing.

To better characterize sexual dimorphism during wound healing, we first investigated the kinetic of wound repair between male and female animals. We performed a punch biopsy on the back skin of both male and female mice. Wound area was measured on the same mice at D0, 2, 4, 7, 9 and 12 with wounds of both sexes until wound closure around D12. Notably, female mice were found to heal significantly faster than male mice (Fig. 3a-c).

To decipher if this dimorphism was the consequence of a different response of the keratinocytes, we assessed proliferation using EdU and clonal dynamic using lineage tracing. As we and others have previously described, the keratinocyte response to wound healing is divided in two areas (Aragona et al., 2017; Park et al., 2017). There is a non-proliferative ring, consisting of migratory cells (the leading edge), and a proliferative ring (proliferative hub), where cells actively divide to mediate wound closure (Fig. 3d). These two distinct compartments can be observed following an EdU pulse that labels proliferative cells.

To assess the difference in proliferation between male and female animals, we performed a punch biopsy on the back skin of both male and female mice and performed a 4h EdU pulse to study the establishment of the proliferative hub at D2 and D4 post wound from epidermal whole mounts. At the initial stage, D2, there was more proliferative cells (4330 ± 320 EdU+cells/mm² vs. 2860 ± 330 EdU+ cells/mm²) and the ring of proliferative cells was larger (800 ± 37 μ m vs. 588 ± 63 μ m) in female mice compared to males (Fig. 3e-f). This difference of epidermal proliferation observed at D2 vanished at D4.

To assess the impact of this difference in proliferation on the clonal dynamic of epidermal cells during wound healing, we performed lineage tracing of basal cells. K14CreER-ControlMosaic mice were induced at clonal dose, and a wound was performed at D7 post induction. Mice were sacrificed at D2 or D4 post wound. Clone size was measured from epidermal whole mounts inside the proliferative ring (Fig. 3). At D2, female basal clone size was significantly greater than male clones (4.7 ± 0.2 cells vs. 3.0 ± 0.1 cells). This difference was still present but of a lower magnitude at D4 post wound (Fig. 3i). Of note, in homeostasis clone size did not differ between male and female animals at 28 days post induction (Extended Data Fig. 4e). Finally, when looking at the clone size distribution, it appeared that at D4 post wound, female mice presented a notable increase in clones composed of even numbers of basal cells compared to male mice that presented a smoother, exponential-like distribution (Fig. 3i). These results highlighting that wound healing leads to the early appearance of a high degree of cell coordination in female animals, but not in males.

Altogether, these data nicely illustrate the consequences of the sexual dimorphism of epidermal stem cell dynamic during wound healing. Female mice have a faster rate of

wound healing due to more rapid SC activation in the initial stage of wound healing and a higher degree of cell coordination.

Referee #3 (Remarks to the Author):

In this work, the authors performed intravital timelapse imaging to track the long-term fate of individual lineage-labelled cells in the ear skin interfollicular epidermis, and observed enhancement of even basal clone sizes, especially in female mice. Then, the authors proposed a mathematical model with two-proliferative compartment to explain and reproduce the observations. A key discovery is a sex-specific difference in this process: female mice exhibit more synchronized patterns of cell division, differentiation and delamination. Single-cell and bulk RNA-sequencing and clonal analysis following castration revealed that male sex hormones appear to promote stem cell differentiation and delamination.

Overall, the study presented an interesting observation of even basal clone size enhancement and the sex-specific differences, proposing a stem cell lineage model to predict potential mechanisms. However, several key weaknesses limit its current impact. Most critically, the study lacks experimental validation of core model predictions, such as synchronized behaviors in female mice. Furthermore, the connection between molecular mechanisms and the kinetic parameters used in modeling remains insufficiently developed. Additionally, contradictory transcriptomic interpretations and limited exploration of immune-related signaling raise concerns about consistency and depth. Taken together, these issues must be addressed to solidify the conclusions and improve the overall rigor.

We thank the reviewer for the positive assessment of our manuscript study and for the interesting questions and suggestions.

As suggested by the reviewer as well as reviewer#1, we have now strengthened our proposed model by using repeated imaging every 12 hours to assess the coordination of cell division and differentiation in male and female, to provide direct experimental evidence of the coordination between sister cell fates. We have also used novel mouse lineage tracing experiments using distinct CREER mice to link the cell state heterogeneity identified by single-cell sequencing with the clonal dynamic of these distinct cell populations in vivo. We better described the impact of male sex hormones and sex chromosomes using new mouse models. We have improved our bioinformatic analysis of both scRNA-seq and bulkRNA-seq. As suggested, we looked for potential insight in regard of cell-cell communication. The model description, alternative hypothesis and technical description have been improved.

My comments are as follows:

Major comments:

1. A key prediction by the two-proliferative compartment model is that female mice exhibit more synchronized patterns of cell division, differentiation and delamination. Is there any experimental evidence for the prediction? Is it possible to observe/measure the synchronizations in experiment? If so, it would validate the prediction and enhance the conclusion of this study.

We thank the reviewer for raising this important question. To directly assess the coordination of sister cell division and the delamination of differentiated sister cells, as suggested by the reviewer and by reviewer#1, we performed single cell tracking in mice

ear skin, following the fate of the sister cells by repeat imaging of the same area every 12h, tracking individual cells within a given clone for up to 5 days (Extended Data Fig. 7a). This temporal resolution allowed us to resolve lineage trees, alongside clone sizes, from which we could determine the correlation between sister cell events. We assessed 69 clones (188 pairs of unique events where both sister cell fates were observed) in female mice and 60 clones (151 pairs of unique events) in male mice.

The results revealed a strong temporal correlation for all types of sister events, as measured by correlation coefficients between sister lifetimes. For male mice, the sister life-time correlation coefficient was 0.89 ± 0.03 between delaminating sisters, 0.7 ± 0.15 between dividing sisters and 0.75 ± 0.18 between one sister dividing and one sister delaminating. For female mice, the sister life-time correlation coefficient was 0.82 ± 0.14 between delaminating sisters, 0.8 ± 0.12 between dividing sisters and 0.3 ± 0.3 between one sister dividing and one sister delaminating. Further, we frequently observed two delamination events within a clone. For male mice 84% (42/50) of these events were sisters, whereas for female mice 98% (45/46) of these double delamination events were sisters. The results confirm the prediction of strong synchronization between female and male events, but the numerical differences between male and female mice is not significantly different at the present sample size.

As further evidence for the tight temporal correlation between sister cell events (Extended Data Fig. 7c), we considered the frequency of events, starting from basal clone sizes of 2, 3 and 4 cells, aggregating clones across all time-points. From a basal clone size of 2 cells, we observed that the most common event was that both cells divide ($\sim 40\%$ of clones experiencing such events). From a clone size of 3 cells, the most common event was a single cell division ($\sim 35\%$ for M and $\sim 50\%$ for F). Finally, for a basal size of 4 cells, the most common event was that two cells delaminate ($\sim 50\%$ of clones with events). This highlights once again that clones with an even number of basal cells stay coordinated, while clones with an odd number of basal cells tend to go back to evenness, reflecting both the coordination and symmetric (paired) nature of events.

These strong correlations support the predictions of our modelling about the tight coordination between dividing sisters and coordination between delaminating sisters (see Supplementary Theory).

Beyond synchronization, our model predicted that male mice have a greater degree of asymmetric fate outcomes following in TA cell division, which breaks the even-odd correlation. To assess this, we quantified cell fate decisions. The current setting of 5 days imaging was too short to assess SC fate decisions (which would require multiple weeks of daily imaging). We therefore focused on TA cell fate decisions. We classified the putative TA compartment as cells where at least 1 sister delaminates. We found that both sisters delaminated more frequently in female animals at 57/91 ($63 \pm 5\%$) compared to male 46/93 ($49 \pm 5\%$). In the case of one sister delaminating and the other dividing, the remaining cell performed a terminal division more frequently as well in female animals 6/32 ($19 \pm 7\%$) than in male 6/40 ($15 \pm 7\%$). In other cases, we observed that the dividing daughter went through multiple rounds of division ($20/40 = 50\%$ for males and $16/32 = 50\%$ for females) or the fate of the dividing cell's daughters were undetermined ($14/40 = 35\%$ for males and $10/32 = 31\%$ for females). Further, due to the short time window, we observed a proportion of clones where a cell delaminates, and the other cell has an undetermined

fate (7/93 for males and 2/91 for females). These numbers were qualitatively consistent with our fitting of the SC/TA model to long-term data, where male TA cells have a greater capacity for asymmetric divisions.

In our long-term modelling, we assumed that for TA cell asymmetric divisions, the fate timing of the TA and D cell sisters are uncoordinated, a simplifying assumption that is challenged by the findings discussed in the paragraph above. However, if included, such correlations would be indistinguishable at the level of modelling in the long-term data. Moreover, we assumed that TA cells may only asymmetrically divide once, a further simplifying assumption. However, at the predicted frequency of asymmetric divisions, a model in which cells have the capacity to undergo more than one round of division would be indistinguishable based on the long-term data.

In conclusion, these results demonstrate the presence of coordination between dividing sisters and coordination between delaminating sisters, as predicted by the model. Temporal correlation between sister divisions was higher in female compared to male animals (0.8 ± 0.12 for females vs. 0.7 ± 0.15 for males), in line with our predictions. However, the difference does not reach above statistical significance. Moreover, there is a strong correlation between the lifetimes of pairs of delaminating sisters (0.89 ± 0.03 for males vs. 0.82 ± 0.14 for females). Finally, events that could be assumed as arising from an asymmetric division, where one sister divides and one delaminates, seem to occur more frequently in male mice than female mice (0.75 ± 0.18 vs. 0.3 ± 0.3 , with the 95% CI overlapping). These new data are now included and discussed in the revised manuscript in lines 265-283.

2. Similarly, experimentally validating the prediction that “male sex hormones had an impact on the basal cell delamination rate and on the relative frequency of asymmetric TA cell divisions” would strengthen this study.

We thank the reviewer for this important comment. In line with the reviewer’s comment and reviewer 4’s suggestion we turned to the Four Core Genotype (FCG) mice model. This model involves the deletion of the testis-determining gene Sry from the Y chromosome and introducing an autosomal Sry transgene (commonly on chromosome 3). Therefore, gonadal sex is determined by the presence of the transgene rather than by the Y chromosome itself. These mice present the following phenotypes: XX and XY mice with ovaries (Sry-), and XX and XY mice with testes (Sry+).

To study the basal cell delamination rate and the relative frequency of asymmetric TA cell division, we performed similar experiments as in the original manuscript by tracking EdU doublets after 96h of chase. Sry expressing individuals, irrespective of the chromosomal status, had a significantly increased delamination rate ($17.0 \pm 1.8\%$ for Sry+ vs. $7.7 \pm 1.3\%$ for Sry-) (Fig. 4d). When compared, XX and XY individuals did not present any statistically significant differences in terms of delamination, irrespective of Sry status ($13.5 \pm 2.0\%$ for XX vs. $11.2 \pm 3.0\%$ for XY). In terms of division fate outcome, Sry expressing individuals, irrespective of the chromosomal status, had a significantly increased rate of asymmetric division (K14+K10+ EdU doublets, $59.8 \pm 3.3\%$ for Sry+ vs. $42.2 \pm 4.1\%$ for Sry-) (Fig. 4e). When compared, XX and XY individuals did not present any statistically significant differences in term of division outcome, irrespective of Sry status ($53.0 \pm 6.6\%$ for XX vs. $49.0 \pm 3.7\%$ for XY).

These results show that the Sry gene, and by extension male sex hormones, are driving the increased delamination rate and fraction of asymmetric cell divisions.

To further study the role of male sex hormones, we performed hormone replacement using dihydro-testosterone (DHT), the biologically active form of testosterone after castration. Male mice underwent castration and were then treated with DHT implant (Belma, DHT-M, 40 µg/24h). Uncastrated male mice were used as controls. Two weeks post-surgery, we performed EdU pulse chase experiments to assess the fate of EdU doublets after 96h of chase. Compared to castrated males without implant, castrated males with DHT implants presented a statistically significant increase in basal cell delamination at 96h ($10.3 \pm 1.5\%$ for castrated vs. $31.4 \pm 4.4\%$ for DHT) (Fig. 4f). DHT implants allowed to rescue the higher level of delamination than seen in control males ($31.4 \pm 4.4\%$ for DHT vs. $21.4 \pm 2.8\%$ for control males) (Fig. 4f). In terms of division fate outcome, when compared with castrated, DHT males had a significantly increased rate of asymmetric division (K14+K10+ EdU doublets, $41.2 \pm 3.7\%$ for castrated vs. $58.4 \pm 3.3\%$ for DHT) (Fig. 4g) as well as more K10+K10+ EdU doublets in DHT: $1.62 \pm 0.56\%$ for castrated vs. $7.14 \pm 1.53\%$ for DHT vs. $4.6 \pm 1.7\%$ for control males. DHT and control males achieved similar level of asymmetric division ($58.4 \pm 3.3\%$ for DHT vs. $62.8 \pm 3.1\%$ for control males) (Fig. 4g).

Altogether, these results indicate that the increased basal cell delamination rate and the relative frequency of asymmetric divisions is mediated by male sex hormones and are not mediated by other genes presented in the Y chromosome. We demonstrated that testosterone (DHT) can rescue the decreased basal cell delamination rate and the relative frequency of asymmetric divisions present upon castration, showing that testosterone is a key regulator of basal cell delamination and asymmetric division. We have presented and discussed these new data in the revised manuscript.

3. While the authors show that one proposed mathematical model, the two-compartment model, generates dynamics that are more consistent with the observed experimental data, there is no reason that there could not be other mechanisms that could give rise to the same dynamics. It would be worth proposing alternative models in the Discussion and how one may “discriminate” between these models.

The reviewer raises an important question. When developing the model, we considered the simplest dynamic (compromising the fewest discrete cell types and rate parameters, etc.) that could account quantitatively for all the observed features of the clonal data including the full temporal basal and suprabasal clone size distributions and clone survival probabilities. Simpler models – based on a one progenitor paradigm – were quantitatively ruled out as not being compatible with all features of the data (Supplemental Theory). However, by the nature of a modelling-based approach, it is not possible to rule out the validity of more complex models, comprising additional channels of cell fate or intermediate cell states. For example, when TA cell division leads to asymmetric fate outcome, short-term live-imaging suggests that the timing of the division of the dividing TA sister is correlated with the delamination of the differentiated sister (as mentioned above). Such temporal correlations were not incorporated into the model, yet their inclusion would not significantly improve the quality of the fits. Moreover, models with a more fine-grained stem cell compartment (such as one in which cells transition reversibly between multiple SC states) could also rapidly converge to the same effective

dynamics provided there is a strong temporal coordination in the fate behaviour and dynamics of sister cells, the final division before delamination is mostly symmetric (i.e., TA cells mostly terminally divide), and there is on average a bias of the SC pool towards asymmetric fate outcome.

Acknowledging the suggestion of the reviewer, we have sought to explain better the strengths and potential limitations of the proposed model in the Discussion section of the paper, with a more detailed discussion provided at the end of the Supplemental Theory.

4. While the authors proposed a mathematical model to explain the sex-specific difference and performed scRNA-seq and bulk RNA-seq to characterize the transcriptomic difference, there is a gap between the differences in molecule expressions and different kinetic parameters defined in the proposed mathematical model. The rigor of this study would be improved by explicitly establishing the link from molecular characterizations to kinetic parameters, and ultimately to clonal dynamics.

We thank the reviewer for raising this important question. To address the reviewer's comment and link clonal fate mapping, modelling and scRNA-seq, we performed lineage tracing experiments using different CREER mice models based on putative marker genes enriched in SC and TA clusters (Fig. 5d). We used *Lgr6*CreER (Snippert et al., 2010) to target preferentially the SC cluster and *Notch1*CreER (Fre et al., 2011) to target the TA and Diff clusters. As fate decisions and clonal loss in TA and Differentiated cells can arise early, we performed the first imaging time point at D3 instead of D7, as some early events in the *Notch1*CreER lineage would already have been lost at D7. Specifically, to test the prediction that the basal cell layer hosts two distinct, proliferative populations, SCs and TA cells, we imaged from D3 to D28 in mice ear skin using *Notch1*CreER-ControlMosaic and *Lgr6*CreER-ControlMosaic.

By first combining clonal data from all animals, irrespective of sex, we found that the clonal persistence – the probability that a clone retains at least one basal cell – was dramatically different between models, with $44.6 \pm 4.0\%$ of clones persisting for *Lgr6*CreER at D28 (Fig. 5j) compared to $10.0 \pm 0.3\%$ for *Notch1*CreER (Fig. 5k). This major difference already highlighted that using different Cre, targeting the cells-enriched SC marker gene *Lgr6* and the TA (and Diff)-cell enriched marker gene *Notch1*, label functionally discrete basal populations. To further assess whether these targeted cells were rooted in self-renewing populations, we looked at the change in average labelled cell number – equivalent to the mean number of cells per clone including clones of size “zero”, the latter representing those that have become lost through differentiation (Fig. 5l,m). Notably, an early drop in labelled cell number in the *Notch1*-targeted population indicated that initially labelled proliferative cells is not enriched in cells with self-renewal potential, in line with a putative TA cell population (Fig. 5m). In the longer term, the labelled cell density then stabilized, most likely due to a residual labelling of SCs by the *Notch1*CreER. (However, although not included explicitly in the model, we cannot rule out the possibility that TA cells may revert back to a SC identity, albeit at some low frequency.) By contrast, the *Lgr6* population showed an early increase in average labelled cell number, followed by rapid convergence to a constant value, highlighting the self-renewal potential of this population, in line with a putative SC identity (Fig. 5l).

The mean clone size and clonal persistence of *Lgr6*CreER targeted cells were similar between male and female (Extended Data Fig. 13e). However, clones from *Notch1*CreER

female animals expanded more than male animals (5.1 ± 0.4 vs 3.1 ± 0.3 cells at D28) (Extended Data Fig. 13f) and the labelled cell density was higher in female mice compared to male (Extended Data Fig. 13j).

In Notch1CreER tracing, $75.5 \pm 1.0\%$ of clones showed no cell division over the time course, while in Lgr6CreER tracing non-dividing clones represented just $18.7 \pm 5.1\%$ of cases (Fig. 5n). This highlights that Notch1CreER labelled cells comprising both terminally differentiated cells (Diff) and cells able to divide (potentially TA and some SC). By focusing on clones that did divide, the frequency of clones that stayed within the maximum 3-cell threshold (putative TA cells) was around $62.1 \pm 3.7\%$ in Notch1CreER and $38.6 \pm 5.7\%$ in Lgr6CreER (Fig. 5o), consistent with the preferential labeling of TA cells by Notch1CreER compared to Lgr6CreER. The fraction of dividing clones that was lost through differentiation was similar with $6.8 \pm 2.0\%$ for Notch1CreER and $8.9 \pm 1.6\%$ for Lgr6CreER (Fig. 5o). The proportion of clones that kept expanding at D28 was $52.5 \pm 4.7\%$ in Lgr6CreER versus only $32.2 \pm 4.4\%$ in Notch1CreER (Fig. 5o), providing further evidence for the preferential labeling of SC by Lgr6CreER compared to Notch1CreER.

To assess the degree of differentiation of targeted cells and their immediate progeny, we assessed Krt10 expression in basal targeted cells at D3 following TAM administration. The fraction of basal labeled cells expressing Krt10 was $7.7 \pm 2.9\%$ in male Lgr6CreER targeted cells and $9.24 \pm 1.0\%$ in female Lgr6CreER targeted cells (Extended Data Fig. 13k-m). For Notch1CreER, the proportion was $38.2 \pm 4.5\%$ in male and $55.3 \pm 3.8\%$ in female animals (Extended Data Fig. 13m). Regarding the composition of clones at D3, the fraction of suprabasal cells over total labelled cells was 0% in male Lgr6CreER and $1.4 \pm 1.4\%$ in female Lgr6CreER animals, compared to $27.4 \pm 4.4\%$ in male Notch1CreER and $21.3 \pm 1.4\%$ in female Notch1CreER animals (Extended Data Fig. 13n). These results were consistent with the faster delamination of terminally differentiated cells in males, and a higher content of cells committed to differentiation (basal cells expressing Krt10+) on the basal layer of female mice.

Computational modelling of the Notch1CreER and Lgr6CreER lineage tracing showed that the two proliferative-compartment model captures well the dynamics of each population (Extended Data Fig. 14). For the Lgr6 population, we found that 50% of clones from males and 52% of clones from females were SC-like, relative to 41% for K14 Male, 50% K14 Female, 10% for Notch1 Male and 12% for Notch1 female. This indicates that the Lgr6 promoter captures SCs in similar to proportion to K14, but the Notch1 captures only a small number of SCs. For both Notch1 and Lgr6, we found that the model could accurately capture the average size and distribution of SC-like clones (Extended Data Fig. 14a-d). Further, the model captured reasonably the dynamics of TA-like and D-like clones with the exception of the persistence of TA-like clones (Extended Data Fig. 14e-l). In terms of parameters, the homeostatic composition of SC/TA/D is comparable by sex to that found for the K14 promoter for both Lgr6 and Notch1 (Extended Data Fig. 15).

Altogether, these results support the existence of two proliferative basal cell compartments, with SCs preferentially targeted by the Lgr6CreER and TA and differentiated cells preferentially targeted by the Notch1CreER.

These results have been added to the revised manuscript in the molecular characterization section.

5. How is the parameter scan performed for the one- and two-proliferative compartment model? For the scan space, what's the percentage of parameter sets that yield the desired behavior? Which parameters are critical to the model? How robust/sensitive is the model to parameters?

As described in Supplemental Theory, to fit the models we used specific features of the experimental data to constrain parameters. (The plateau in the average labelled cell density was used to set the proportion of SCs, and the ratio between basal and suprabasal clone sizes was used to constrain the delamination rate of cells from the basal layer.) We then performed a coarse grid-search to map the parameter landscape. After this, a simulated annealing algorithm was used to find the optimum parameters, and the uncertainties in each-parameter were calculated using bootstrapping of experimental samples and comparing to simulations from the grid-search, resulting in the confidence intervals provided in Table 1 and Supplementary Table 2 and 3 from Supplemental Theory

For the model, all parameters included influence the quality of the model fits (using log-likelihood broken into SC-like, TA-like, D-like clones), but certain parameters and parameter combinations have greater influence. Here, for clarity we specify the influence and sensitivity of each model parameter, before summarizing the most critical model parameters. For both the SC-TA-D and SD model, the dynamics of the SC compartment are well-constrained by the data:

- The relative proportion of stem cells in the basal layer (f_s) is fully constrained by the data and hence not fit, as the plateau in the labelled cell density of SC-like clones provides a read out of $1/f_s$.
- The proportion of SC divisions that result in asymmetric fate (r) is critical to capturing the profile of average clonal growth, with the observed fast initial growth followed by a slower growth rate requiring a significant bias towards asymmetric fate outcome. The particular optimal value of r is influenced by the correlation parameters below, which have a strong influence on the initial rate of clonal growth.
- Given f_s and r , the division rate λ is constrained by the data, since linear-like long-term clonal growth occurs at rate $r\lambda/f_s$, as reported in prior studies. (Clayton et al., 2007) In our fitting procedure, with r and f_s fixed, the optimal λ is obtained by finding the temporal rescaling between simulation time and experimental data that makes average clone sizes match.
- Shape parameters of the cell lifetime distribution (CV_s) are constrained by the data, with exponential lifetimes overestimating the number of clones in the tail of the clone size distribution. However, the particular values are not constrained by the data since there is a broad uncertainty interval due to the collective influence of other parameters.
- Correlation parameters for daughters from stem cell divisions (C_{ST} , $C_{SS}=C_{TT}$) are not precisely constrained by the data. Since most SC divisions are asymmetric, C_{ST} must be high (~ 1) otherwise even-odd features would be rapidly erased. For simplicity, we fixed this parameter to unity to reduce the dimensionality of the parameter space. Conversely, the correlation for symmetric stem cell divisions C_{SS} is poorly constrained by the data since the proportion of symmetric SC events is small (95% CI is $[0.1, 1.0]$ for males and $[0.0, 1.0]$ for females).

For the TA and D cell populations, the parameters are not as precisely constrained by the data, with degeneracies along certain parameter combinations:

- Since the short-term growth characteristic of TA cell clones is similar to SC-derived clones, it follows that the division rate of TA cells is $\sim\lambda$, the rate of SC division. Further, for simplicity, we assume that $CV_T = CV_S$.
- The proportion of asymmetric TA cell divisions is considered as a free parameter (1-q). The model is sensitive to this parameter as, without it, one cannot explain the observation of 3 or 5 cells clones observed in female mice. For the log-likelihood fit, there is some degree of degeneracy as q is covaried with certain parameters (C_{DD} , CV_D).
- The D cell delamination rate (γ) is uniquely defined once λ , f_s and q are fixed. The quality of fits is sensitive to the delamination rate if C_{DD} is small since a slower rate of delamination would erase even-odd features. Further, the fits are relatively insensitive to CV_D , given other parameter combinations of C_{DD} and γ/q .
- For the correlation parameters, we fixed $C_{TD}=0$, and treated C_{DD} as a fitting parameter. For male mice, the fits were relatively insensitive to C_{DD} and C_{TD} as there is no even-odd fine structure in the clonal data. In contrast, female mice fits are strongly sensitive to C_{DD} requiring a high degree of sister cell coordination to maintain the observed even-odd features. The presence of coordination between delamination and TA cell divisions following asymmetric division (C_{TD}) alters the value of the best fit parameters of q/γ and C_{DD} , but does not increase the quality of fits from the present data.

For the suprabasal layer, parameters are well-constrained by the data:

- The suprabasal delamination rate (Γ) is fixed by the data with the quality of suprabasal layer fits highly sensitive to the value.
- The suprabasal shedding variability CV_{sb} influences the degree of even-odd features in the suprabasal layer, so is strongly constrained in female data. (Low variability is needed for even-odd suprabasal features to persist.) But CV_{sb} is not well constrained by the male data.

The proportion of parameter sets yielding the desired behaviour can be assessed via bootstrapping on the grid. The free fitting parameters, r and q, were sampled over a uniform 20 point grid, and the parameters C_{SS} and C_{DD} were sampled over a 10 point grid. Over 1000 resamples of experimental data, we found that 0.5% of the unique parameter combinations were best fits for male mice and 0.1% for female mice. Of these parameters, Principal Component Analysis of male bootstrap best fit parameters revealed that C_{SS} is a “nuisance parameter” with 40% of the total parameter variance explained by varying it alone. In contrast, 58% of the parameter variance is explained by a combination of q and C_{DD} , reflecting a sensitivity of fits to sister delamination correlation if the delamination rate is slow, but higher C_{DD} is required if the delamination rate is slower (q is smaller). Variation in r explains only 0.5% of total parameter variance, reflecting the proportion of asymmetric divisions being strongly constrained by the profile in the average basal clone size. For female mice the picture is strikingly different, 95% of parameter variance is explained by a principal component, which depends mostly on C_{SS} , with weak dependence on q. This means that equally high-quality fits can be found along a manifold where C_{SS} increases and q decreases. This suggests these 4 fitting parameters

correspond to 2 soft parameter directions in male mice and 1 soft parameter direction in female mice.

To summarize, the average model dynamics for SC-like clones are determined by the division rate (λ), proportion of stem cells (f_s), proportion of asymmetric divisions (r) and shed rate (Γ). To capture the fine-grained features of the clone size distributions, fits are strongly sensitive to C_{ST} and C_{DD} (with reductions in either value destroying even-odd correlations), CV_s (which sets the maximum clone sizes), with a contribution from CV_D and q (where decreasing q destroys even-odd clone size correlations when C_{TD} is not high). To simultaneously capture the TA-like and D-like dynamics with SC-like dynamics, the fits are sensitive to q , C_{VD} , C_{TD} and C_{DD} along particular parameter combinations. Overall, the model robustly explains the male dynamics over a wider parameter regime, whereas female mice require fine-tuning to maintain the fragile even-odd features via a high degree of coordination with a low degree of TA cell division asymmetry.

6. Line 296: the justification of the 3D manifold is a little speak, considering that either 2D or 3D visualizations could lead to misinterpretation of the data, as is well known about visualizations such as UMAP, diffusion maps, etc.

We thank the reviewer for this important comment. We fully agree that any low-dimensional visualization inevitably entails information loss as it compresses tens of thousands of dimensions into 2D or 3D, which can lead to potential misinterpretations. Among the available options, however, 3D provides the highest representational capacity that can be directly visualized. This can be supported by the Johnson–Lindenstrauss (JL) lemma.

The JL lemma establishes that a set of n high-dimensional points can be embedded into a k -dimensional Euclidean space while preserving all pairwise distances within a factor of $(1 \pm \epsilon)$, provided that $k = O(\log n / \epsilon^2)$. For typical single-cell datasets with thousands of cells, this bound implies that dozens of dimensions would be needed for low-distortion embeddings; both 2D and 3D visualizations therefore inevitably lose information. Nonetheless, the JL framework and concentration-of-measure arguments show that the expected distortion decreases approximately as $1/\sqrt{k}$. Hence, even a modest increase in dimension improves fidelity: moving from 2D to 3D reduces the expected distortion by about $18\% \approx 1 - \sqrt{2/3}$. This provides a theoretical basis for why 3D embeddings more faithfully preserve the underlying geometry than 2D.

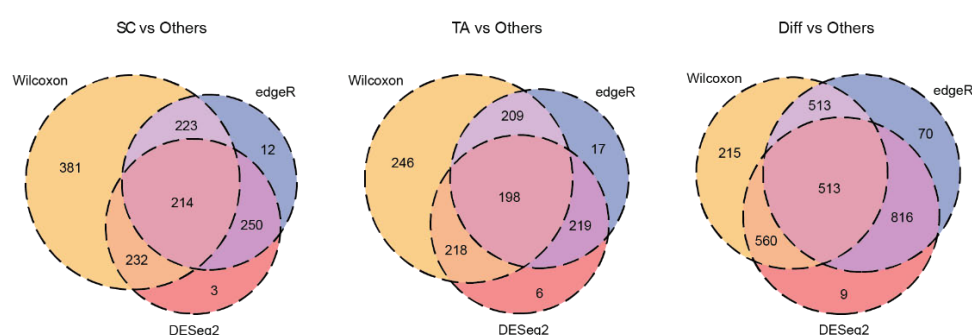
In our case, the strength of the 3D manifold is its ability to simultaneously preserve both self-renewal (looped) trajectory and differentiation (linear) trajectory. The 3D manifold successfully captures both structures within a single representation. By contrast, in 2D UMAP, G2/M and S-phase cells appear artificially separated, requiring prior biological knowledge to interpret their relationship.

In summary, while visualization is always approximate, we believe that the 3D manifold provides a more faithful surrogate than 2D. In our study it yields a representation that aligns with the hierarchical stem cell model and captures both self-renewal and differentiation dynamics.

7. Considering that replicates for male and female were sequenced for scRNA-seq analysis, it would be better to perform pseudobulked differential expression analysis per

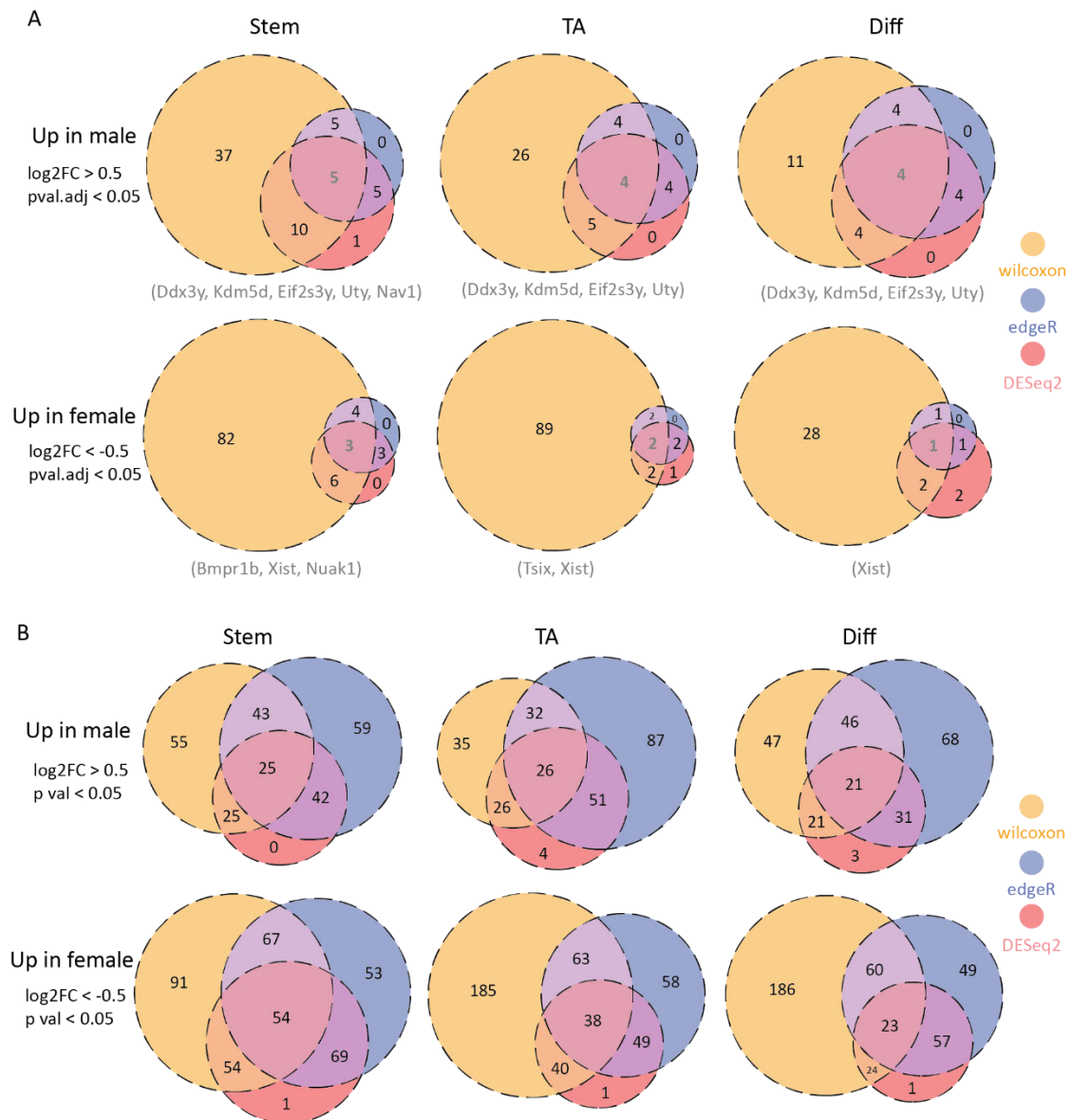
cell type to identify more cell-type-specific differences, using statistical models that can be specified using, e.g., DESeq2 edgeR. This is the more “consensus” way of performing differential analysis using scRNA-seq analysis these days.

We thank the reviewer for this helpful suggestion. In the revised analysis, we performed pseudobulk differential expression (DE) for each cell type using both DESeq2 and edgeR, in addition to our original Wilcoxon test. Using the same thresholds ($\log_2FC > 0.5$ and adjusted $pval < 0.01$), we found that the DE genes identified by all three approaches showed highly significant overlaps, as showed in the Venn diagram. Pairwise hypergeometric tests of overlap among Wilcoxon, DESeq2, and edgeR yielded p -values $< 1.0 \times 10^{-280}$ across SC-up, TA-up and Diff-up gene sets (see Table below). These results demonstrate strong consistency across methods. We have included the complete DE gene lists from all three methods in the supplementary materials for the readers’ reference.



Hypergeometric test p-value	SC-up genes	TA-up genes	Diff-up genes
Wilcoxon vs edgeR	1.54e-283	1.17e-300	0.0
Wilcoxon vs DESeq2	2.22e-304	0.0	0.0
DESeq2 vs edgeR	0.0	0.0	0.0

In addition, we performed pseudobulk differential expression (DE) between female and males for each cell type using both DESeq2 and edgeR, in addition to our original Wilcoxon test.



Using consistent thresholds ($\log_2\text{FC} > 0.5$ for “up in male”, $\log_2\text{FC} < -0.5$ for “up in female”, and adjusted $p < 0.05$ with BH correction), we compared the DE genes identified by the three methods. As shown in Panel A of the figure above and the Table below, there is significant overlap among Wilcoxon, DESeq2, and edgeR results, supporting the robustness of the detected signals.

Up in Male	Stem		TA		Diff	
	pval.adj	pval	pval.adj	pval	pval.adj	pval
Wilcoxon vs edgeR	6.67e-13	4.49e-63	4.27e-11	2.92e-44	2.71e-12	9.56e-67
Wilcoxon vs DESeq2	2.71e-24	3.11e-43	9.99e-14	8.80e-46	2.71e-12	6.07e-36
DESeq2 vs edgeR	2.01e-16	8.10e-89	6.78e-15	1.21e-96	1.99e-15	1.83e-58
Up in Female	Stem		TA		Diff	
	pval.adj	pval	pval.adj	pval	pval.adj	pval

Wilcoxon vs edgeR	1.24e-13	3.61e-95	6.15e-05	3.54e-71	2.86e-3	2.69e-61
Wilcoxon vs DESeq2	1.24e-13	7.53e-92	0. 1.84e-4	1.79e-57	4.73e-05	2.89e-24
DESeq2 vs edgeR	2.61e-10	3.40e- 143	4.51e-08	6.18e-96	3.81e-4	2.10e- 109

Given that our dataset includes only two biological replicates per sex, the statistical power of pseudobulk methods is inherently limited. As expected, DESeq2 and edgeR identified fewer significant genes under multiple testing correction, and the commonly detected genes were predominantly canonical sex-linked genes (e.g., Y chromosome genes and *Xist*). However, several non-sex chromosome genes shared across methods are biologically informative. For example, *Nav1* is upregulated in male SCs. *Nav1*, a member of the neuron navigator family, is involved in the some inflammatory skin diseases as it was significantly upregulated in the epidermis of rosacea and psoriasis skin lesions. (Zhang et al., 2022). Among genes upregulated in female SCs, *Bmpr1b*, a BMP receptor, may play a role in regulating cell proliferation (Lewis et al., 2014). These observations suggest intrinsic regulatory differences between male and female SC populations.

To further explore broader trends under limited statistical power, we performed a complementary analysis using a relaxed threshold ($p < 0.05$ without multiple testing correction). As shown in Panel B of the figure above, this approach increases sensitivity and reveals consistent trends across methods. All of the genes highlighted in the revised manuscript main text and figures (such as *Krdap*, *Ehf*, *Skint1* in males or *Smoc2*, *Postn*, *Il20ra*, *Il34* in females) are supported by at least one method other than the Wilcoxon test.

Taken together, these results support the presence of sex-dependent regulatory differences across IFE cell states, despite limited sample size. Importantly, both stringent (adjusted p) and exploratory (unadjusted p) analyses yield consistent biological themes. All DE gene lists and enrichment results have been included in the supplementary materials for transparency and reproducibility.

8. Two contradictory pieces of information appeared in the results section “Molecular characterization of the IFE reveals sexual dimorphism at the transcriptomic level”. At lines 315-316, “Male IFE SC cluster showed increased levels of *Skint1* and differentiation-related genes such as *Ehf* and *Krdap* (Fig. 5n)...” However, at lines 342-343, “In contrast, many differentiation markers were strongly upregulated in female basal cells compared to male...” It would be necessary to interpret the two contradictory pieces, in order to avoid confusion and improve the rigors.

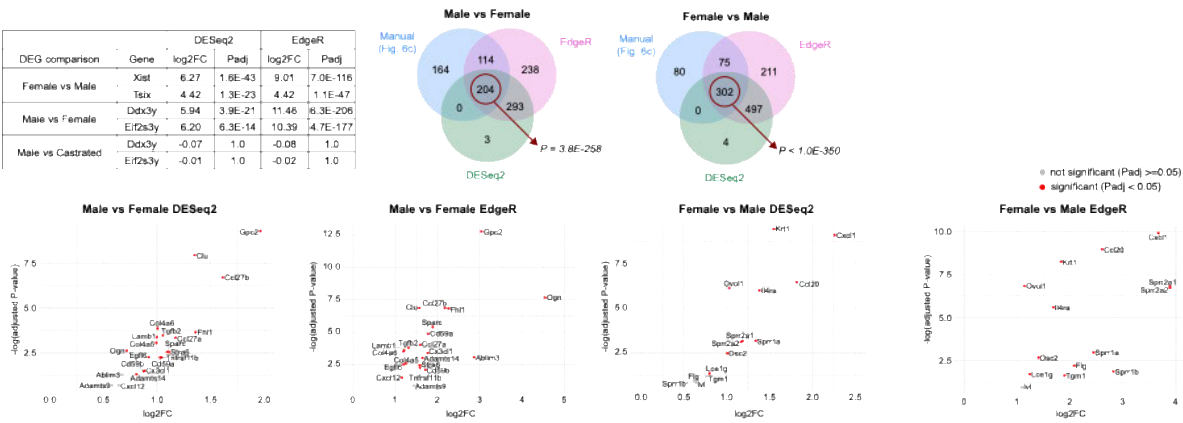
We thank the reviewer for raising this point. In the current manuscript one of the main claims is regarding male basal cell delamination. We view single-cell data as a precise analysis of single-cell fate. Therefore, a cell delaminating faster would be expected to express a higher degree of differentiation marker, as seen in male basal cells by scRNA-seq (Fig. 5p-r). However bulk RNA-seq of the basal layer is used to measure the average composition of the basal layer and is therefore an average of the transcriptome of all different cell states presented in the basal layer including SC, TA and differentiated cells rather than an assessment of the single-cell transcriptome. Since differentiated cells are

delaminating faster in male mice (Fig. 4b), they stay for a shorter time on the basal layer. Therefore, the basal layer in males contains a lower proportion of differentiated cells, which will lead to a decrease in the level of differentiation genes among all basal cells containing SC, TA and differentiated cells (Fig. 6b). In summary, the seemingly difference in the single-cell and bulk transcriptome is, we believe, the consequence of a different proportion of SC, TA and differentiated cells in the basal layer between male and female animals due to the faster cell delamination of differentiated cells in male mice (Figs. 4b).

We have added a sentence in lines 508-512 of the revised manuscript to clarify this point.

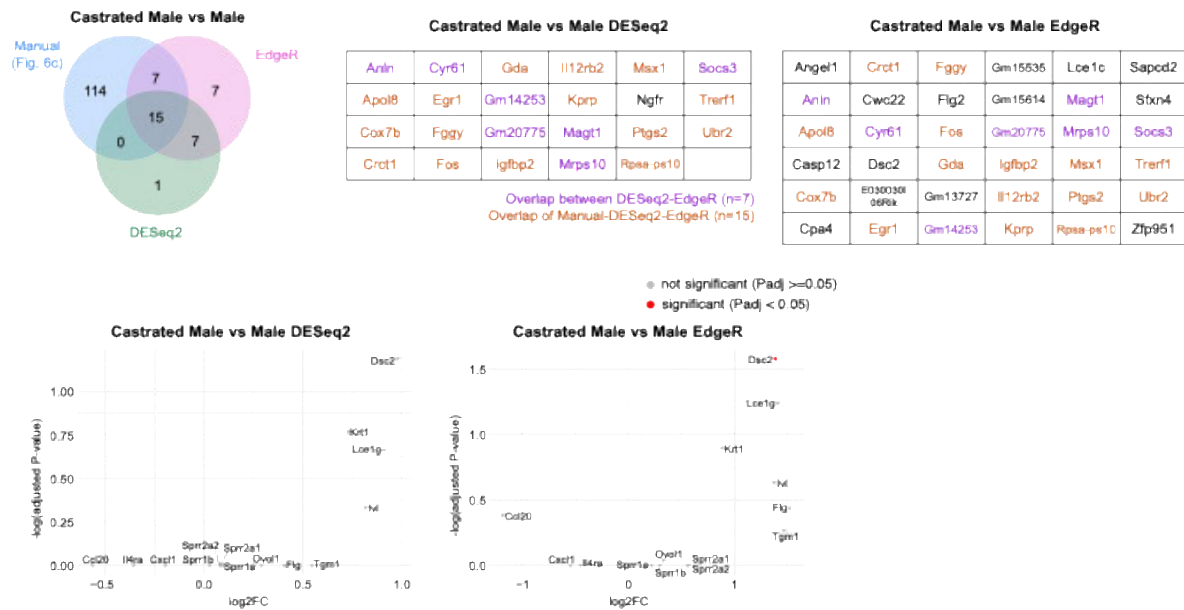
9. Given that bulk RNA-seq was generated a statistical model built using, e.g., DESeq2 or edgeR, should be used over mere log-fold-change calculation to test for differential expression.

We thank the reviewer for this important comment. In the revised analysis, we performed differential gene expression analysis using both DESeq2 and edgeR (Extended Data Fig. 17e-m).



Key sex chromosome–linked genes included in Fig. 6a and 6b of the first submission (*Xist*, *Tsix*, *Ddx3y*, *Eif2s3y*) displayed the same expression tendencies across DESeq2, edgeR, and our initial evaluation.

For the male versus female comparison, the majority of genes highlighted in our original Fig. 6c were confirmed to be significantly upregulated by both DESeq2 and edgeR. The two models produced highly consistent results, with strong overlap in significantly upregulated genes (three-way intersections: $P = 3.8 \times 10^{-258}$ for male-up genes; $P < 1.0 \times 10^{-350}$ for female-up genes). Furthermore, other genes highlighted in Fig. 6c and 6d were robustly identified as significantly differentially expressed by both methods. The corresponding volcano plots illustrate that DESeq2 and edgeR consistently reproduce the fold-change directionality observed in the initial evaluation, with stronger statistical support.

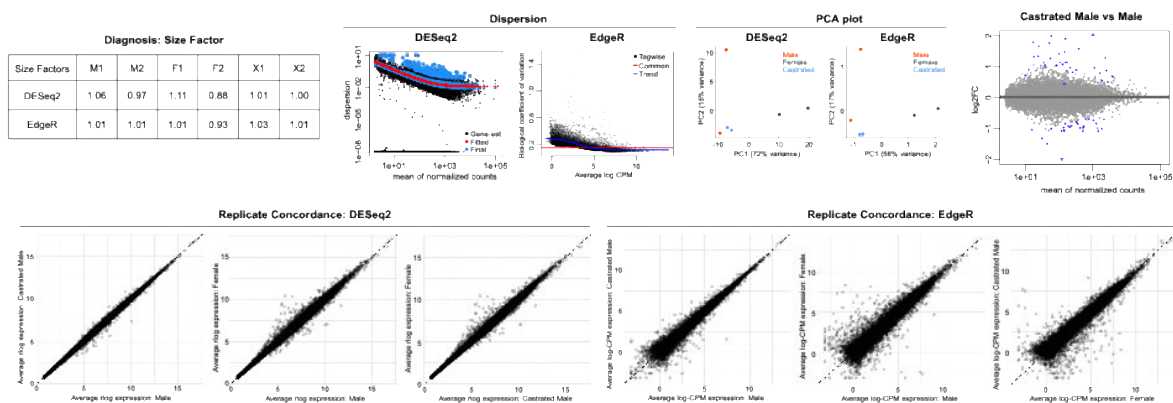


Altogether, these results demonstrate that the strong differences between males and females are robustly captured by both DESeq2 and edgeR.

For the castrated male versus control male comparison highlighted on Fig. 6b, only a small number of genes such as *Dsc2*, *Krt1* and *Lce1g* were differentially expressed, and the overall number of significant DE genes was markedly lower than in the male versus female comparison. However, most if not all genes with increased log₂FC identified by those pipelines were related to differentiation (*Dsc2*, *Lce1g*, *Krt1*, *lxl*, *Flg*, *Tgm1*, *Ovol1*, *Spr2a1*, *Spr2a2*, *Spr1b*, *Spr1*) (Extended Data Fig. 17k-m) but lacked statistical significance calculated by those tools.

To rule out technical artifacts resulting from this weaker signal, we performed extensive quality control and diagnostic analyses for both DESeq2 and edgeR. These confirmed that:

- Normalization and size factors were balanced across all samples (range ~0.88–1.11), excluding compositional or depth-related bias.
- Dispersion and variance trends followed the expected patterns, with no evidence of inflated variability masking DE calls.
- Global transcriptome structure showed castrated and intact male samples clustering closely together in PCA space, in contrast to the clear separation seen in male versus female comparisons. MA plots indicated that most genes lie near log₂FC = 0, with only a handful of modest-effect outliers.
- Replicate concordance (rlog/CPM expression) confirmed strong agreement between castrated and intact male samples, without systematic bias.

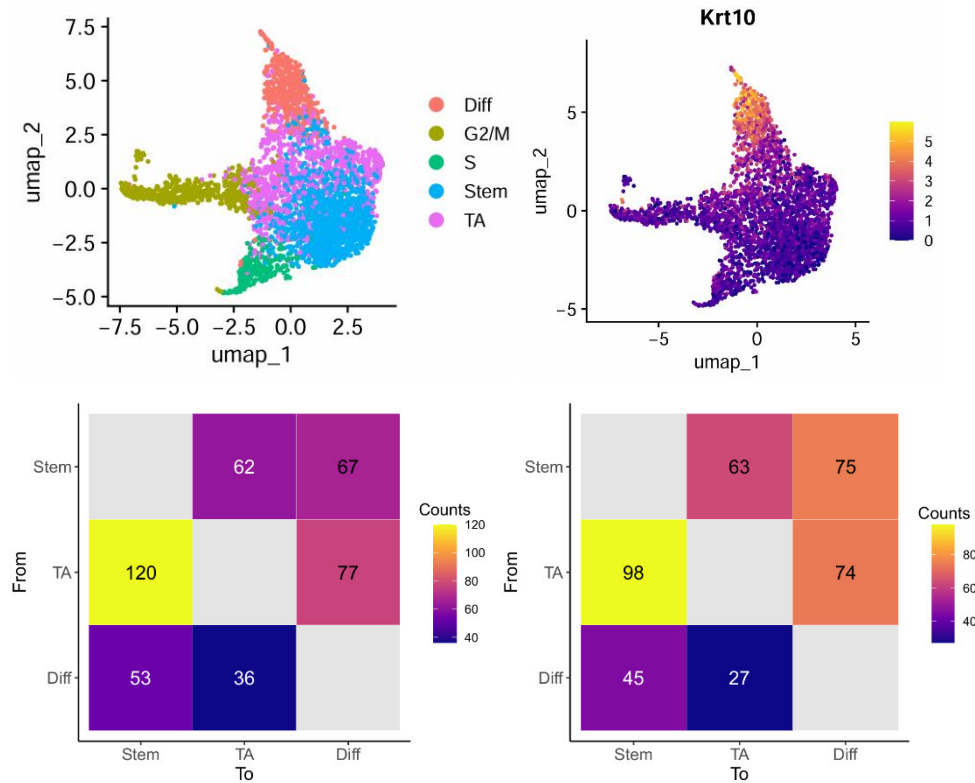


This lack of significance is likely due to the limited number of biological replicates ($n = 2$ per condition), which reduce statistical power and limits reliable estimation of gene-wise dispersion. Indeed, while several genes show consistent differences in average expression between conditions, these differences do not reach statistical significance when applying standard frameworks such as DESeq2 or limma, which implement conservative variance modelling and multiple testing correction. Therefore, the absence of statistically significant genes should not be interpreted as a lack of biological differences, but rather as a limitation in statistical power. In this context, we focused on overall expression trends and complementary analyses to support our conclusions.

The initial reason for including castrated samples was to show that the difference between male and female differentiation was mediated by male sex hormones.

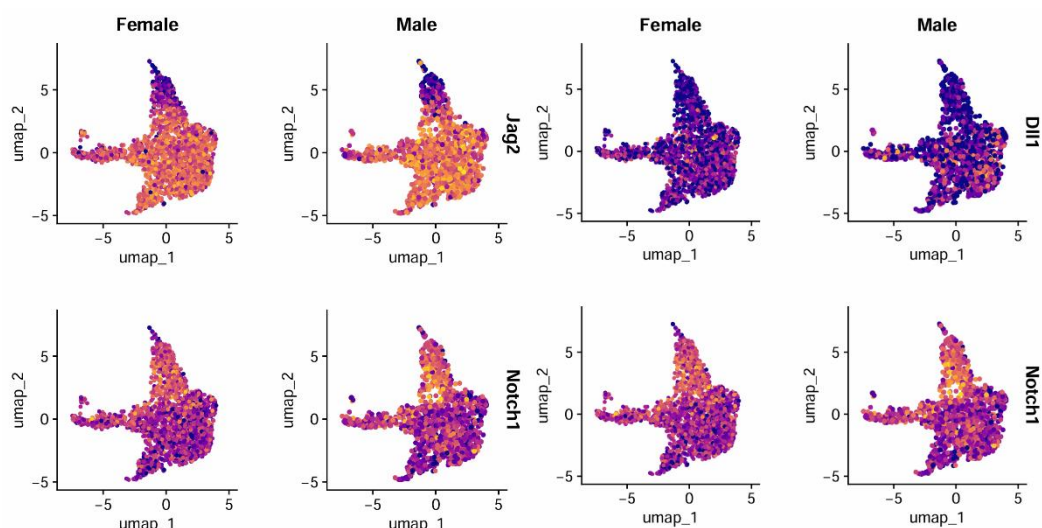
10. It would be interesting to investigate how cell-cell communications may shape the differentiation landscape in male and female mice.

We thank the reviewer for this comment. To address this point, we systematically investigated sex-specific cell-cell communications across the SC-TA-Diff axis using CellPhoneDB (v5). We applied this strategy independently to male and female samples to identify common and sex-specific ligand-receptor (L-R) pairs. CellPhoneDB identified putative L-R pairs between all 3 clusters (SC-TA-Diff) with the highest number between TA to SC in female mice (120 vs. 98 significant TA to SC interactions, and total interaction of 415 in females and 382 in males). For other directions, including TA to Diff, Diff to TA and SC to TA, the number of interactions was either similar or greater in females. Even if fitting with our narrative of a greater coordination between female and males, we first wanted to curate the list for biological relevant interactions, as these tools are known to infer some ligand-receptor pairs with limited biological translation.

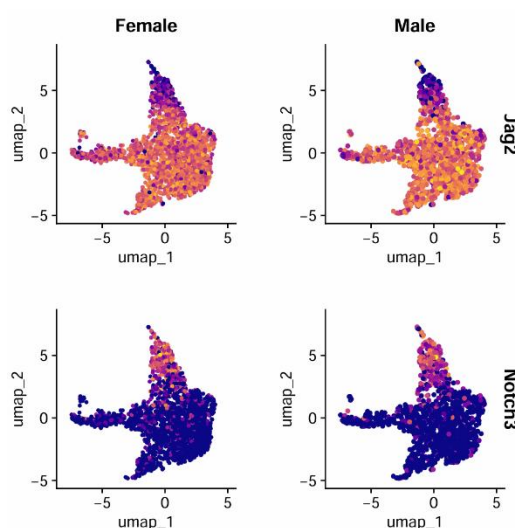


In the setting of IFE basal cells, most putative ligand–receptor genes are expressed across both dividing (SC and TA) basal cells. However, when comparing between dividing cells (SC and TA) and the post-mitotic Diff cluster, we identified some cluster-specific ligand–receptor pairs.

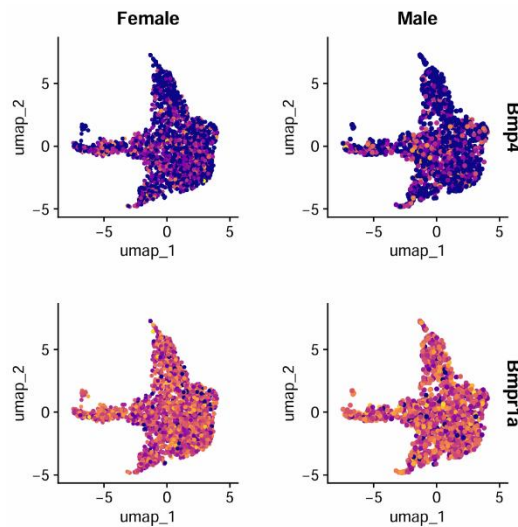
The Jag2-Notch1 interaction was identified in both SC to TA, TA to Diff and SC to Diff. Among all Notch pathway interactions, this was the one with the higher LR_means (representing ligand and receptor expression level in each cell type). This interaction is in our opinion biologically valid, as the activation of the Notch pathway is crucial for keratinocyte differentiation and epidermal stratification (Rangarajan et al., 2001; Blanpain et al., 2006; Williams et al., 2011). This interaction was more highly scored between SC to Diff and TA to Diff, than between SC to TA. Interestingly, this prominent axis of differentiation presented a higher LR_means in males (SC to Diff at 1.92 and TA to Diff at 1.87) than in female (SC to Diff at 1.79 and TA to Diff at 1.78). These higher scores reflect higher expression of this ligand-receptor pair in male basal cells than in females. The fact that this known ligand–receptor pair, relevant for basal cell differentiation, was higher in males, can potentially be linked to a higher degree of differentiation of male basal cells on average. Other ligand–receptor pairs presented a higher score in males, such as DLL1-Notch1 from SC to Diff and from TA to Diff (with SC to Diff at 1.29 and TA to Diff at 1.25 in males compared to SC to Diff at 1.19 and TA to Diff at 1.13 in females).



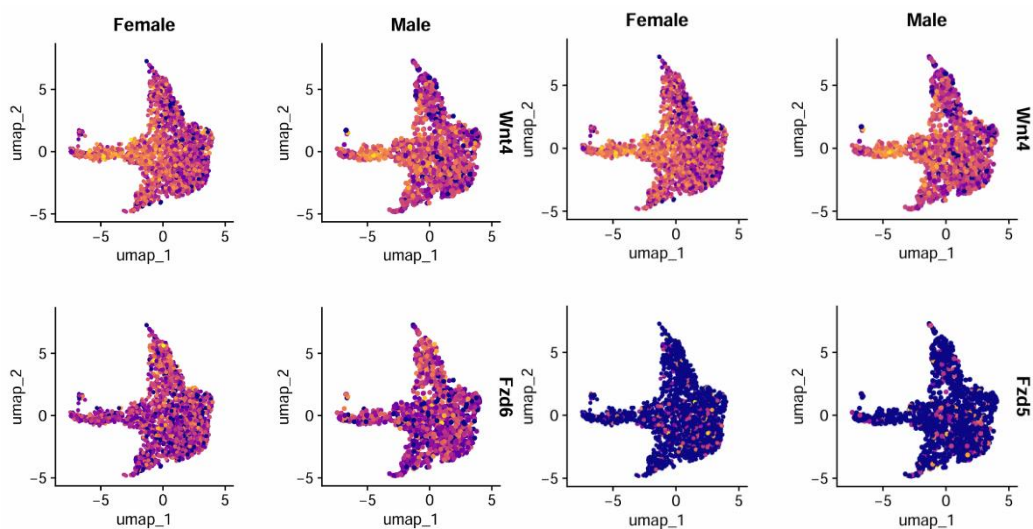
However, albeit with a lower LR-means, some other Notch pathway pairs presented a similar score between males and females, such as Jag2-Notch3 (1.56 from SC to Diff in females vs. 1.54 from SC to Diff in males).



Bmp signaling in the epidermis has mostly been studied in regard to the hair follicle, where it regulates HF cycle (Blanpain and Fuchs, 2009). Some data suggest that activation of Bmp signaling through Smad1 overexpression leads to decreased proliferation during homeostasis and wound healing in the IFE (Lewis et al., 2014). In our analysis the highest ranked Bmp ligand–receptor pair in female was Bmp4-Bmpr1a from TA to SC and from TA to Diff. This could be one of many putative communications between TA and other basal cells in order to coordinate their divisions, as Bmp4 expression seemed quite specific to TA cells, and was described as being able to modulate basal cell proliferation.



Whereas canonical Wnt signaling is mostly known to regulate hair follicle activation, Wnt expression in the IFE can positively regulate basal cell proliferation and stemness (Choi et al., 2013). The highest ranked interactions were found between Wnt4 and Fzd6 associated with the non-canonical Wnt pathway which was never studied in the regulation of proliferation and differentiation of the IFE. Most of the other ligand-receptor pairs were also involved in non-canonical signaling, limiting our ability to draw conclusions regarding the impact of differential expression of Wnt pathway ligand-receptor pairs between SC and TA cells.



Based on this suggested analysis alone, it is difficult to make strong biological claims. The higher number of interactions between female basal cells seemed appealing. However, this number is based on many unproved biological interactions. The higher activity of the pro differentiation pathway such as Jag2-Notch1 or Dll1-Notch1 in males could be related to higher degree of differentiation observed in male basal cells and their faster delamination. Due to the lack of clear message of this analysis, we therefore did not add these cell-cell communication data to the revised manuscript.

11. Something that would help the study is addressing in the Discussion why no previous study of stem cell lineages in IFE has identified these sex-specific differences, i.e., what was missing from previous work, or what was “done correctly” in this study.

We thank the reviewer for this comment. As mentioned in the manuscript, the key to detecting sex-specific differences lay in the enrichment of clones of even numbers of basal cells. This was noted previously in the mouse skin epidermis where, under injury conditions, such correlations become pronounced (Aragona et al., 2020). Moreover, although not highlighted in the study, it is also readily apparent in a long-term lineage tracing study of mouse oesophagus (Piedrafita et al., 2020). However, these signature features are extremely fragile: If sample sizes are small, and when data from male and female mice are pooled, the even-odd correlations can become easily erased. Notably, long-term, sex-specific data has been missing from previous studies – and this is the reason why these features have been overlooked.

Once recognized, we think that our intravital microscopy setup brought sufficient statistical power and precision to our lineage tracing analysis to be sure that the enrichment was not an artefact but a statistically robust and sex-related feature of the dynamics. Key to this success is both ensuring that the data is sufficiently powered between male and female “controls”, and the ability to trace the fate of individual clones over time.

In summary, it is the integration of the precision of intravital microscopy long-term lineage tracing of the same clones, the fact that number of clones analyzed here is much higher than in previous studies, and the inter sex comparisons that enable us to detect and decipher these sex-specific differences.

We have made the proposed modification at the line 583-585 of the revised manuscript.

12. Lines 820–823: considering that the samples were integrated using Harmony to generate the diffusion map (lines 860–861), why weren’t the samples integrated for the “original” analysis?

We thank the reviewer for this comment. We agree that datasets with multiple samples may be confounded by batch effects that reflect technical variation rather than true biology, and therefore integration (i.e., batch correction) is generally required before downstream analysis. The recommended workflow in the field is to first perform integration across samples, and then apply clustering or visualization techniques such as diffusion maps or UMAP. (Heumos et al., 2023; Luecken and Theis, 2019)

Our “original” analysis did include integration. In the Seurat pipeline (R), we used canonical correlation analysis (CCA) for integration (line 839 in original version; line 1261 in the revised version), whereas in the Python workflow we used Harmony-corrected principal components. Both CCA and Harmony are widely used and recommended approaches for single-cell integration, valued for their simplicity and stability (Luecken and Theis, 2019; Tran et al., 2020).

In summary, we did not analyze unintegrated samples. Instead, we consistently applied integration prior to clustering, annotation and visualization. We have clarified this point in the revised text to avoid possible confusion.

Minor comments:

1. At line 41, change “comprises” to “is”.

We thank the reviewer for this comment. We have made the proposed modification at line 49 of the revised manuscript.

2. Line 49, ‘...in the 1960s’ should have citations to the original works.

We thank the reviewer for this comment. We have added a reference in line 57 of the revised manuscript.

3. At line 74, the term “CreER” is used without explanation. Consider briefly defining this term for readers who may not be familiar with genetic engineering tools, even if it is common jargon in the field.

We thank the reviewer for this comment. We have added a sentence in lines 81-84 of the revised manuscript.

4. In the paragraph beginning with (line 73), “However, several observations challenged the one-compartment model. First, lineage tracing using different CreER mice results in differences in the long-term survival and growth potential of clones.^{13–18} Second, fate mapping studies of back skin epidermis under perturbed conditions showed evidence of an enrichment of clones with even cell numbers, a feature difficult to explain within the framework of a one-compartment model. Finally, fate mapping of the ear skin epidermis based on a Keratin 10 (Krt10) reporter showed evidence of proliferative cells with strictly limited proliferative potential,” the relation of the first and final points to one-compartment model is not clear.

These statements have been refined in the main text to improve clarity, in lines 89-90 of the revised manuscript.

5. Typos and grammar errors: “beside” at line 86 should be “besides”; “recording of” to “recording” at line 118; “some 1171 clones” at line 127; “5-months” to “5-month” at line 134; “Further, we the model could not capture temporal features...” at line 199.

We thank the reviewer for pointing out the typos. We have corrected all the typos mentioned and carefully checked the revised manuscript for any new ones.

6. The paragraph “Finally, beside reproductive organs, sex has been associated with differences in organ morphology, tissue composition, and brain specific circuits.^{31–33}. However, little is known about the role of sex hormones in adult SC fate decisions. In the skin, morphological difference between male and female have been described.^{34,35} However, whether sex affects stem and progenitor cell dynamics during epidermal homeostasis is not known” should be reorganized.

We thank the reviewer for this comment. We have reorganized the paragraph, now available in lines 96-100 of the revised manuscript.

7. All figure panels should be cited in the main article text and numbered in the order of their first appearance. For example, Figure 1a and 1b are cited in reverse order.

We thank the reviewer for this comment. We have modified *Figure 1* panels order.

8. It's not clear how the clonal persistence is calculated from the data (Fig. 1f). Given a time point, does it mean the fraction of clones that retains at least one basal cell?

As inferred by the reviewer, the clonal persistence is the fraction of clones labelled at Day 7 that have at least one basal cell. We have modified the figure legend on *Figure 1*.

9. Lines 150-151: was any statistical test performed to confirm this difference was significant?

We have now quantified this difference with a z-test ($p < 0.001$).

10. It seems that the enrichment of even cell numbers in female mice is not obvious at D6 (Fig. 2l). How to interpret this?

We thank the reviewer for this comment. Indeed, it does not seem that the EdU pulse and chase experiment was able to decipher strong evidence for sexual dimorphism based on the enrichment of even cell numbers at this time point. However, this assay should be interpreted with caution. Lineage tracing labels single cells randomly, independently of their link to cell cycle. Therefore, at D7 post labeling, the mean clone basal size during lineage tracing is around 1.48 ± 0.03 cells for females and 1.46 ± 0.05 cells for males (Fig. 2a) whereas in the EdU pulse chase experiment at D6 the pseudo-clone's basal size was already of 2.27 ± 0.08 for females and 2.28 ± 0.07 for males (Extended Data Fig. 3h). This increased size indicates a bias in the labelling of pseudo-clone by EdU due to labeling S phase only cells. Therefore, we suppose that the preferential labeling of proliferative cells is artificially increasing enrichment of even cell numbers. However, we should make clear that, in our proposed model, the coordination between dividing cells and delaminating cells is present in both sexes. The magnitude is just higher in female. It is therefore not that surprising that, under certain conditions, it is possible to see the enrichment of even cell numbers in males.

11. Lines 195-196: "Formally, to capture the abrupt increase followed by the shallow linear-like growth in the average basal clone size (Fig. 2e-h)..." . It's unclear how Fig. 2e-h is related to the sentence. Maybe Fig. 2a?

We have corrected the figure reference to Fig.2a.

12. Lines 286-287: "As previously described, we found three G0/G1 clusters." The sentence is confusing because the "three G0/G1 clusters" seems not mentioned before.

We have corrected the wording, now shown in line 385 of the revised manuscript.

13. Lines 298-299: "Leiden clustering with a resolution of 0.5 identified three clusters not related to cell cycle..." It's not clear whether the clustering is based on G0/G1 cells or all cells. In addition, justifications are needed why these three clusters are not related to cell cycle.

We thank the reviewer for this important comment. To clarify, clustering was performed on all cells, not restricted to G0/G1 cells. Specifically, we first applied Harmony to integrate multiple samples and obtained Harmony-corrected principal components (PCs). We then regressed out cell cycle effects from these PCs and performed Leiden clustering (resolution = 0.5) on the corrected space. Because the clustering was based on PCs that were both batch-corrected and cell cycle-regressed, this approach enabled the

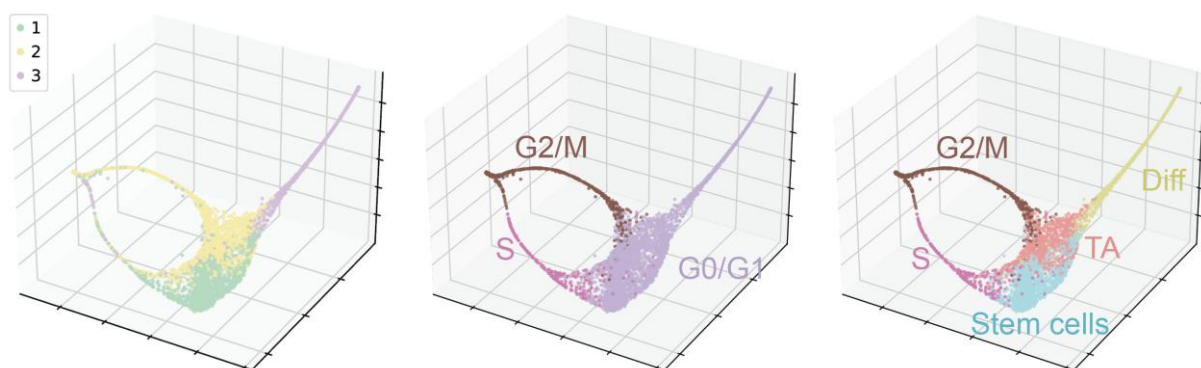
identification of cell populations that are largely independent of sample-specific variation and cell cycle states.

To further examine the relationship between cell identity and cell division, we performed diffusion map analysis using Harmony-corrected PCs without cell cycle regression, followed by 3D FLE manifold construction. This representation preserves cell cycle-related structure and allows us to assess how cell identity transitions relate to proliferative states.

As shown in the figure below, the left panel displays the clustering results, while the middle panel shows predicted cell cycle stages. The three identified clusters are not driven by cell cycle phase, as cells from different cell cycle stages are distributed across clusters. Instead, based on canonical marker gene expression and transcriptomic profiles, these clusters correspond to stem-like, transit-amplifying (TA)-like, and differentiated (Diff)-like states, respectively.

For downstream differential expression analysis, to minimize confounding effects from cell cycle-related genes, we retained cells annotated as S and G2/M phases but assigned all cells to their corresponding biological clusters (Stem, TA, and Diff) based on the cell cycle-regressed clustering. This ensures that DE comparisons reflect differences in cell identity rather than cell cycle status.

We have clarified these procedures in the Methods section of the revised manuscript.



14. It's not clear how the conclusion “Cells from both the SC and TA cluster were able to enter the cell cycle loop, while most of the differentiated cells were post-mitotic (Fig. 5i)” is made from Fig. 5i, as there is no trajectory direction or pseudotime information. It would be better to incorporate Extended Data Figure 6e (i.e, the RNA velocity analysis) to support this conclusion.

We thank the reviewer for this useful remark. We have corrected the wording, as shown in line 405 of the revised manuscript.

15. How to interpret the increase of the differentiation markers in male SC and TA clusters, such as *Krt14*?

We thank the reviewer for raising this point. This reply goes in line with the one given to this reviewer question 8. In the current manuscript, one of the main claims concerns male

basal cell delamination. We view single-cell data as a precise analysis of single-cell fate. Therefore, a cell delaminating faster would be expected to express a higher degree of differentiation marker, such as *Krt14* (Bazzi et al., 2007), as seen in male basal cells by scRNA-seq (Fig. 5p-r). However bulk RNA-seq of the basal layer is used to measure the average composition of the basal layer and is therefore an average of the transcriptome of all different cell states present in the basal layer including SC, TA and differentiated cells rather than an assessment of the single-cell transcriptome. Since differentiated cells are delaminating faster in male mice (Fig. 4b), they stay for a shorter time on the basal layer and therefore the basal layer contains a lower proportion of differentiated cells in males that will lead to a decrease in the level of differentiation genes among all basal cells containing SC, TA and differentiated cells (Fig. 6b). In summary, the seemingly difference in the single-cell and bulk transcriptome is the consequence of a different proportion of SC, TA and differentiated cells in the basal layer between male and female animals due to the faster cell delamination of differentiated cells in male mice (Fig. 4b).

16. Lines 832–836: what was the p.adjust threshold used to select for marker genes?

We have used the threshold $p.adjust < 0.01$. That has now been added to the revised Methods.

Referee #4 (Remarks to the Author):

This paper employs an elegant long-term *in vivo* imaging approach to revisit a longstanding question regarding clonal stem cell dynamics in the mouse epidermis. The findings suggest sex-specific differences in the hierarchy of stem and transit-amplifying (TA) cells in the interfollicular epidermis of mouse ears, with male and female mice displaying distinct patterns. Analysis of castrated males supports the argument that these differences are driven by androgen signaling. As it is, the study is highly descriptive and of questionable general significance. There are several important limitations:

We thank the reviewer for highlighting the elegant long-term *in vivo* imaging approach and how it addresses a longstanding question regarding epidermal stem cell dynamics. To address the reviewer's remark regarding general significance, we have performed multiple novel experiments. As suggested by the reviewer, we have made great use of the Four Core Genotype mouse model to better describe the respective role of sex chromosomes and sex hormones. We also used primary human keratinocyte 3D culture to assess the human relevance of our findings and assess the direct effect of male sex hormones to keratinocyte differentiation. We performed lineage tracing to expand our findings to two other skin locations. Finally, as suggested by the reviewer #2, we assessed the relevance of the sexual dimorphism in epidermal stem cell dynamics during wound healing, thereby increasing its general significance.

1. While the imaging technique is technically impressive, it provides only incremental insights relative to an extensive body of prior work, without introducing a conceptually novel framework.

We thank the reviewer for this comment. We have tried to better highlight the conceptual novelty in the revised manuscript. Here is a summary of why we think the present work should be considered of important value and brings significant conceptual novelties:

- The existence of TA cells has been speculated, but any connection with SCs was never demonstrated. This work is the first demonstration of the coordination of SC and TA cells in a defined hierarchy in the epidermis. This work is now validated by both long- and short-term lineage tracing and single-cell sequencing. This provides a novel framework to consider the cellular organization and functional fate dependences of the epidermis for a vast community of stem cell biologists.
- Although skin sexual dimorphism has been proposed before, it has not been observed in the context of quantitative lineage tracing studies, nor has been interpreted through the modelling of individual cell fate decisions. Here, we uncovered strong, consistent and hormonally mediated differences that have not been hitherto described. Our findings from the first submission have since been validated by genetic lineage tracing, EdU short term lineage tracing, human *in vitro* experiment and sequencing experiments. This study will be of high importance for all researchers performing clonal or tumor initiation analysis in the epidermis, as potential sex differences should now be considered in future work.
- Those two major novelties will be crucial to better understand skin diseases, such as potential sex differences in skin cancers and inflammatory skin diseases. Indeed, through our response to reviewer 2, our new data have shown how it can be used to better understand sexual dimorphic response during wound healing.

2. The conclusion that androgens directly influence epidermal stem and TA cell dynamics is rather questionable, as the observed effects could stem from indirect influences. Androgens are known to affect immune cell populations in the skin as well as dermal fibroblasts, which in turn can impinge on epidermal cells.

We thank the reviewer for this very important question. We tried to improve our demonstration that androgen can modulate basal cell fate.

First, we performed hormone replacement using DHT, the biologically active form of testosterone after castration. Male mice underwent castration and were then treated with DHT implant placement (Belma, DHT-M, 40 µg/24h). Uncastrated male mice were used as controls. Two weeks post-surgery, we performed EdU pulse chase experiments to assess the fate of EdU doublets after 96h of chase. Compared to castrated males without implant, castrated males with DHT implants presented a statistically significant increase in basal cell delamination at 96h ($10.3 \pm 1.5\%$ for castrated vs. $31.4 \pm 4.4\%$ for DHT) (Fig. 4f). DHT implants allowed to rescue the higher level of delamination than seen in control males ($31.4 \pm 4.4\%$ for DHT vs. $21.4 \pm 2.8\%$ for control males) (Fig. 4f). In terms of division fate outcome, when compared with castrated, DHT males had a significantly increased rate of asymmetric division (K14+K10+ EdU doublets, $41.2 \pm 3.7\%$ for castrated vs. $58.4 \pm 3.3\%$ for DHT) (Fig. 4g) as well as more K10+K10+ EdU doublets in DHT: $1.62 \pm 0.56\%$ for castrated vs. $7.14 \pm 1.53\%$ for DHT vs. $4.6 \pm 1.7\%$ for control males. DHT and control males achieved similar level of asymmetric division ($58.4 \pm 3.3\%$ for DHT vs. $62.8 \pm 3.1\%$ for control males) (Fig. 4g).

Altogether, these results indicate that the increased basal cell delamination rate and the relative frequency of asymmetric divisions is mediated by male sex hormones and not by the sex chromosome. In particular, we demonstrated that testosterone (DHT) can rescue the decreased basal cell delamination rate and the relative frequency of asymmetric divisions present upon castration. Therefore, testosterone seems to be a key regulator of basal cell commitment to delamination and asymmetric division.

However, this experiment was performed *in vivo* and is therefore, as suggested by the reviewer, subject to immune cell interaction. To exclude any impact of the immune system we studied *in vitro* 3D skin equivalent (3D SE) culture of human keratinocytes in collaboration with Michele De Luca group (Tham et al., 2025). This method is one of the only that allows to study a properly stratified tissue *in-vitro*, with minimal and controlled media composition. Primary human keratinocytes, from male et female donors were seeded on 3T3-J2 mixed with collagen gels (final concentration of 4 mg/mL) (Extended Data Fig. 17n). Human keratinocytes were culture in presence or absence of 100nM of DHT. At D1 after keratinocytes seeding, they were moved to air-liquid interface to allow for proper stratification of the skin equivalent. At D9 after air lift, once the skin equivalent was properly stratified, a pulse of EdU was administrated and chased for 72h. Trying to reproduce *in vivo* EdU pulse and chase experiment presented in the initial manuscript. 3D skin equivalent was then fixed and embedded in paraffin to allow delamination quantifications. No significant modification of proliferation rate was observed between control and DHT treated culture (Fig. 6-i). However, following a chase of 3days, DHT treated human keratinocytes, presented a significantly greater fraction of EdU labelled suprabasal cells ($37.16 \pm 1.99\%$ Vs $24.61 \pm 2.04\%$) - number of EdU+ suprabasal cell over

total EdU+ cells (Fig. 6j). This *in vitro* experiment is consistent with a direct effect of DHT on human keratinocyte delamination, in the absence of any immune cells.

Finally in order to validate that DHT can have a molecular impact on human keratinocyte, we perform *in vitro* culture of human derived skin epithelioid (Herms et al., 2024). In contrast to the 3D skin equivalent, this technique allows us to perform keratinocyte culture without a support of 3T3 gel (Extended Data Fig. 17o). The drawback being a lower level of stratification, not allowing pulse and chase experiments but still sufficient to study the initial step of differentiation. Human skin samples were harvested during planned surgical interventions and fresh samples were cultured. Once confluency reached, the biopsy was removed and the epithelioid was lifted to air-liquid interface to allow proper stratification. After two weeks, the epithelioids were placed in a starvation medium for 3 days, and then 3 days with or without 100nM of DHT. Whole epithelioid, including basal and suprabasal layers were collected for RNA extraction. Out of the 3 patients (2 male – including 1 facial and 1 eyelid biopsy and 1 female facial biopsy) 2 did present an important increase of differentiation associated transcripts (EVPL, IVL, KRTDAP, OVOL1 and TGM1) upon testosterone treatment (Fig. 6k). The increased expression of differentiation associated genes goes along with the putative role of DHT to promote basal cell delamination and stratification. In contrast to the mouse samples where only basal cells were analyzed the human *in vitro* assays, basal and differentiated suprabasal cells were collected. With this we analyzed the impact of DHT on basal and suprabasal cells which increased differentiation of culture keratinocyte.

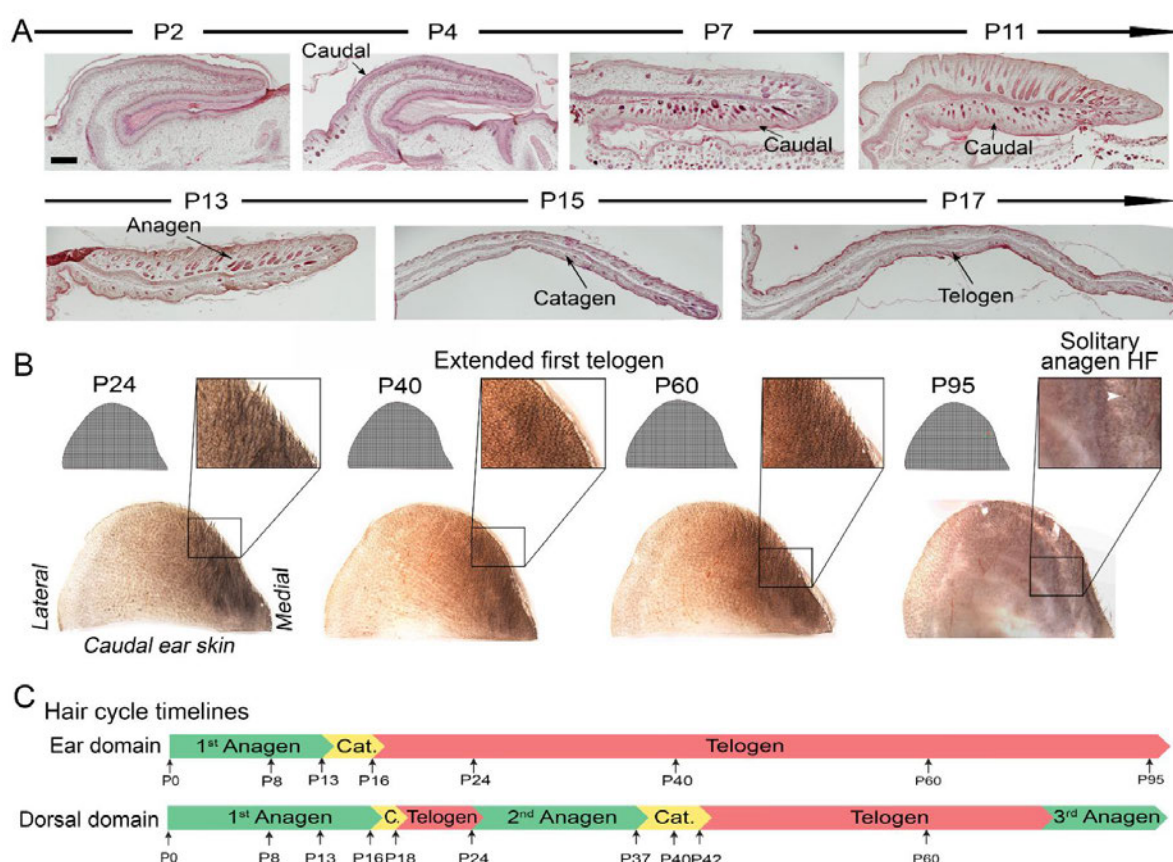
To exclude potential confounding factor coming from modification in the dermis and hair follicle *in vivo*, we have performed the following experiment, suggested by reviewer 1, to study modification in dermal thickness and hair follicle cycle.

Concerning dermal thickness, some previous papers have reported differences of dermal thickness between male and female animals (Azzi et al., 2005). In dorsal skin of adult mice, males have thicker dermis than females. In the same study, castration (with or without hormonal replacement) did not significantly modify male dermal thickness. To address the reviewer's question, we measured dermal thickness in the ear skin of control male and female mice, together with castrated mice with or without hormone replacement (DHT for male, E2 for female). In line with back skin data, male ear dermis was significantly thicker than female ($53.9 \pm 3.5 \mu\text{m}$ vs. $28.2 \pm 4.0 \mu\text{m}$) (Extended Data Fig. 16a,b). However, the castration of male mice did not significantly modify dermal thickness ($53.9 \pm 3.5 \mu\text{m}$ vs. $49.9 \pm 3.9 \mu\text{m}$) (Extended Data Fig. 16b). Only exogenous DHT replacement in castrated males significantly increased dermal thickness compared to castrated males ($65.7 \pm 2.8 \mu\text{m}$ vs. $49.9 \pm 3.9 \mu\text{m}$) but not compared to control males ($65.7 \pm 2.8 \mu\text{m}$ vs. $53.9 \pm 3.5 \mu\text{m}$) (Extended Data Fig. 16b). Therefore, as castrated males did not present significant difference compared to control males, it is unlikely that the differences observed in the epidermal dynamic arise from modification of dermal thickness. We have added and discussed these data in our revised manuscript.

Therefore, as castrated males did not present significant difference compared to control males, it is unlikely that the differences observed in the presented data arise from modification of dermal thickness.

Concerning hair follicle (HF) cycle, it should be noted that HF have a very specific behaviour in the ear skin compared to other regions of the mouse skin. Indeed, as

highlighted below, in contrast to HFs in mouse back skin that undergo hair cycle, the HF in the ear skin only performs one initial cycle (known as morphogenesis: anagen-catagen-telogen) and are considered afterwards as refractory to cycling (Wang et al., 2017). Few solitary anagen HFs could be observed, but the vast majority of HFs stay in the resting, telogen phase. To verify that castration did not interfere with this established, global telogen, we performed HF cycle assessment in both control and castrated males. Two weeks after castration, males had $96.7 \pm 3.3\%$ of HF in telogen phase, while control males presented $96.0 \pm 2.0\%$ (Extended Data Fig. 16d). Therefore, as castrated males did not present significant differences compared to control males, it is unlikely that the differences observed in the presented data arise from modification of the HF cycle. We have added and discussed this data in our revised manuscript.



Source: Figure 5 in Wang et al., 2017, eLife

In addition, a clarification on potential impact of immune cells and fibroblast was added to the discussion, line 660.

3. The potential contribution of sex chromosome-linked genes has not been explored. This could be addressed using the “four core genotypes” (FCG) model, which has previously been applied to studies of mouse skin, including the interplay between androgens and sex chromosomes in control of ILC2 and dendritic cells.

We thank the reviewer for this important comment. In line with the reviewer’s comment and reviewer 3’s suggestion we turned to the Four Core Genotype (FCG) mice model.

This model involves the deletion of the testis-determining gene *Sry* from the Y chromosome and introducing an autosomal *Sry* transgene (commonly on chromosome

3). Therefore, gonadal sex is determined by the presence of the transgene rather than by the Y chromosome itself. These mice present the following phenotypes: XX and XY mice with ovaries (Sry-), and XX and XY mice with testes (Sry+).

To study the basal cell delamination rate and the relative frequency of asymmetric TA cell division, we performed similar experiments as in the original manuscript by tracking EdU doublets after 96h of chase. Sry expressing individuals, irrespective of the chromosomal status, had a significantly increased delamination rate ($17.0 \pm 1.8\%$ for Sry+ vs. $7.7 \pm 1.3\%$ for Sry-) (Fig. 4d). When compared, XX and XY individuals did not present any statistically significant differences in terms of delamination, irrespective of Sry status ($13.5 \pm 2.0\%$ for XX vs. $11.2 \pm 3.0\%$ for XY). In terms of division fate outcome, Sry expressing individuals, irrespective of the chromosomal status, had a significantly increased rate of asymmetric division (K14+K10+ EdU doublets, $59.8 \pm 3.3\%$ for Sry+ vs. $42.2 \pm 4.1\%$ for Sry-) (Fig. 4e). When compared, XX and XY individuals did not present any statistically significant differences in term of division outcome, irrespective of Sry status ($53.0 \pm 6.6\%$ for XX vs. $49.0 \pm 3.7\%$ for XY). These results show that the Sry gene, and by extension male sex hormones, are driving the increased delamination rate and fraction of asymmetric cell divisions.

Altogether, these results indicate that the increased basal cell delamination rate and the relative frequency of asymmetric divisions is mediated by male sex hormones and are not mediated by other genes presented in the Y chromosome. As discussed above, in question number 2, we demonstrated that testosterone (DHT) can rescue the decreased basal cell delamination rate and the relative frequency of asymmetric divisions present upon castration, showing that testosterone is a key regulator of basal cell delamination and asymmetric division. We have presented and discussed these new data in the revised manuscript.

In addition to study sex chromosome and androgen a missing part of our study was the role played by female sex hormones and estrogens. To this end we studied their role in proliferation. To better understand how proliferation was controlled in male and female mice, we performed 4h EdU pulse experiments in homeostatic conditions. In the ear skin IFE, female mice presented a higher degree of proliferation compared to male mice ($7.2 \pm 0.3\%$ vs. $5.6 \pm 0.4\%$) (Fig. 4j-k). Modulation of male sex hormones by castration with or without supplementation with DHT did not influence proliferation of basal IFE cells (Fig. 4k). However, modulation of female sex hormones by ovariectomy decreased significantly the proliferation rate ($7.2 \pm 0.3\%$ vs. $4.9 \pm 0.3\%$), and estradiol administration with a slow release implant at the same time as the ovariectomy rescued the higher proliferation rate ($7.2 \pm 0.3\%$ vs. $7.6 \pm 0.8\%$) (Fig. 4k). This goes in line with previously published data that high estrogen administration can mediate epidermal thickness and proliferation through Era (Movérare et al., 2002). Of note, ovariectomy did not have any impact on basal cell delamination (Fig. 4b). Finally, to characterize the impact of female sex hormone on cell coordination we performed long term clonal analysis using intravital imaging on ovariectomized K14CreER control mosaic mice. The absence of female sex hormone led to a nearly total disappearance of enrichment of clones composed of even number of basal cells (Fig. 4m and Extended Data Fig. 9j), suggesting that the control of estrogen on the proliferation of basal cells has an impact on the female-specific higher degree of basal cell coordination.

4. The observed differences may be limited to the mouse ear epidermis, and the broader relevance of the findings to other anatomical sites and to human biology remains unexplored.

We thank the reviewer for raising these important questions.

To assess, as the reviewer suggested, the broader relevance of the findings to other anatomical sites we have now studied 3 different skin anatomical sites presenting slightly different histology and differentiation: ear, back and tail skin. As applied to ear skin, we performed lineage tracing and clonal analysis experiments using K14CreER-ControlMosaic mice (Extended Data Fig. 4a-f). As with the ear-skin, the clone size distribution for female mice differs from male mice for both back skin (Extended Data Fig. 4b) and tail skin (Extended Data Fig. 4d), with even basal clone sizes being more enhanced in females relative to males. These results demonstrate that the higher level of temporal coordination in sister cell fate decisions is a hallmark of female epidermal cells across multiple skin regions. We have now presented and discussed these data in the revised manuscript.

In addition, as shown in question number 2 and suggested by the reviewer, we assessed the human relevance of our data. To this end, we studied *in vitro* 3D skin equivalent (3D SE) (Tham et al., 2025). This method is one of the only that allows to study a properly stratified tissue in-vitro, with minimal and controlled media composition. Primary human keratinocytes, from male et female donors were seeded on 3T3-J2 mixed with collagen gels (final concentration of 4 mg/mL) (Extended Data Fig. 17n). Human keratinocytes were culture in presence or absence of 100nM of DHT. At D1 after keratinocytes seeding, they were moved to air-liquid interface to allow for proper stratification of the skin equivalent. At D9 after air lift, once the skin equivalent was properly stratified, a pulse of EdU was administrated and chased for 72h. Trying to reproduce *in vivo* EdU pulse and chase experiment presented in the initial manuscript. 3D SE was than fixed and embedded in paraffin to allow delamination quantifications. No significant modification of proliferation rate was observed between control and DHT treated culture (Fig. 6-i). However, following a chase of 3days, DHT treated human keratinocytes, presented a significantly greater fraction of EdU labelled suprabasal cells ($37.16 \pm 1.99\%$ Vs $24.61 \pm 2.04\%$) - number of EdU+ suprabasal cell over total EdU+ cells (Fig. 6j). This *in vitro* experiment is consistent with a direct effect of DHT on human keratinocyte delamination, in the absence of any immune cells.

Finally in order to validate that DHT can have a molecular impact on human keratinocyte, we perform *in vitro* culture of human derived skin epithelioid (Herms et al., 2024). In contrast to the 3DSE, this technique allows us to perform keratinocyte culture without a support of 3T3 gel (Extended Data Fig. 17o). The drawback being a lower level of stratification, not allowing pulse and chase experiments but still sufficient to study the initial step of differentiation. Human skin samples were harvested during planned surgical interventions and fresh samples were cultured. Once confluency reached, the biopsy was removed and the epithelioid was lifted to air-liquid interface to allow proper stratification. After two weeks, the epithelioids were placed in a starvation medium for 3 days, and then 3 days with or without 100nM of DHT. Whole epithelioid, including basal and suprabasal layers were collected for RNA extraction. Out of the 3 patients (2 male – including 1 facial and 1 eyelid biopsy and 1 female facial biopsy) 2 did present an

important increase of differentiation associated transcripts (EVPL, IVL, KRTDAP, OVOL1 and TGM1) upon testosterone treatment (Fig. 6k). The increased expression of differentiation associated genes goes along with the putative role of DHT to promote basal cell delamination and stratification. In contrast to the mouse samples where only basal cells were analyzed the human *in vitro* assays, basal and differentiated suprabasal cells were collected. With this we analyzed the impact of DHT on basal and suprabasal cells which increased differentiation of culture keratinocyte.

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We thank the reviewers for their positive assessment of our work. In this revised manuscript, we have addressed all their remaining questions, added clarifications, and refined the scope of our claims as requested. We have primarily enhanced the description of the Results sections and added supplementary information to the Methods section. Some more technical descriptions in the results section have been moved to the Methods to meet the editorial recommendations on the manuscript length.

Referee #1 (Remarks to the Author):

The revised manuscript is substantially strengthened by the extensive new experiments and analyses. The additional live-imaging experiments provide direct evidence for temporally coordinated sister-cell fate events, and the new Lgr6CreER and Notch1CreER lineage-tracing studies establish an important connection between transcriptionally defined populations, clonal behaviour, and the proposed mathematical framework. Together, these additions address several of the major concerns raised in the original review and significantly improve the manuscript. I therefore find the central conclusion that epidermal homeostasis is regulated in a sexually dimorphic manner to be well supported. The manuscript makes an important contribution to our understanding of epidermal dynamics and stem-cell regulation.

After reading the revised version, however, I find one remaining gap between the experimental observations and the model-based explanation of the reported sexual dimorphism. I am not requesting additional experiments, but I think further discussion would help readers better understand how the proposed mechanism is supported by the available data.

The revised live-imaging experiments demonstrate that temporally coordinated sister-cell behaviour occurs within the experimental observation window (12-h imaging intervals over 5 days). At the same time, the modelling framework, particularly the analyses presented in Extended Data Fig. 7, suggests that coordinated division and delamination events play a central role in generating and maintaining the characteristic even-odd clonal structure. However, it remains less clear how the experimentally measured coordination relates quantitatively to the coordination parameters inferred by the model, and how observations made over a relatively short temporal window are linked to the long-term clonal statistics analysed over several months. It would be valuable for the authors to discuss more clearly and directly how the experimentally observed coordination events map onto the corresponding model parameters, and how these measured and inferred quantities together account for the persistent enrichment of even-numbered clones and the reported sexual dimorphism. Such discussion would help readers distinguish more clearly between features that are directly supported by experimental observation and those that rely more heavily on model-based inference.

This point does not diminish the importance of the study or the substantial advances achieved through the revision. Rather, a more unambiguous discussion of this issue would help readers better appreciate both the strengths of the experimental findings and the role of the analytical framework in interpreting their biological significance.

We thank the reviewer for the positive evaluation of our work and kind remarks.

For a non-invasive probe, short-term imaging provides the ideal modality for measuring temporal coordination parameters. Indeed, the coordination parameters measured in the short-term live-imaging data directly map almost directly onto the parameters of the model. The correlation of sister lifetimes between delaminating sisters corresponds to the parameter c_{DD} for the model (which is found to be heavily constrained by the data for female mice but has a broad error bar for male mice). The correlation of the lifetimes between one sister dividing and another sister delaminating in the short-term live-imaging corresponds to the parameter c_{TD} in the model. (We assume that $c_{TD}=0$ in the model to keep the modelling framework minimal, noting that adding this parameter does not significantly improve fits, with the parameter being unconstrained by the long-term time-lapse data). Finally, the correlation in lifetimes between dividing sisters is a combination of the different stem cell division modes, with $rc_{SS}+(1-r)c_{ST}$ corresponding to the

experimentally measured parameter. (For the model, there is a single correlation parameter for daughters from symmetric divisions, $c_{TT}=c_{SS}$).

However, in practice, we find that short-term live-imaging protocol leads to an overall acceleration in the proliferation rate of basal cells, potentially a consequence of the repeat imaging possibly leading to local inflammation. Beyond estimates of the cell division rates, this can lead to quantitative differences in the inferred parameters as compared to those obtained from the long-term live-imaging. In particular, from the fine-grained even-odd features in the clonal distributions obtained from short-term live-imaging (Figure 1m, Extended Data Figure 3e) or wholemount (Extended Data Figure 3g-h and 4a-d), the modelling-based analyses indicate differences in the capacity of TA cells to asymmetrically divide (as indexed by the parameter q) and the temporal coordination between delaminating sister cells (c_{DD}). Notably, the model shows that a reduction in the degree of temporal coordination between sister cells or an increase in the proportion of asymmetric TA divisions will diminish and eventually erase the even-odd signature, which tightly constrains these parameters for female mice (where these correlations are strong), and limits the confidence in these parameters (reflected by broader error bars) in the male mice (where these correlations are weak). The Supplemental Theory (pg 21, subsection titled “Determination of spatiotemporal correlation parameters”) has been modified to highlight these relationships.

Referee #2 (Remarks to the Author):

The authors have addressed all the issues raised from the first revision. The new results added on the wound healing and new mouse models of sexual dimorphism have greatly strengthened the work. I think this is an outstanding study highlighting a previously unknown sex dimorphic adult stem cell behavior with clear consequences over tissue homeostasis and overall physiology. As such, I strongly recommend its publication in Nature.

[We thank the reviewer for the positive evaluation of our work and kind remarks.](#)

Referee #3 (Remarks to the Author):

The authors have done commendably well to address reviewer comments and revise the initial submission substantially. However, there are still some areas that remain unresolved.

Response to previous comments:

Major comment #1:

Not fully addressed. I thank the authors for the additional experiments and analyses quantifying synchronization between sister cell events. The lineage tracking and the correlation analysis provide useful evidence for strong temporal coordination of sister cell fates. However, it remains somewhat unclear whether the synchronization metrics support the model's predicted sex difference. Among the three correlation measures reported, only the correlation between dividing sisters appears slightly higher in females, without reaching statistical significance, whereas the correlations for delaminating sisters do not show a consistent trend favoring females, and the confidence intervals overlap substantially. It would therefore be helpful if the authors could further clarify how these measurements support the predicted sex-specific difference in synchronization of cell division, differentiation and delamination, or explicitly acknowledge that the current data primarily demonstrate strong synchronization overall rather than a clear sex difference.

Otherwise, the phrase "female mice showing a highly synchronized pattern of cell division, differentiation and delamination" may overstate the strength of the evidence, given the lack of consistent, statistically supported differences between sexes.

We thank the reviewer for this important comment. We do agree that, in this specific experiment, no clear significant difference was observed, in contrast with the longer-term tracing originally performed.

We initially opted for longer-term tracing only, as in our experience daily imaging and manipulation may lead to a certain degree of inflammation. This leads to a slightly increased cell division rate in bi-daily imaging (cell division every ~2 days on average for dividing cells) compared to long-term imaging (cell division every ~6 days on average for dividing cells). Therefore, although bi-daily imaging has enabled us to gain valuable insight into the qualitative behaviour by showing directly features such as the tight coordination between sister cell events, one should be cautious extrapolating the results from the quantitative analysis. For quantitative parameter estimates, we tend to favour the results from the longer-time time-lapse imaging, which is subject to less manipulation and invasiveness. Indeed, given the emphasis that has been placed on short-term live-imaging in the literature, we view this as potentially an important message for the community, that due care is required in drawing conclusions from quantitative analysis in a "image intensive" setting.

We have now made sure that this discrepancy is clearly stated (Supplemental Theory pg 21-22) and have explained why we rely on long-term tracing rather than short-term tracing to estimate clonal dynamic parameters such as coordination between sister events, and the cell division and delamination rates. In addition, we would like to remind the reviewer that the clone size distribution pattern, which could only be explained by a higher degree of coordination of cell division, differentiation, and delamination in female mice, was observed in two independent body areas and during wound healing.

Major comment #3:

Not fully addressed. The additional lineage-tracing experiments strengthen the evidence for distinct SC and TA compartments and support the two-proliferative-compartment model. However, the original concern regarding the link between molecular characterization and the kinetic parameters of the model remains unaddressed. It would be helpful if the authors could further clarify how the sex-specific transcriptomic differences relate to the sex-specific model parameters, e.g., correlations between cell sisters, proliferation, differentiation, and delamination rate.

We thank the reviewer for this comment. If we understand correctly, this question relates to Major comment 4 and not 3.

Multiple sex-specific differences in transcriptomic data (single cell and bulk RNAseq) support sex-specific differences uncovered by clonal analysis and biophysical modeling.

ScRNA-seq shows that differentiation-related genes were higher in male basal cells. Consistent with this notion, SCENIC analysis reveals that regulon activity of Klf4, a key transcription factor promoting epidermal differentiation, was increased in males compared to females (Fig. 4p-r and Extended Data Fig. 12g-i).

Second, we found several sex-specific transcriptional difference in bulk RNA-seq of basal cells demonstrated that:

(1) Due to the faster delamination rate mediated by the testosterone and the resulting lower content of differentiated cells in the basal layer in male mice, bulk-RNA-seq of all basal cells shows a lower expression of differentiation-related genes, reflecting the lower content of differentiated basal cells, as demonstrated by Krt10 staining (Fig. 5c-f).

(2) Female mice had higher expression of proliferation-related genes, directly linked with higher proliferation in female IFE found by EdU pulse experiments (Fig. 3k and 5b).

(3) Transcriptomic data *per se*, does not directly provide the molecular explanation of the higher degree of coordination of stem cell division and differentiation.

At a broader level, the regulation of cell division timing – and specifically the length of the G0/G1 phase - and the factors that drive entry into S phase is an intensively studied field, yet insights into such control in tissues remain limited. Beyond transcription, multiple post-translational effects could impact on timing and the coordination of sister cell events, such as protein degradation and the segregation of fate determinants, could be hypothesized. We believe that the current observation provides a rationale for further mechanistic studies to unravel the causes of the coordination of stem cell division and differentiation. However, to interrogate the molecular mechanisms of such coordination in sister cell division and differentiation would require profiling sister cell lineages calling for spatial omics-based approaches. We agree that such questions would be of great interest to the community, but we would argue that they fall beyond the scope of the current study.

Major comment #7:

Mostly addressed. The authors are correct in their response that 2 biological replicates per sex is not a lot to use methods like edgeR or DESeq. Given the amount of extra (and arguably, much harder) work the authors did to address other reviewer points, these underpowered experimental

designs do detract from the credence of the results, even if multiple bioinformatic methods were applied.

We thank the reviewer for this comment. The scRNA-seq experiment was initially performed to investigate the cellular heterogeneity of the basal layer and to identify Stem, TA, and Diff populations. In our view, these findings are now well supported by both methods such as edgeR and DESeq2, as well as by lineage tracing experiments. The inclusion of each sex was done as a secondary objective, mainly to assess and control for any major sex-related bias, as discussed above. We have decided to include differential gene expression on scRNA-seq data as it was supported by some lineage tracing experiments, showing higher speed of delamination in males, as well as by some regulon activity by SCENIC analysis, showing higher expression of pro-differentiation regulons such as Klf4 in male mice. It would be difficult to predict whether increasing the number of samples would mitigate the limitations of our scRNA-seq approach, especially in detecting differences in a single cell type (keratinocyte), given the coverage depth constraints. We believe that bulk RNA-seq—already validated using edgeR or DESeq2 (Major Question 9 of the previous round)—is the most effective method for assessing sex-based transcriptional differences. Therefore, we believe that additional male and female samples for further scRNA-seq analyses would be of limited scientific value.

We made sure that the purposes and conclusions of each transcriptomic experiment were clarified in the revised manuscript.

Major comment #8:

Not fully addressed. First, the authors state that the “Male IFE SC cluster showed increased levels of Skint1 and differentiation-related genes...”, whereas the response appears to attribute the differentiation marker signal primarily to differentiated cells.

We thank the reviewer for this comment. There is probably a misunderstanding regarding our conclusions. In our interpretation, the increased levels of differentiation-related genes in the scRNA-seq clusters, although limited, are present across all three clusters and reflect cell behaviours and faster commitment to differentiation. In contrast, in the bulk RNA-seq, we mostly measure the consequence of this faster delamination, leading to an increased content of post-mitotic differentiated cells in the basal layer of female mice. However, the interpretation of scRNA-seq sex-specific cell fate is indeed based on a limited number of genes and of limited power, unlike the analysis performed on bulk RNA-seq that is much more robust. In our current revision, we made sure to interpret scRNA-seq sex differences with the necessary caution to avoid overclaiming and provide only potential explanations for these results.

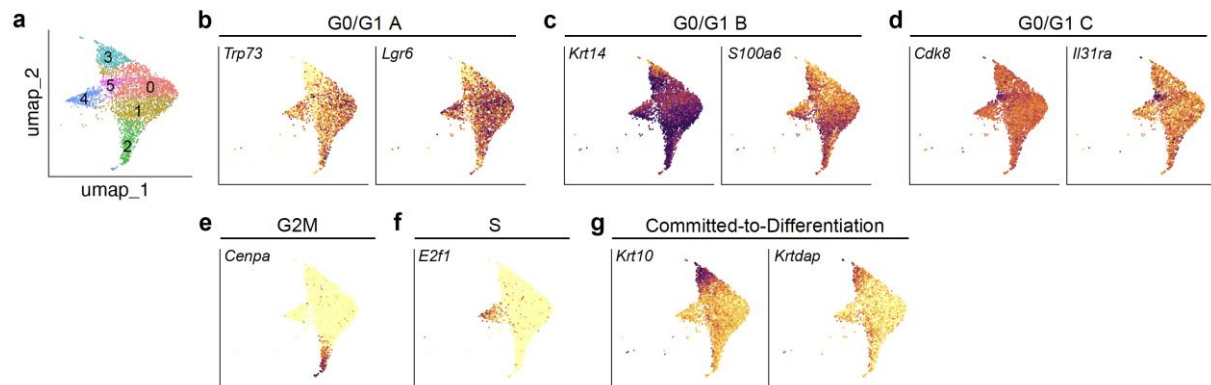
Major comment #12:

Why wasn't only CCA or only Harmony used for integration? Both methods have different underlying assumptions and potentially different effects on downstream analyses. It is fair that there is no single “best” integration method, but at least ensuring consistency with bioinformatic analyses would help strengthen the validity of the results.

We thank the reviewer for this important comment. We agree that different integration approaches rely on distinct assumptions and may influence downstream analyses. In this study, CCA-MNN and Harmony were used for different analytical purposes rather than interchangeably within the same workflow.

For two-dimensional visualization and cell-state annotation, we followed the established analysis framework used in our previous studies of the mouse interfollicular epidermis (IFE) (Bansaccal et al., 2023; Sarate et al., 2024). This approach, based on Seurat integration using canonical correlation analysis (CCA) and mutual nearest neighbors (MNN), has been extensively used to define and characterize the major IFE cell states, including G0/G1 A, G0/G1 B, G0/G1 C, S, G2/M, Committed, and Differentiated populations. Maintaining consistency with this established framework allowed direct comparison with previous datasets and ensured that cell-state annotations remained aligned with prior studies.

To evaluate the robustness of our results, we additionally performed integration using Harmony. While the overall cellular organization was broadly preserved, Harmony produced stronger mixing between transcriptionally distinct cell states. In particular, the committed-to-differentiation population, characterized by expression of *Krt10* and *Krt14*, became less distinctly separated from neighboring populations. This most probably lead to the “Committed” cluster disappearance in harmony integrated data. However, the biological relevance of this state was never investigated. Likewise, the G0/G1 A (*Trp73*, *Lgr6*) and G0/G1 B (*Krt14*, *S100a6*) states showed reduced segregation and less specific marker gene localization following Harmony integration. By contrast, the CCA-MNN workflow preserved these biologically interpretable cell states while still effectively correcting for sample-specific variation. We therefore selected the CCA-MNN integrated dataset for initial 2D visualization and comparison with previous studies. Importantly, none of the biological conclusions of the study rely solely on the two-dimensional UMAP representation.



For downstream manifold learning analyses, including diffusion map construction and three-dimensional force-directed embedding (3D FLE), we used the Pegasus framework. Harmony was employed in this context because it is natively integrated within the Pegasus workflow and provides efficient batch correction prior to diffusion-based manifold construction. Diffusion-map-based approaches are widely used for studying continuous cellular transitions because they preserve nonlinear relationships between cell states and capture the global organization of differentiation manifolds. The resulting diffusion manifold was visualized using three-dimensional force-directed embedding (FLE), which facilitates exploration of the manifold topology and cellular trajectories in three dimensions. The previously established cell-state annotations were then projected onto the three-dimensional manifold for interpretation of the resulting trajectories and cellular relationships.

Importantly, the major biological conclusions of the study do not depend on the specific integration method. The principal cell populations, marker-gene expression patterns, and inferred biological relationships were consistently observed across integration approaches. Furthermore, differential expression analyses were not based on the two-dimensional UMAP representation.

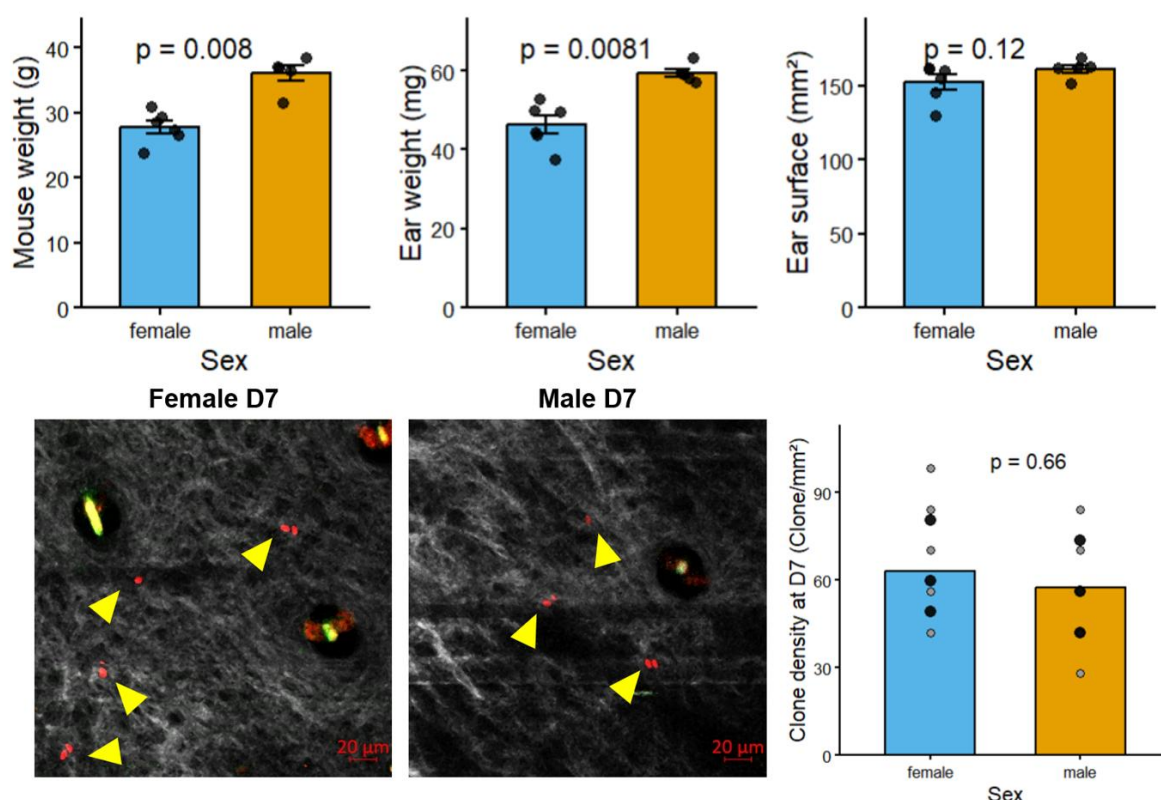
Therefore, our conclusions are unlikely to be driven by the choice of either CCA-MNN or Harmony integration.

To clarify this point, we have revised the Methods section to more explicitly describe the rationale for using CCA-MNN for annotation to maintain consistency with the established annotation framework, and Harmony for downstream manifold analyses.

Other major comments:

- How much is clone size affected by the size of the mouse? Male and female mice differ significantly in weight, bone structure, and so forth. Does that confound the clone sizes estimated? Is there a way to look at “relative” clone size or clone density (per area)?

We thank the reviewer for raising this concern. Indeed, male mice tend to be significantly bigger than female mice. We have now measured P60 mice body weight and ear weight (as our main tissue of interest) (N=5 male and 6 female mice from the same litter). As predicted, male mice tend to be significantly heavier than females in both total weight (36±1 g vs. 28±1 g) and ear weight (59±1 mg vs. 46±2 mg). The ear surface area was however not significantly different between sex (161±3 vs. 152±5 mm²). To our understanding, this means that extra weight originates from the dermis (previously demonstrated in Extended Data Fig. 16b), adipose tissue or cartilage and not from a larger ear skin. Since the body weight imbalance was previously known, we always normalized the dose of tamoxifen (0.2 mg/30 g) to obtain similar clonal density in all animals (63±9 vs. 57±9 clone/mm², in N=3 male and female at D7).



In our setting, as IFE surface area and clone density are the same in both sexes, such consideration should therefore not apply.

- In these types of Voronoi models, usually the energy to be minimized includes a term describing interfacial tension (based on adjacent center-to-center lengths). Is there a reason it was

neglected in this model, other than the fact that it wasn't included in the original proposed framework? That would also help reduce numerical instabilities when modelling cell division, as one could introduce a rule that the reference length between newly divided cells slowly increases to the mature reference length.

The present model includes interfacial tension. In vertex models, linear terms in junction length ($\Lambda_i l_i$) capture the balance between interfacial tension (positive contribution to the linear term), and cell-cell adhesion (negative contribution to the linear term). In the case where the interfacial tension is assumed to be homogeneous across all cells in the layer – as we assume here – then the interfacial tension terms across all junctions for a cell can be combined into the linear perimeter term ($-k_P P_i P_0$). A comment has been added to the Supplemental Theory (pg. 5) to clarify this important point:

“The first term corresponds to volume incompressibility (cell height can fluctuate). If we expand the second perimeter term, the quadratic perimeter term ($k_P P_i^2$) corresponds to the active contractility of the cell cortex, and the linear perimeter term ($-2k_P P_i P_0$) captures the balance between cortical tension (reducing perimeter) and cell-cell adhesion (cells sticking together try to increase the length of interface).”

- Considering the epidermis consists of several confluent layers, I am not fully convinced that modeling a top-down view of a monolayer interspersed with basal and suprabasal cells is a suitable abstraction of the skin epidermis, as the cellular neighborhoods would be significantly different. As an alternative, the authors may want to consider a model of 2D cross-sectional view of the epidermis and model the different layers or implement a 3D Voronoi model. That way, detachment and subsequent removal of suprabasal cells would be clearer.

The present spatial Voronoi model is only of the basal layer of the epidermis, as was stated in the main text (line 161), with the differentiated cells representing those that are committed to differentiate and delaminate but are yet to delaminate. The suprabasal compartment is modelled as “zero-dimensional” (i.e., spatially unconstrained) with the shedding time drawn from a Gamma distribution (pg 6-7 of Supplemental Theory).

A 2D cross-sectional view (Fiore et al., 2020) or 3D Voronoi model may be able to better capture model spatial correlations in processes across cell layers but would require a more detailed consideration of how mechanical properties change as cells transit through the layers, as well as mechanical changes preceding delamination, as was considered in recent work (Sahu et al., 2025; Hassikpezi et al., 2026; Villeneuve et al., 2026). These details would make the model much more complex, and would add new parameters that are unlikely to be constrained by the present data. At long times, it is likely that mechanical influences on cell delamination and shedding can be captured by effective rates and coordination parameters. Therefore, for simplicity, we therefore opted against turning to a more complex 3D vertex-type model in this study. We have added a short discussion on 3D models to the Supplemental Theory (subsection titled “Mechanics of cell delamination” on pg.20).

- Considering the authors already modelled mechanical feedback on cell division, did the authors consider density-dependent cellular extrusion?

It is not possible to include density-dependent cellular extrusion from the suprabasal cell layer in the present model as we consider only a zero-dimensional description of that layer (with cells being shed with a fixed lifetime distribution). However, we have previously considered mechanical feedback on cell delamination from the basal to suprabasal layer in a variant of the one-progenitor

model (results not shown). This feedback modulates the delamination rate locally, so influences the distribution and spatial correlations of delamination times. In natural implementations of such a model variant (where cell delamination is triggered when cells fall below an area threshold), it is challenging to engineer a very strong coordination in the delamination time between sister cells. This happens since once a cell delaminates more space is created for its sister cell, slowing down the sister cell's propensity to delaminate.

An alternative model of delamination could be implemented, where cells transiently shrink to a point before removal or using a 3D model (Sahu et al., 2025; Hassikpezi et al., 2026; Villeneuve et al., 2026) where cells transfer from the basal to suprabasal layer with the commitment to delamination corresponding to a switch in the mechanical properties that leads to a reduction of the basal footprint (for example, having a target cell area of $A_0=0$ in a 2D model). In such a variant, having a mechanical feedback rule governing the initiation of a basal differentiated cell delamination could still maintain a relatively high degree of synchrony between sister cells, since sister cells could be triggered to initiate delamination at a similar time.

In either variant of mechanical feedback on delamination, further free parameters would need to be added, which are not well constrained by the data. We therefore opted against adding this additional complexity. In contrast, the density-dependent feedback on stem cell fate influences the profile of the average basal clone size in the long-term, which can affect the fits of the data when the proportion of stem cells is not too low.

We have included discussion of this point in Supplemental Theory (subsection titled “Mechanics of cell delamination” on pg.20).

- “3D visualization allowed to remove the confounding effect of cell cycle on G0/G1 cells clustering, while leaving the opportunity to study the ability of G0/G1 cells to divide.” I am not sure how 3D visualization removes a confounding effect rather than, say, additional statistical analysis (like the regression step performed by the authors prior to clustering) or removing the non-G0/G1 clusters from further analysis.

We agree with the reviewer that the original wording was imprecise. Our intention was not to imply that the three-dimensional visualization statistically removed or corrected for cell-cycle effects. Rather, the diffusion-map-based three-dimensional manifold provided a representation that better preserved the continuity of cellular state transitions. In the two-dimensional UMAP embedding, S-phase and G2/M cells appeared artificially separated, which could give the impression of discrete rather than continuous proliferative states. In contrast, the three-dimensional manifold revealed a continuous trajectory linking G0/G1, S-phase and G2/M populations, consistent with the expected biology of cell-cycle progression. This improved representation allowed us to interpret which and how G0/G1 populations were connected to proliferative states while preserving the underlying topology of the cellular manifold. We have revised the text accordingly to clarify this point.

- Throughout the paper, the adjusted p-value threshold to determine significance or differential expression changes between analyses. What is the main reasoning behind these choices?

- The previous comment applies to assessment of “differential regulon activity”.

We thank the reviewer for this comment. The significance thresholds were chosen according to the objective and statistical characteristics of each analysis rather than applying a single cutoff throughout the study. For differential gene expression between cell states or clusters, where a

large number of genes were tested simultaneously, we used a more stringent adjusted P-value threshold (0.01) to minimize false positives when defining cell-state-specific markers. In contrast, analyses of sex-associated transcriptional differences were designed to detect more subtle biological effects while still controlling the false discovery rate, and therefore an adjusted P-value threshold of 0.05 was used. Similarly, differential regulon activity was evaluated across only 221 inferred regulons, representing a substantially smaller multiple-testing burden than gene-level analyses. Under these conditions, an adjusted P-value threshold of 0.05 provides an appropriate balance between statistical stringency and sensitivity while maintaining FDR control.

Importantly, we provide the complete differential gene expression and differential regulon activity results in Supplementary Data, together with the SCENIC AUCCell scores for all regulons across all cells. Therefore, the significance thresholds were used primarily for visualization and presentation (e.g., volcano plots) rather than to define the complete set of results. Readers can readily apply alternative significance thresholds to the full datasets if desired. Importantly, the key findings highlighted in the manuscript remain significant under more stringent significance thresholds, indicating that the principal biological conclusions are robust and are not dependent on the specific thresholds chosen for visualization. We have clarified the rationale for the different significance thresholds in the revised Methods.

- Considering that other organs can produce sex steroids, like adrenal glands, adipose tissue, was it confirmed that castrated males and ovariectomized females do indeed have reduced sex steroid levels?

We thank the reviewer for this important question. Castration of male mice and ovariectomy of female mice are two well-established and recognized ways of studying the role of testosterone and estrogen deprivation (Van Nas et al., 2009). While it is true that adrenal glands and other organs can produce androgens, the vast majority are produced by the testes. As demonstrated by measurements performed in mice, testosterone levels decrease from 1.0 ng/ml to 0.1 ng/ml starting one week after castration (Song et al., 2017). This 90% reduction is therefore sufficient to produce the observed phenotype.

Similar data are available for estrogen, which is mainly produced by the ovaries and, to a lesser extent, by the adrenal glands and peripheral tissues. Different studies have shown that upon ovariectomy, estrogen levels drops significantly between 2 to 5 fold depending on the studies and the estrus cycle (Moran et al., 2007; Villablanca et al., 2009). Nevertheless and despite the low level of residual estrogen, ovariectomy leads to the abrogation on the estrus cycle and other estrogen related changes in peripheral tissues such as the vagina and the uterus (Haisenleder et al., 2011).

In addition, since testosterone can be locally converted into estrogen by the enzyme 5 α -reductase, whereas dihydrotestosterone (DHT) cannot, we used DHT in our implant experiments to avoid any confusion.

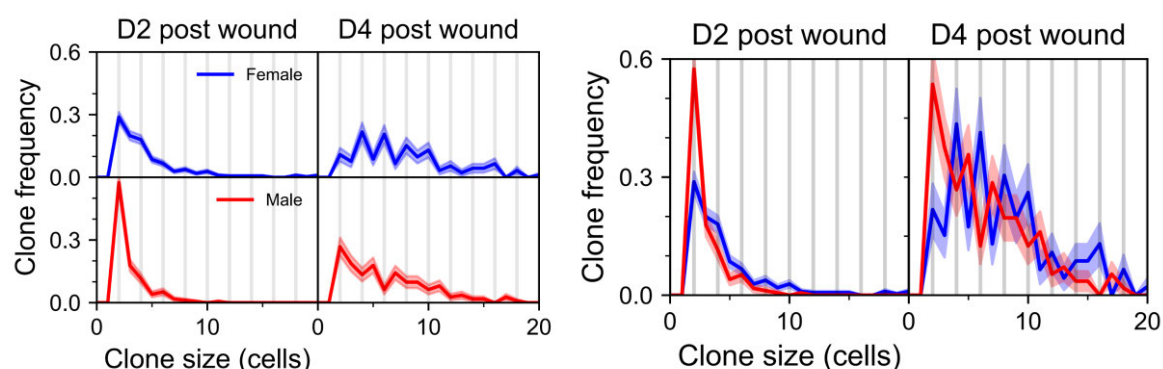
Minor comments:

- Some of the manuscript text was difficult to follow. Granted, I understand that adding all of these revisions has significantly expanded the scope of the manuscript, but I think taking some time to make the results text session as clear as the Introduction (which is very well written) would help improve clarity a lot.

We acknowledge this important point and have sought to improve clarity of the results section in the current revision. Should the manuscript be accepted, we will ensure that the final revision is presented as clearly as possible, given the editorial requirements on overall length.

- Fig. 2f-k: It would may make the results easier to interpret if the clone size fractions of males and females (basal vs. basal, suprabasal vs. suprabasal) were overlaid on top of each other, like what was done for Fig. 2a-d. This comment also applies to Fig. 3i, and Extended Data Figures 3, 4, 5, 6, and 14.

We thank the reviewer for this suggestion. However, given that the panels are small, we feel that visually the results look clearer if the plots are shown on separate graphs. For example, in Fig. 3, if we would overlay the male and female curves, it makes it more difficult to interpret and compare the dependencies as the lines partially obscure one another and shape differences become harder to discern. As a compromise to ensure an easier visual comparison we have instead adjusted panels so that the corresponding male data is stacked vertically directly above/below the female mice.



- Fig. 4a and Fig. 4c have typos: ‘Casrtated’

We have corrected the typos.

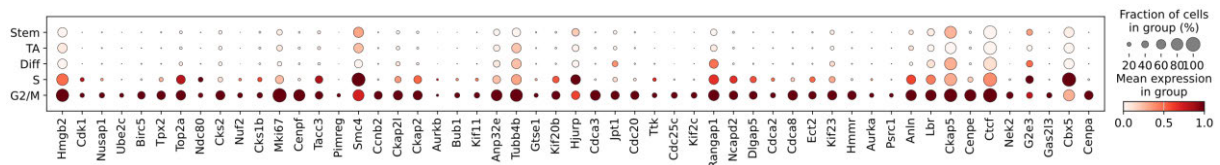
- Is the Voronoi model used in this work a cell-center (one point per cell) or a cell-vertex model (multiple points per cell)? The mathematical descriptions imply that it’s the former, but the text description make it sound like it’s the latter.

A Voronoi model is cell-center based (one point per cell). The wording in the main text and Supplemental Theory has been refined to make this clearer (pg 4 of Supplemental Theory):

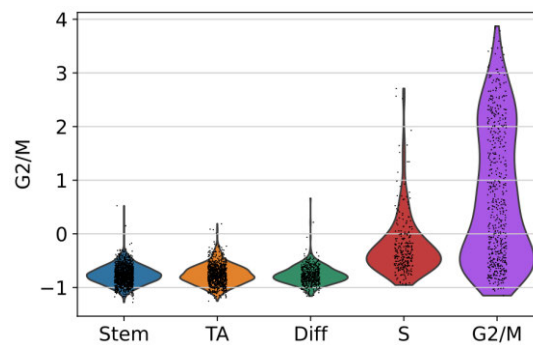
“Within the Voronoi model, the arrangement of cells i are defined by the spatial coordinates $\mathbf{r}_i$ of their cell centres.”

- Fig. 5: Does the G2/M population more resemble the stem or TA population? This can be resolved by examining the expression of the marker genes shown in Fig. 5d in this cluster.

We thank the reviewer for this suggestion. We examined the expression of a panel of canonical G2/M cell-cycle genes (*Top2a*, *Mki67*, *Cdk1*, *Ccnb1*, *Ccnb2*, *Cenpf*, *Ube2c*, etc.), selected from the established G2/M gene set implemented in the Scanpy and Seurat cell-cycle scoring workflows (originally described by (Tirosh et al., 2016)). As shown in the figure below, canonical G2/M markers are broadly detected in both the Stem and TA populations, without evidence of preferential enrichment in either compartment.



These observations are further supported by quantitative analysis of the Scanpy G2/M cell-cycle scores (see the violin plot below), which showed no significant difference between the Stem and TA populations (t-test, P value = 0.59; Wilcoxon rank-sum test, P value = 0.99), indicating comparable cell-cycle activity in the two compartments. Together, these findings are fully consistent with our clonal modelling, which predicts comparable proliferative capacities of Stem and TA cells, as well as with the RNA velocity analysis, which demonstrates that cells from both populations can progress into the S-phase and G2/M states. Thus, four independent lines of evidence—canonical G2/M cell-cycle marker expression, G2/M cell-cycle scoring, RNA velocity, and clonal dynamics—consistently support the conclusion that both Stem and TA populations actively contribute to epidermal proliferation. Accordingly, we do not find evidence that the G2/M population is preferentially associated with either the Stem or the TA compartment.



- Lines 1234–1239: technically differentially.

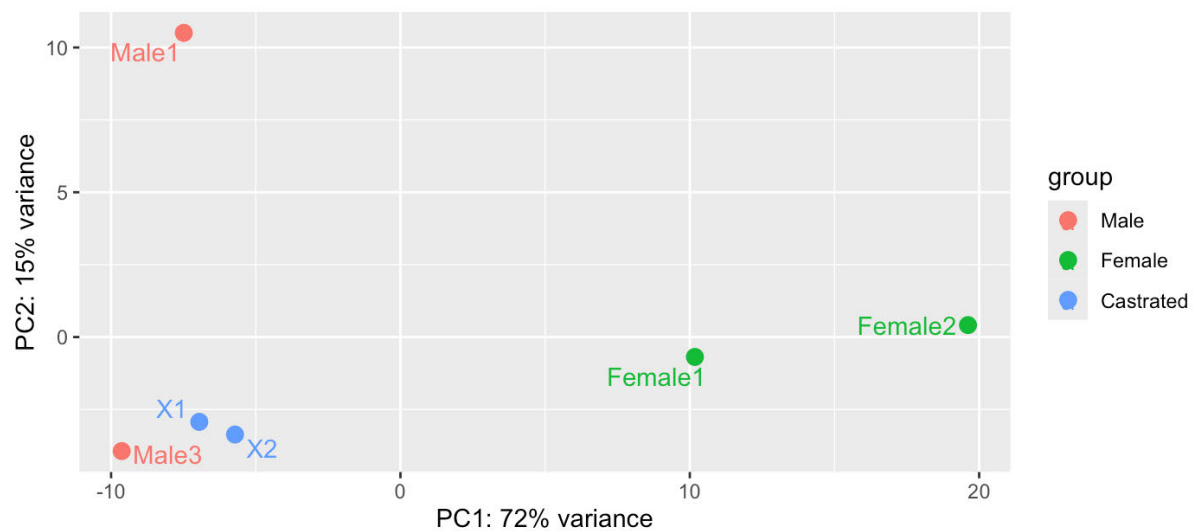
We thank the reviewer for this comment. We agree that the term "differentially regulated" is not technically accurate in this context. The text has been revised to “differentially expressed”.

- The phrase ‘RNA velocity’ is more widely used than ‘cell velocity’ in the field.

We thank the reviewer for pointing this out. We have revised the manuscript to use the term “RNA velocity” throughout.

- It would be nice to see the PCA plot of the bulk RNA-seq samples as described in the Methods.

We thank the reviewer for this suggestion. The PCA plot of the bulk RNA-seq samples is provided below. The analysis shows that samples separate according to biological condition, with female samples clearly segregating from male and castrated samples (X1 and X2) along PC1, which explains 72% of the variance. As expected, PC1 is driven by sex chromosome related genes (Xist, Tsix, Ddx3y, Uty, Eif2s3y or Kdm5d) and terminal differentiation genes (Sprr2a2, Sprr2a1, Sprr2b, Sprr2a3). These results indicate that the major source of transcriptional variation is associated with the sex differences.



- What are the cluster markers in Extended Fig. 11a?

We thank the reviewer for this comment. The clusters shown in Extended Data Fig. 11a correspond to the same epithelial populations that are annotated in Extended Data Fig. 11d with the exception of the tissue dissociation cluster (circled). In panel a, we specifically highlight the expression of tissue dissociation-associated genes (*Egr1*, *Atf3*, *Fosb*, and *Jun*) to identify and remove clusters enriched for dissociation-induced transcriptional signatures. Following this filtering step, the remaining cells were annotated into G0/G1A, G0/G1B, G0/G1C, G2/M, S, committed and differentiation populations, as shown in Extended Data Fig. 11d. Representative marker genes for each annotated cluster are presented in Extended Data Fig. 11e–j. No new clustering step was run after tissue dissociation cluster removal.

Referee #4 (Remarks to the Author):

The authors have addressed my concerns and should be congratulated for their comprehensive study.

We thank the reviewer for the positive evaluation of our work and kind remark.

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Referee #3 (Remarks to the Author):

The authors have answered all comments thoroughly and carefully, clarifying several points of confusion, and have improved the work significantly from its initial submission. I am happy for the manuscript to be accepted for publication.

We thank the reviewer for the positive evaluation of our work and kind remarks.

There are some final, minor comments:

- The axes of some plots across figures, e.g. like the ones shown in Fig. 1m, look strange with the '0, 5, 0', and '0.0, 0.3, 0.0' tiling. Maybe stagger 10/0 and 0.6/0.0, respectively, or add small double ticks.

The panel has been modified based on the reviewer's suggestions.

- As it is hard to draw meaningful conclusions about tissue composition from scRNA-seq data, which is biased in cell capture ability across cell type, I would tone down the conclusions in lines 549-552 in the Discussion section.

This sentence has been removed