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Peer Review File

Multilayered control of mRNA delivery to cell protrusions drives localized mini-cytoplasm establishment for stress-induced cell activation

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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:

Reviewer #1

(Remarks to the Author)
The manuscript by Duval et al. presents a compelling and methodologically innovative study that leverages a pooled genetic screening platform (ReGenT-seq) to identify chromatin regulators of EpSC activation. Their discovery of a cytoplasmic role for Cbx3 in directing subcellular mRNA localization within polarized protrusions of migrating EpSCs, and its role in establishing a localized "mini-cytoplasm," represents a conceptual advance. The authors demonstrate that Cbx3-dependent mRNA transport is critical for the spatial organization of organelles such as the ER and lysosomes in mature protrusions. The study is highly relevant to the wound healing and stem cell fields, offering new insights with potential translational implications. My main comments are:

1. While the ReGenT-seq approach is elegant, the manuscript does not provide sufficient detail on the shRNA design strategy. How many shRNAs were employed per epigenetic factor in the pooled screen? More importantly, how was knockdown efficiency verified? Given that off-target effects can significantly impact screen outcomes, a systematic validation of shRNA specificity and efficiency is essential.
2. The observation that Cbx3 promotes migration but inhibits proliferation is intriguing and reminiscent of the pathological state of chronic wounds, where keratinocytes often exhibit hyperproliferation but impaired migratory capacity. It would be valuable for the authors to explore Cbx3 expression patterns in human chronic wound tissues. Does Cbx3 expression correlate inversely with keratinocyte migration status in these contexts? Such data could enhance the clinical relevance of the findings.
3. Although the primary results are based on murine EpSCs, the study would benefit from a more explicit assessment of whether the Cbx3-dependent mechanisms are conserved in human primary keratinocytes. The authors briefly mention SCC and human samples, but is there functional evidence showing that Cbx3 similarly regulates mRNA localization, organelle recruitment, or protrusion formation in human EpSCs or keratinocytes?
4. Primary EpSCs typically lose their stemness and begin differentiating after 5–6 passages. However, the experiments described involve 3–4 weeks of culture. Can the authors provide data or morphological/marker-based evidence that the cells retained their EpSC characteristics throughout the course of these experiments? This clarification is essential to interpret whether the observed phenomena truly reflect stem cell biology or partially differentiated cell behavior.
5. In Figure 3K, the authors claim differential Cbx3 expression between basal and suprabasal layers. However, the identification of these layers appears to rely on histological inference rather than specific markers. I recommend the inclusion of basal (e.g., ITGA6, KRT14) and suprabasal (e.g., KRT10) markers to confirm the cellular identity. Additionally, the color coding in Figure 3K does not seem to align with the expression intensity presented in Figure 3J, raising questions about consistency in data representation.
6. The authors claim to use epidermal stem cells (EpSCs); however, based on the methods described, it is challenging to discern whether the isolated cells are indeed bona fide epidermal stem cells, transient amplifying progenitors, or simply basal keratinocytes. The terminology surrounding these populations remains somewhat ambiguous and inconsistently applied across the field. I encourage the authors to clarify whether their purification strategy truly enriches for epidermal stem cells. Have they performed any validation—such as functional assays, stem cell markers, or lineage tracing—that supports the identity of these cells as EpSCs rather than a broader basal or progenitor population?

Reviewer #2

(Remarks to the Author)
This manuscript claims to study cell protrusions in the context of stress in skin epithelial stem cells. To perform the studies, the authors used a novel screen called ReGEnT-seq, which they apply to chromatin factors controlling epidermal stem cell activation. Using this technique they identified Cbx3 as a critical protrusion factor. The authors also found that Cbx3 is in the cytoplasm of cells in wounds and cancers and governs the subcellular localization of specific mRNAs with polarizing protrusions with in vitro experiments suggesting that Cbx3 is plays a role in epidermal stem cell stress in protrusions. Altogether, the manuscript presents an impressive amount of next generation sequencing data and contains novel approaches, with detailed functional and mechanistic characterization of Cbx3 in epidermal stem cells. The identification of Cbx3 as a cytoplasmic regulator of mRNA localization and protrusion maturation is intriguing and represents a potentially important shift in the understanding of chromatin factors.
However, despite the richness of the data in the manuscript, it is extremely difficult to read. The central logic is obscured by dense language and an unclear narrative structure. Additionally, the manuscript contains numerous grammatical errors throughout, to the extent that it was difficult to catalog them all. Consequently, my enthusiasm for the publication is quite diminished.
Major:

Conceptual framing issue:

The manuscript frames the entire study around ‘stress induced cell activation’, but the experimental models do not test stress conditions. The main screen uses K6 promoter activity as a proxy for stress, yet no external stressors are applied in the foundational in vitro experiments. At its core the manuscript investigates how epigenetic regulators modulate K6 promoter activity, not stress induced K6 promoter activity. For example, K6 is expressed in the palmar plantar skin of the hand epidermis, hair follicles and sweat and sebaceous glands (Wang et al. 2018 and numerous other publications). Without experimental validation that the authors are inducing stress it is impossible to claim that the K6 promoter in their experimental system is activated by stress.

Misleading/confusing figures:

Importantly, the representation of the data in the manuscript is difficult to ascertain how and what experiments were performed. One example of many, the experimental setup in Figure 1a–c is fundamentally unclear and misrepresents the actual experiment that the authors performed. Specifically, Figure 1a misleadingly suggests that the K6-Blast and K6-DTR reporters were delivered into the same population of EpSCs, followed by pooled shRNA transduction and sequencing. If that were the case, it would make the selection logic uninterpretable, as cells could simultaneously express opposing survival/death signals. However, from the methods and description, it appears that the two reporter systems were used in separate, parallel experiments with otherwise identical conditions and shRNA pools. This distinction is critical to understanding the design and stringency of ReGenT-seq and must be explicitly clarified in both the figure and the legend. Otherwise, readers will be confused about how the reciprocal screening logic was applied and how hits were called. This one of many examples throughout the manuscript.

Reviewer #3

(Remarks to the Author)
In this paper, Duval et al. investigate epigenetic mechanisms involved in epidermal stem cell (EpSC) activation during wound healing and squamous cell carcinoma (SCC). Using a novel sequencing protocol, ReGenT-seq, the authors identified Kmt5c, Ehmt2, and Cbx3 as key epigenetic regulators for cell activation and cell protrusion. The authors utilized several molecular techniques to dissect how these epigenetic regulators mediate cell activation and cell protrusion. RNA-sequencing analysis in combination with GSEA analysis revealed that while Ehmt2 acts as a negative regulator of cell plasticity, Kmt5c and Cbx3 act as positive regulators of cell plasticity. To better understand how Cbx3 drives cell plasticity upon wound induction, the authors performed an in vitro scratch assay on Cbx3 KD cells, where they conclude that Cbx3 is a regulator of cell migration. To better understand the role that Cbx3 may play in wound healing and SCC, the authors investigated expression of Cbx3 in both contexts. They found that Cbx3 is in the migratory front of wounds, however in SCC, they found that Cbx3 was present in the cytoplasm of deep SCC areas, suggesting that Cbx3 may also play a role in the cytoplasm. Next, to study the role of Cbx3 in RNA-splicing, the authors utilized seCLIP-sequencing and found that Cbx3 mainly targets mature RNA at various sites. Next, the authors investigated the role of Cbx3 in mediating RNA nuclear export in EpSC plasticity and protrusions. To do this, the authors fractionated cells to separate nuclear and cytoplasm to perform RNA-seq and investigate differences in between RNA in the nucleus versus the cell protrusions. The authors performed a similar experiment in Cbx3 KD cells to interrogate which genes are regulated by Cbx3, which they conclude are genes associated with positive regulation of wound healing, collagen-containing ECM, focal adhesion, and establishment of cell organelles. Collectively, the authors conclude that Cbx3 plays distinct roles in the nucleus and cytoplasm that mediates cell plasticity and cell protrusion during both wound healing and SCC.

In summary, the authors describe a novel sequencing protocol and identify several potential functions of Cbx3 in regulating cell activation and protrusion in both wound healing and SCC. Most of the data for a great story are present in their paper, however, the rationale and story need to be reorganized. The authors should focus on making one main argument with several supporting details.

Major comment(s):

1) The author describes a very interesting and novel method to identify key epigenetic modulators; however, the validation is slightly unclear – are there previous reports using this method? Are you the inventor of this method? If so, a reference to previous work is needed.
2) The authors use RNA-seq to dissect the potential role of three epigenetic regulators in cell activation, Ehmt2, Kmt5c, and Cbx3 – while I understand the rationale, I think the explanation of the results is a little confusing. Which specific genes are dysregulated in the KD conditions? Which genes display splice variants or lack thereof due to the loss of Cbx3. If you start the argument with three potential roles for Cbx3: (1) transcriptional repression, (2) transcriptional activation, and (3) RNA-splicing, then walk us through each situation and conclude how Cbx3 is playing a role in this system. At this point, I would also try to make a strong argument to dissect only Cbx3 and no longer investigate the other two epigenetic regulators, as the jumping back and forth is confusing.
3) The authors claim that “Cbx3 promotes cell migration and invasion while it negatively impacts cell proliferation.” While in this results section, the authors describe the expression distribution of Cbx3 in both the wound front and SCC, they do not show any functional data to determine that Cbx3 promotes cell migration and invasion or that it impacts cell proliferation. To do this, I would suggest developing models, either in vivo or in vitro, and test the effects of the loss of Cbx3 on cell migration, invasion, and proliferation. Something like the 2D and 3D models that are described later in the paper.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed my comments thoroughly and satisfactorily. I have no further concerns and recommend acceptance of this manuscript.

Reviewer #2

(Remarks to the Author)
The authors have clarified the figures particularly Figure 1a to more accurately represent their new and novel screening technique addressing that concern. Similar corrections have also been addressed in subsequent figures. The grammatical errors have been corrected and the revised manuscript is much improved.

A issue on K6a/b use as exclusively a marker of activation of cellular stress. The authors have tried to address my concern experimentally by generating a new K6b-GFP promoter and exposed cells to multiple physiologically relevant stressors showing through flow cytometry analysis that K6 is upregulated. These experiments show that stress induces K6b expression. It does not show that K6b expression is exclusively linked to stress as keratinocytes in sweat glands in humans and other non stress conditions can and may express K6a and k6b (simply google searching and investigating webtools to search for k6a/b expression in scRNA-seq data sets reveals this). Consequently, I'm not convinced that K6 expression exclusively marks stress conditions but rather different states of keratinocytes differentiation, including stress. The fact that K6 expression is not exclusive to stress does not invalidate the manuscripts premise but k6 expression outside of stress should not be ignored. To address my concern (no new experiments are needed) the authors can add this critical discussion point to their introduction and in their discussion as a caveat and/or alternative interpretation to their study. This would greatly enhance the manuscript.

Reviewer #3

(Remarks to the Author)
In the manuscript “Multilayered control of mRNA delivery to cell protrusions drives localized mini-cytoplasm establishment for stress-induced cell activation,” Duval et al. examine mechanisms that regulate epidermal stem cell (EpSC) activation during wound healing and squamous cell carcinoma (SCC). Using a newly developed sequencing approach (ReGenT-seq), the study identifies Kmt5c, Ehmt2, and Cbx3 as epigenetic regulators involved in cell activation and protrusion formation. A combination of genomic and molecular approaches is then used to explore how these regulators contribute to EpSC plasticity.

RNA-seq combined with GSEA analysis suggests that Ehmt2 acts as a negative regulator of cell plasticity, whereas Kmt5c and Cbx3 function as positive regulators. To investigate the role of Cbx3 during wound activation, the authors perform scratch assays in Cbx3 knockdown cells and report reduced migratory capacity, suggesting a role for Cbx3 in cell migration. Expression analysis further shows that Cbx3 is enriched at the migratory front of wounds. In SCC samples, however, Cbx3 appears predominantly cytoplasmic in deeper tumor regions, raising the possibility that it also functions outside the nucleus.

To better understand the molecular activity of Cbx3, the authors perform seCLIP-seq and find that Cbx3 primarily binds

mature RNAs at multiple sites. They then explore whether Cbx3 regulates RNA nuclear export during EpSC activation and protrusion formation. Nuclear and cytoplasmic fractions are isolated and analyzed by RNA-seq to compare RNA localization patterns. Similar analyses in Cbx3 knockdown cells identify gene sets associated with wound healing, extracellular matrix organization, focal adhesion, and organelle establishment. Based on these findings, the authors propose that Cbx3 plays distinct roles in the nucleus and cytoplasm that contribute to cell plasticity and protrusion dynamics during wound repair and SCC.

The manuscript introduces an interesting sequencing strategy and presents several observations suggesting roles for Cbx3 in cell activation and protrusion biology. The dataset is substantial and potentially supports a compelling story. However, the overall narrative would benefit from clearer focus and organization. At present, multiple mechanistic directions are explored in parallel, which makes the central message somewhat difficult to follow.

Major comments

1. The RNA-seq and ChIP-seq analyses are often described in broad terms, which makes it difficult to evaluate the conclusions. For example, the statement that “ChIP-seq peaks with deregulated genes in CBX3-depleted cells revealed only a limited direct role for CBX3 in regulating migration-associated genes” is quite general. It would help to highlight specific genes identified in these analyses and explain how they support the proposed functional model. Providing concrete examples throughout the Results section would make the analysis more convincing and easier to follow.

2. The manuscript explores several possible functions of CBX3, including transcriptional regulation, RNA processing, RNA export, and cytoplasmic roles. While each of these analyses is interesting, the overall narrative becomes somewhat fragmented. A more focused story would strengthen the paper. For example, the manuscript reports that splicing alterations mainly affect genes linked to proliferation and RNA processing rather than migration, yet earlier sections emphasize a role for CBX3 in cell migration. Clarifying this discrepancy—or narrowing the analysis to mechanisms directly linked to migration—would improve the overall coherence of the study. In addition, the discussion frequently moves between wound healing and SCC, which can make the flow harder to follow. Structuring the manuscript so that SCC is discussed primarily toward the end may help maintain a clearer narrative.

3. It remains somewhat unclear whether nuclear CBX3 activity directly contributes to migration phenotypes. The data appear to suggest a stronger role in protrusion formation and focal adhesion dynamics. If this is the central mechanism, the manuscript could benefit from emphasizing these processes more clearly. The relevance of the RNA-processing analyses to migration should also be clarified.

4. The data analyses are generally thorough, but the Results section would benefit from clearer guidance through the experimental logic. In several places, figure panels, experimental descriptions, and data interpretation are presented somewhat separately. Integrating these elements more tightly would help readers follow how each experiment supports the overall model.

Point-by-point Response to Reviewers' comments

Authors' comments:

- We thank all the reviewers for their thorough analysis of our manuscript and for the constructive suggestions.
- For an easier assessment by the editor and the reviewers, the changes in the main text with respect to the first submission are highlighted in blue.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript by Duval et al. presents a compelling and methodologically innovative study that leverages a pooled genetic screening platform (ReGenT-seq) to identify chromatin regulators of EpSC activation. Their discovery of a cytoplasmic role for Cbx3 in directing subcellular mRNA localization within polarized protrusions of migrating EpSCs, and its role in establishing a localized "mini-cytoplasm," represents a conceptual advance. The authors demonstrate that Cbx3-dependent mRNA transport is critical for the spatial organization of organelles such as the ER and lysosomes in mature protrusions. The study is highly relevant to the wound healing and stem cell fields, offering new insights with potential translational implications.

We are grateful for the Reviewer's enthusiasm and for recognizing the "innovative" methodology of our study. We appreciate the assessment that our work is compelling, highly relevant to the field, and offers new potential translational implications.

My main comments are:

1. While the ReGenT-seq approach is elegant, the manuscript does not provide sufficient detail on the shRNA design strategy. How many shRNAs were employed per epigenetic factor in the pooled screen? More importantly, how was knockdown efficiency verified? Given that off-target effects can significantly impact screen outcomes, a systematic validation of shRNA specificity and efficiency is essential.

We recognise that details regarding the shRNA library were insufficient in the original submission and have now addressed this in the revised manuscript.
Although ReGenT-seq is a newly developed approach, it was performed using a previously generated and well-characterised shRNA library. Specifically, we employed an shRNA library developed by the Johannes Zuber Laboratory (Research Institute of Molecular Pathology, Vienna BioCenter, Vienna, Austria), which has been extensively validated and previously published (Cheloufi et al., *Nature* 2015; already cited in the Results section).

As described in Cheloufi et al., this is a miR-E–based, chromatin-focused library comprising 5,049 sequence-verified miR-30 shRNAs targeting 615 known and predicted chromatin regulators. To clarify the origin, composition, and key features of the library, we have added detailed information in the revised manuscript, both in the Results (**Fig.1A and 1F)** and in the Methods section.

With respect to specificity, the shRNA library was originally designed to minimise off-target effects through a systematic optimisation strategy. Cheloufi et al. refer to prior work from the Zuber laboratory describing optimisation of the experimental miR-30 backbone and hairpin structure, which is essential for robust single-copy activity in pooled RNAi screens (Fellmann et al., *Cell Reports* 2013). For target sequence selection, shRNAs were predicted *de novo* using the DSIR algorithm, focusing on regions common to all known transcript variants. These predictions were further filtered using "Sensor rules" to enrich for sequence features associated with efficient shRNAmir processing and potent knockdown (Fellmann et al., *Cell Reports* 2013). In addition to this prior validation, we directly assessed shRNA specificity in the context of the present study. As shown in **New Supplementary Fig.1A,B**, alignment of each shRNA against all known transcript variants revealed that 96.4% of shRNAs display a strong primary alignment to a single expected target gene, with no comparably strong secondary alignments. Moreover, gene-level hits in our screening are derived from the combined activity of multiple shRNAs per gene. As detailed in the Methods section (ReGenT-seq paragraph), shRNAs were grouped into gene sets and analysed using ROAST (mroast function in the edgeR package), which increases robustness and further reduces off-target effects. An additional level of stringency is provided by the ReGenT-seq design itself, as gene hits are only called when identified in both reciprocal pooled sub-screens using complementary reporters (K6-blasticidin and K6–diphtheria toxin receptor; **Fig.1B)**.

Regarding shRNA knockdown efficiency, this was addressed at both the library-design and experimental levels. The miR-E backbone used to express shRNAs was specifically optimised to enhance shRNA processing and potency. Fellmann et al. (*Cell Reports* 2013) identified a conserved element located 3′ to the basal stem that is required for optimal shRNAmir processing, leading to increased mature shRNA abundance and substantially improved knockdown efficacy. In our study, we experimentally validated shRNA knockdown efficiency for the three ReGenT-seq hits selected for downstream analysis (Cbx3, Kmt5c, and Ehmt2) as shown in **Supplementary Fig.5A,B**. In addition, following the reviewer's suggestion, we systematically predicted knockdown efficiency across the shRNA library using the web-based SplashRNA tool (Pelossof et al., *Nature Biotechnology* 2017). High SplashRNA scores (>1) have been shown to correspond to >85% protein knockdown from a single genomic integration. Using this approach, we found that 76% of shRNAs in the library achieve high predicted efficiency scores. These results are now included in the revised manuscript as **New Supplementary Fig.1C**.

2. The observation that Cbx3 promotes migration but inhibits proliferation is intriguing and reminiscent of the pathological state of chronic wounds, where keratinocytes often exhibit hyperproliferation but impaired migratory capacity. It would be valuable for the authors to explore Cbx3 expression patterns in human chronic wound tissues. Does Cbx3 expression correlate inversely with keratinocyte migration status in these contexts? Such data could enhance the clinical relevance of the findings.

We appreciate the reviewer's insightful comment highlighting the potential relevance of our findings to chronic skin injuries. To address this point, we analysed acute and chronic human wound samples and examined CBX3 expression.

Consistently with our observations in murine acute skin injury (**Fig.3B,C**), acute human wounds display a well-defined epidermal organisation that is required for proper functional healing (Park et al., *Nature Communications* 2017). Specifically, we identified distinct proliferative regions, characterised by actively dividing epidermal cells (increased Ki-67 positivity, densely packed cells, and nuclei predominantly oriented perpendicular to the basement membrane), and migratory regions, which are largely Ki-67-negative and exhibit nuclei oriented more parallel to the basement membrane. Having established conservation of this structural organisation between mouse and human acute wounds, we next compared acute skin injuries with chronic venous ulcers and pyoderma gangrenosum-associated ulcers in human samples. In line with our findings in murine acute wounds, CBX3 expression in human acute injuries was significantly higher in migratory regions than in epidermal areas exhibiting enhanced proliferation (**New Fig.3F,G**). Importantly, this association between CBX3 expression and migratory versus proliferative regions was lost in both types of chronic wounds analysed (**New Fig.3F,G**). This loss of correlation reflects the altered architectural organisation of chronic wounds, in which the proliferative compartment predominates while the migratory edge is reduced or even absent. This observation is consistent with the prevailing model that epidermal cells in chronic wounds frequently exhibit hyperproliferation but impaired migratory capacity. Taken together, the selective association of elevated CBX3 expression with migratory regions in acute wounds, and its disruption in chronic wounds, further supports a potential role for CBX3 in regulating epidermal cell migration.

3. Although the primary results are based on murine EpSCs, the study would benefit from a more explicit assessment of whether the Cbx3-dependent mechanisms are conserved in human primary keratinocytes. The authors briefly mention SCC and human samples, but is there functional evidence showing that Cbx3 similarly regulates mRNA localization, organelle recruitment, or protrusion formation in human EpSCs or keratinocytes?

We thank the reviewer for highlighting the importance of determining whether the Cbx3-dependent mechanisms identified in murine epidermal cells are conserved in human cells. To directly address this point, we performed analogous functional experiments in HaCaT immortalised keratinocytes and primary human keratinocytes. These analyses demonstrate that CBX3-dependent regulation of protrusion formation and associated organelle recruitment is conserved in human keratinocytes.

In 2D culture conditions, CBX3 overexpression significantly increased the size of cellular protrusions (**New Supplementary Fig.15A,B,C)**. In parallel, we observed enhanced recruitment of intracellular organelles into protrusions, as evidenced by increased localisation of WGA-positive Golgi elements and Lamp1-positive lysosomes within protrusive structures (**New Supplementary Fig.15D-H**).

To further assess CBX3 function in a dynamic and physiologically relevant context, we next used a 3D culture system that allows temporally controlled induction and maturation of protrusions. In this setting, CBX3 knockdown resulted in a marked impairment in the formation of mature protrusions, whereas CBX3 overexpression significantly enhanced protrusion formation (**New Supplementary Fig.16B-E)**. These results indicate that CBX3 is required for efficient protrusion establishment in human keratinocytes, mirroring its role in murine epidermal progenitor cells.

Together with our murine data, these findings support a conserved role for CBX3 in regulating protrusion dynamics and migratory behaviour across species.

4. Primary EpSCs typically lose their stemness and begin differentiating after 5–6 passages. However, the experiments described involve 3–4 weeks of culture. Can the authors provide data or morphological/marker-based evidence that the cells retained their EpSC characteristics throughout the course of these experiments? This clarification is essential to interpret whether the observed phenomena truly reflect stem cell biology or partially differentiated cell behavior.

We thank the reviewer for raising this important point regarding the maintenance of epidermal stem/progenitor cell identity during extended culture. To directly address this concern, we performed new experiments to assess both stemness maintenance and differentiation capacity of our epidermal cultures over the full duration of the ReGenT-seq protocol.

Specifically, we maintained our epidermal cells in culture for 4 weeks, corresponding to the maximum experimental duration used in ReGenT-seq, and longitudinally assessed the expression of established epidermal stem/progenitor cell markers (Krt5, Krt14, Itgb1, and Col7a1). Across the culture period, expression levels of these markers remained overall stable (**New Supplementary Fig.1G**).

To determine whether long-term cultured cells also retained functional differentiation capacity, we induced suspension-mediated differentiation, as previously described (Vietri Rudan et al., *PNAS* 2020). Analysis of mRNA expression revealed comparable induction of the differentiation markers Involucrin and Keratin 10 at week 0 and week 4 (**New Supplementary Fig.1H**). In parallel, expression of the stemness marker Krt5 decreased upon differentiation induction, with a similar magnitude of reduction at both time points (**New Supplementary Fig.1H)**, indicating preserved responsiveness to differentiation cues.

Collectively, these data indicate that the cells employed in this study preserve epidermal stem/progenitor cell properties over the entire course of the ReGenT-seq protocol and remain fully competent to differentiate.
(Please also check our response to point 6)

5. In Figure 3K, the authors claim differential Cbx3 expression between basal and suprabasal layers. However, the identification of these layers appears to rely on histological inference rather than specific markers. I recommend the inclusion of basal (e.g., ITGA6, KRT14) and suprabasal (e.g., KRT10) markers to confirm the cellular identity. Additionally, the color coding in Figure 3K does not seem to align with the expression intensity presented in Figure 3J, raising questions about consistency in data representation.

We thank the reviewer for this suggestion and agree that, particularly in the context of epidermal tumours, distinguishing basal from suprabasal compartments based solely on histological features can be challenging.

As suggested, we performed additional histological analyses using layer-specific markers. We stained tumour sections for Keratin 14 (KRT14), a marker of epidermal stem/progenitor and basal cells, and FABP5, a marker of early epidermal differentiation. FABP5 is expressed in suprabasal layers of the interfollicular epidermis but can also be detected in basal cells that have initiated differentiation (Donati et al.,Nat Cell Biol 2017), making it well suited to capture early suprabasal identity.

Our analyses revealed that although KRT14 is a highly specific basal marker under homeostatic conditions, it is not reliable in the tumour context, as it also labels terminally differentiated epidermal cells in squamous cell carcinomas (**New Supplementary Fig.8F**). In contrast, FABP5 robustly and consistently marked suprabasal cells in both homeostatic and tumour settings, allowing clear discrimination between superficial (differentiated) and deeper tumour regions (**New Supplementary Fig.8F**).

To validate our original observations of differential CBX3 expression (**Fig.3H-L**), we therefore quantified CBX3 protein levels based on tumour depth, comparing superficial versus deep tumour regions rather than relying on inferred basal/suprabasal morphology alone. Consistent with our previous results, CBX3 expression was significantly higher in deeper tumour regions compared with superficial regions (**New Supplementary Fig.8G**), supporting the data in **Fig.3K**.

Finally, to address concerns regarding consistency and transparency of data representation, including colour coding, we have attached the script used for all histological quantifications to this point-by-point response letter. This clarifies how quantitative values shown in **Fig.3J** and **Fig.3K** were derived.

6. The authors claim to use epidermal stem cells (EpSCs); however, based on the methods described, it is challenging to discern whether the isolated cells are indeed bona fide epidermal stem cells, transient amplifying progenitors, or simply basal keratinocytes. The terminology surrounding these populations remains somewhat ambiguous and inconsistently applied across the field. I encourage the authors to clarify whether their purification strategy truly enriches for epidermal stem cells. Have they performed any validation—such as functional assays, stem cell markers, or lineage tracing—that supports the identity of these cells as EpSCs rather than a broader basal or progenitor population?

We thank the reviewer for highlighting the ongoing ambiguity and inconsistent use of terminology surrounding

murine epidermal stem and progenitor populations in vitro. We agree that, based solely on isolation and culture conditions, it is challenging to unequivocally distinguish bona fide epidermal stem cells from transit-amplifying progenitors.
In this study, cells were isolated using an established and widely adopted protocol (Jensen et al., *Nature Protocols*, 2010) that enriches for basal epidermal populations with stem/progenitor-associated properties. This approach has been used extensively in prior studies, in which similar cultures are commonly referred to as "epidermal stem cells" (e.g., Boonekamp et al., *PNAS*, 2019; Baksh et al., *Nature Cell Biology*, 2020).
However, we acknowledge that these cultures are heterogeneous and comprise a mixture of stem cells and transit-amplifying progenitors. Importantly, such heterogeneity is not unique to our system, as even highly purified epidermal stem cell populations rapidly generate progeny under in vitro culture conditions. Consistent with previous reports using this methodology, our cultures retain differentiation capacity and express stem/progenitor-associated markers (new Supplementary Fig. 1G–H), supporting the presence of epidermal stem/progenitor populations.
To avoid overstating the purity or identity of the isolated cells, we have revised the manuscript to remove the term "epidermal stem cells" and now refer to these cultures as "epidermal progenitor cells" (EPCs), reflecting epidermal cell populations enriched for stem/progenitor-associated features.

Reviewer #2 (Remarks to the Author):

This manuscript claims to study cell protrusions in the context of stress in skin epithelial stem cells. To perform the studies, the authors used a novel screen called ReGEnT-seq, which they apply to chromatin factors controlling epidermal stem cell activation. Using this technique they identified Cbx3 as a critical protrusion factor. The authors also found that Cbx3 is in the cytoplasm of cells in wounds and cancers and governs the subcellular localization of specific mRNAs with polarizing protrusions with in vitro experiments suggesting that Cbx3 is plays a role in epidermal stem cell stress in protrusions.
Altogether, the manuscript presents an impressive amount of next generation sequencing data and contains novel approaches, with detailed functional and mechanistic characterization of Cbx3 in epidermal stem cells. The identification of Cbx3 as a cytoplasmic regulator of mRNA localization and protrusion maturation is intriguing and represents a potentially important shift in the understanding of chromatin factors.
However, despite the richness of the data in the manuscript, it is extremely difficult to read. The central logic is obscured by dense language and an unclear narrative structure. Additionally, the manuscript contains numerous grammatical errors throughout, to the extent that it was difficult to catalog them all. Consequently, my enthusiasm for the publication is quite diminished.
Major:

We thank this Reviewer for recognising that our work contains an "impressive amount of NGS data", "novel approaches" and "detailed functional and mechanistic characterization of CBX3".
We are pleased that the reviewer finds our data on CBX3-mediated mRNA transport compelling and agrees that they represent "a potentially important shift in the understanding of chromatin factors."

We apologize for the grammatical errors and lack of clarity in the original manuscript and believe these issues have now been addressed in the revised version.

Conceptual framing issue:

The manuscript frames the entire study around 'stress induced cell activation', but the experimental models do not test stress conditions. The main screen uses K6 promoter activity as a proxy for stress, yet no external stressors are applied in the foundational in vitro experiments. At its core the manuscript investigates how epigenetic regulators modulate K6 promoter activity, not stress induced K6 promoter activity. For example, K6 is expressed in the palmar plantar skin of the hand epidermis, hair follicles and sweat and sebaceous glands (Wang et al. 2018 and numerous other publications). Without experimental validation that the authors are inducing stress it is impossible to claim that the K6 promoter in their experimental system is activated by stress.

We thank the reviewer for this thoughtful critique regarding the conceptual framing of the study around "stress-induced cell activation." While our foundational in vitro experiments do not involve the acute application of artificial stressors, it is important to note that the transition of epidermal stem/progenitor cells from their native, homeostatic niche into in vitro culture constitutes a well-established physiological stress that leads epidermal activation programmes.
Keratin 6 expression is widely recognised as a hallmark of epidermal activation in response to stress, particularly during wound healing and in epidermal tumours (Aragona et al., 2017; Ge et al., 2017; Levra Levorn

et al., 2023). Accordingly, activity of our K6-based reporter reflects entry into an activated cellular state rather than basal epidermal homeostasis.

To address the concern that Keratin 6 may be expressed in homeostatic conditions in certain epidermal cell populations, we specifically used the promoter of *Krt6b*, rather than *Krt6a*. Single-cell RNA-seq analyses demonstrate that *Krt6a* is expressed in hair follicles under homeostatic conditions and is further induced upon activation, whereas *Krt6b* is not detectably expressed in homeostatic epidermis and is specifically induced during wound healing (**New Supplementary Fig.1D**). Consistent with this, the 2 kb *Krt6b* promoter used in our study was selected based on its increased chromatin accessibility during wound healing, as revealed by ATAC-seq analyses (Ge et al., *Cell* 2017). Thus, *Krt6b* promoter activity provides a selective and biologically grounded readout of stress-associated epidermal activation.

To directly assess the activation state of epidermal cells in culture, we compared RNA-seq profiles from our cultured epidermal cells with published transcriptomic datasets of epidermal stem cells in vivo, including stem cells from the hair follicle and interfollicular epidermis in homeostasis, as well as stem cells during wound healing and in SCC. These analyses revealed that our epidermal cultures exhibit transcriptional signatures that more closely resemble activated epidermal cells during wound healing and squamous cell carcinoma (SCC) than epidermal stem cells under homeostatic conditions (**New Supplementary Fig.2A,B**). In response to the reviewer's comment, we further interrogated shared gene expression programmes using gene set enrichment and Gene Ontology analyses. These revealed robust enrichment of pathways associated with stress-induced activation, including survival, migration, proliferation, metabolic reprogramming, and innate immune responses, that are hallmarks of epidermal stem cell plasticity during tissue repair (Levra Levorn et al., 2023) (**New Supplementary Fig.2C,D**).

To provide direct experimental validation that the *Krt6b* promoter is stress responsive, we generated a new *Krt6b* reporter in which promoter activity drives GFP expression and exposed cells to multiple physiologically relevant stressors, including hypoxia, UV irradiation, oxidative stress (hydrogen peroxide), viral infection, mechanical stress simulation, and inflammatory TNF signalling. In all cases, stress exposure resulted in a robust and time-dependent increase in GFP expression (**New Supplementary Fig.1E,F**), confirming that the *Krt6b* promoter functions as a bona fide stress-responsive regulatory element in vitro.

Collectively, these data demonstrate that epidermal cells isolated from homeostatic tissue undergo a pronounced stress-associated activation programme upon adaptation to in vitro culture, and that *Krt6b* promoter activity faithfully reports this state. Thus, while our screen interrogates epigenetic regulation of *Krt6b* promoter activity, it does so within a biologically relevant stress-activated cellular context. This clarification strengthens the conceptual framework of the study and supports the interpretation of our findings as mechanisms regulating stress-associated epidermal cell activation.

Misleading/confusing figures:

Importantly, the representation of the data in the manuscript is difficult to ascertain how and what experiments were performed. One example of many, the experimental setup in Figure 1a–c is fundamentally unclear and misrepresents the actual experiment that the authors performed. Specifically, Figure 1a misleadingly suggests that the K6-Blast and K6-DTR reporters were delivered into the same population of EpSCs, followed by pooled shRNA transduction and sequencing. If that were the case, it would make the selection logic uninterpretable, as cells could simultaneously express opposing survival/death signals. However, from the methods and description, it appears that the two reporter systems were used in separate, parallel experiments with otherwise identical conditions and shRNA pools. This distinction is critical to understanding the design and stringency of ReGenT-seq and must be explicitly clarified in both the figure and the legend. Otherwise, readers will be confused about how the reciprocal screening logic was applied and how hits were called. This one of many examples throughout the manuscript.

We thank the reviewer for highlighting the need for greater clarity in the presentation of the experimental design, figures, and figure legends. We agree that the distinction between parallel versus combined reporter screens is critical for understanding the ReGenT-seq strategy, and we have revised the manuscript to address this concern.

We have modified the schematics in **Fig.1A and Fig.1B** to explicitly indicate that the K6-Blasticidin (K6-BL) and K6-DTR reporters were used in separate, parallel pooled sub-screening experiments. Importantly, the two reporters were not introduced into the same cells. This clarification is now clearly depicted in the revised schematic and explicitly stated in the figure legend, resolving the potential misinterpretation raised by the reviewer.

To further enhance clarity and improve the overall flow throughout the manuscript, we implemented the following changes:

- We added explicit information on the origin and composition of the pooled shRNA library (Johannes Zuber laboratory) in **Fig.1A** and **Fig.1F**, as well as in the Results and Methods sections.

- We reorganized **Fig.2A** to clearly indicate that the Venn diagrams represent differentially expressed genes (DEGs) derived from individual knockdowns (CBX3, KMT5C, and EHMT2) compared with control cells.
- The original Supplementary Fig.1 has been reorganized and split into **New Supplementary Figs. 2** and **New Supplementary Figs.3** to accommodate additional panels and to improve logical organization.
- Several panels from **Fig.2** have been moved to the **Supplementary Information** to streamline the main figure and improve readability. **Fig.2** now concludes with Gene Ontology analysis of DEGs in CBX3 knockdown cells, providing a clear transition to the subsequent CBX3-focused analyses.
- Similarly, selected panels from **Fig.3** have been moved to the **Supplementary Information**, while new analyses requested by the reviewers were added to better illustrate the physiological relevance of CBX3.
- We clarified in the legend of **Fig.4A** that the schematic represents a summary of the nuclear analyses performed to dissect CBX3 molecular functions, and supporting panels were moved to the **Supplementary Information** to maintain focus on the main conceptual framework.
- Overall, the number of **supplementary figures** has increased from **9** to **16**, allowing us to present additional experimental details, improve figure clarity, and include new functional data on CBX3 in human cells.

Together, these revisions substantially improve the clarity and interpretability of the experimental design and data presentation, particularly with respect to the reciprocal screening logic underlying ReGenT-seq.

Reviewer #3 (Remarks to the Author):

In this paper, Duval et al. investigate epigenetic mechanisms involved in epidermal stem cell (EpSC) activation during wound healing and squamous cell carcinoma (SCC). Using a novel sequencing protocol, ReGenT-seq, the authors identified Kmt5c, Ehmt2, and Cbx3 as key epigenetic regulators for cell activation and cell protrusion. The authors utilized several molecular techniques to dissect how these epigenetic regulators mediate cell activation and cell protrusion. RNA-sequencing analysis in combination with GSEA analysis revealed that while Ehmt2 acts as a negative regulator of cell plasticity, Kmt5c and Cbx3 act as positive regulators of cell plasticity. To better understand how Cbx3 drives cell plasticity upon wound induction, the authors performed an in vitro scratch assay on Cbx3 KD cells, where they conclude that Cbx3 is a regulator of cell migration. To better understand the role that Cbx3 may play in wound healing and SCC, the authors investigated expression of Cbx3 in both contexts. They found that Cbx3 is in the migratory front of wounds, however in SCC, they found that Cbx3 was present in the cytoplasm of deep SCC areas, suggesting that Cbx3 may also play a role in the cytoplasm. Next, to study the role of Cbx3 in RNA-splicing, the authors utilized seCLIP-sequencing and found that Cbx3 mainly targets mature RNA at various sites. Next, the authors investigated the role of Cbx3 in mediating RNA nuclear export in EpSC plasticity and protrusions. To do this, the authors fractionated cells to separate nuclear and cytoplasm to perform RNA-seq and investigate differences in between RNA in the nucleus versus the cell protrusions. The authors performed a similar experiment in Cbx3 KD cells to interrogate which genes are regulated by Cbx3, which they conclude are genes associated with positive regulation of wound healing, collagen-containing ECM, focal adhesion, and establishment of cell organelles. Collectively, the authors conclude that Cbx3 plays distinct roles in the nucleus and cytoplasm that mediates cell plasticity and cell protrusion during both wound healing and SCC.

In summary, the authors describe a novel sequencing protocol and identify several potential functions of Cbx3 in regulating cell activation and protrusion in both wound healing and SCC. Most of the data for a great story are present in their paper, however, the rationale and story need to be reorganized. The authors should focus on making one main argument with several supporting details.

We thank this reviewer for acknowledging the “great story” potential for our data. We agree that reorganising the data will help improve the clarity and overall flow of the manuscript. Therefore in the new manuscript we reorganised the figures, the supplementary figures and the result paragraph with a clear top-down approach. Specifically:
Figure 1 - The ReGenT-seq genetic screening;
Figure 2 - Exploratory targeted functional characterization of the 3 ReGenT-seq hits;
Figure 3 - CBX3 function on cell migration/invasion and its in vivo relevance in wound healing and SCC;
Figure 4 - Molecular and functional characterization of CBX3 nuclear activities;
Figure 5 - Integration of omics to prediction that CBX3 is functional in mRNA transport in cell protrusion;
Figure 6 - Validation that CBX3 indeed promotes protrusion maturation controlling mRNA localization, including mRNAs for organelle establishment in the protrusions;

Figure 7 - Identification that CBX3 is required for mature protrusions in epidermal progenitor cells and invasive protrusions in cutaneous SCCs.

Major comment(s):

1) The author describes a very interesting and novel method to identify key epigenetic modulators; however, the validation is slightly unclear – are there previous reports using this method? Are you the inventor of this method? If so, a reference to previous work is needed.

We apologize for the lack of clarity in the original manuscript and appreciate the reviewer's comments. In the revised version, we now provide a clearer and more detailed description of the ReGenT-seq strategy and its implementation.

ReGenT-seq is a screening method newly developed in our laboratory that integrates multiple pooled genetic screening modalities to substantially reduce false discovery rates. Unlike conventional pooled screens that primarily rely on cell survival or proliferation as readouts, ReGenT-seq uses transcriptional activity as a high-content functional output, thereby enabling direct identification of regulators of stress-induced cell activation.

To perturb chromatin regulators, we employed a well-established and extensively validated pooled shRNA library targeting 615 chromatin factors, originally generated and optimized by the Johannes Zuber laboratory (Cheloufi *et al.*, *Nature* 2015; Fellmann *et al.*, *Cell Reports* 2013) (**modified Fig.1A; new Supplementary Fig.1A-C**). This same shRNA library was used in two independent pooled sub-screens, each incorporating a distinct engineered reporter system.

Both reporters are driven by the *Krt6b* (K6) promoter, which was selected based on its increased chromatin accessibility *in vivo* during wound healing (ATAC-seq data from Ge *et al.*; see also our response to Reviewer 2, "conceptual framing issue"). The K6 promoter drives either (i) blasticidin resistance (BL) or (ii) diphtheria toxin receptor (DTR) expression, which confers diphtheria toxin (DT) susceptibility (**modified Fig.1A**). Because Keratin 6 is robustly induced by stress in epidermal stem/progenitor cells, including during wound healing and in epidermal tumors, these reporters serve as sensitive and biologically relevant readouts of stress-induced cell activation.

Importantly, the two reporter systems yield complementary selection outcomes. shRNAs that suppress K6 promoter activity are depleted in the blasticidin-resistance sub-screen but enriched in the DTR/DT-susceptibility sub-screen. Intersection of hits from these reciprocal sub-screens markedly increases screening stringency and reduces false-positive identification (**Fig.1B,C**).

Prior to performing the ReGenT-seq, we validated the approach by targeting *c-Jun*, a key AP-1 transcription factor component and a well-established activator of epidermal cell activation that positively regulates *Krt6* expression. Consistent with expectations, *c-Jun* knockdown reduced K6 promoter activity and impaired cell survival under blasticidin selection, confirming the sensitivity and functional relevance of the ReGenT-seq system (**Supplementary Fig.3A-C**).

To improve clarity and accessibility, we have revised the main text, figure legends, and figures accordingly, as indicated in Reviewer 1, point 1. Additional revisions addressing the conceptual framing and clarification of figures have been implemented as detailed in Reviewer 2's comments under "conceptual framing issue" and "misleading/confusing figures".

2a) The authors use RNA-seq to dissect the potential role of three epigenetic regulators in cell activation, Ehmt2, Kmt5c, and Cbx3 – while I understand the rationale, I think the explanation of the results is a little confusing. Which specific genes are dysregulated in the KD conditions?

We thank the reviewer for their comment regarding the clarity of our exploratory, targeted analysis of three selected ReGenT-seq hits. Differentially expressed genes (DEGs) identified for each knockdown relative to control cells, together with the corresponding comparative analyses, are presented in **Fig.2A-H, Supplementary Fig.5C-G,** and **Supplementary Table 3**. To improve clarity, we have revised the relevant Results section and reorganized the figure panels to better explain and contextualize these findings.

2b) Which genes display splice variants or lack thereof due to the loss of Cbx3. If you start the argument with three potential roles for Cbx3: (1) transcriptional repression, (2) transcriptional activation, and (3) RNA-splicing, then walk us through each situation and conclude how Cbx3 is playing a role in this system. At this point, I would also try to make a strong argument to dissect only Cbx3 and no longer investigate the other two epigenetic regulators, as the jumping back and forth is confusing.

We thank the reviewer for these helpful suggestions and for highlighting the need for clearer organization and mechanistic focus. We have now substantially revised the manuscript to address these points.

First, to clarify the initial comparative analysis, we reorganized the Results section such that the exploratory analysis of the three selected ReGenT-seq hits (CBX3, EHMT2, and KMT5C) is now consolidated in **Fig.2**. This section presents the Gene Ontology analysis and GSEA of differentially expressed genes (DEGs) for each knockdown relative to control cells, allowing inference of their functional roles in the context of cell activation. This analysis highlights both shared and divergent regulatory features among the three factors.

Second, in direct response to this reviewer's suggestion, we now focus exclusively on CBX3 from **Fig.3** onward, eliminating back-and-forth comparisons with the other epigenetic regulators. This restructuring was implemented to improve clarity and narrative coherence and to allow a deeper mechanistic dissection of CBX3 function.

Regarding the reviewer's specific question about RNA splicing, **Fig.4** now explicitly examines the nuclear functions of CBX3, including its roles in transcriptional activation, RNA splicing, and nuclear export. In this section, we identify transcripts that display altered splicing patterns upon CBX3 depletion and distinguish these from genes whose expression changes are attributable primarily to transcriptional effects. This organization allows us to sequentially address the proposed roles of CBX3 (transcriptional activation, RNA splicing and nuclear export) and to conclude how these layers contribute to the regulation of epidermal cell activation.

Finally, the decision to focus on CBX3 is now explicitly justified in the text. Unlike EHMT2 and KMT5C, CBX3 emerged from ReGenT-seq as a uniquely multifunctional regulator of gene expression. Moreover, CBX3 represents a particularly intriguing and, in part, controversial factor: while initially characterized as a chromatin protein involved in heterochromatin-dependent gene silencing, subsequent studies demonstrated its association with actively transcribed chromatin and later implicated it in RNA splicing regulation. These multifaceted and context-dependent properties, together with the strong phenotypic effects of CBX3 perturbation on protrusion formation and invasive behavior, motivated our in-depth cellular and molecular characterization of CBX3 in **Fig.3-7**.

3) The authors claim that "Cbx3 promotes cell migration and invasion while it negatively impacts cell proliferation." While in this results section, the authors describe the expression distribution of Cbx3 in both the wound front and SCC, they do not show any functional data to determine that Cbx3 promotes cell migration and invasion or that it impacts cell proliferation. To do this, I would suggest developing models, either in vivo or in vitro, and test the effects of the loss of Cbx3 on cell migration, invasion, and proliferation. Something like the 2D and 3D models that are described later in the paper.

We thank the reviewer for this important comment and agree that the functional evidence supporting CBX3's role in migration, invasion, and proliferation needed to be more clearly emphasized in the manuscript.

While the original submission already included functional data addressing these points using both knockdown and overexpression approaches, we recognize that the previous organization of the Results section did not sufficiently highlight these experiments. To address this, we have now both added new functional data and reorganized the manuscript to clearly connect CBX3 expression patterns with functional outcomes.

Specifically, **Fig.3A** and **New Supplementary Fig.6B,C** demonstrate that CBX3 promotes cell migration. In addition, **Supplementary Fig.6D,E** shows that CBX3 negatively impacts cell proliferation in epidermal progenitor cells. Importantly, we further extended these analyses to cancer models: CBX3 overexpression enhances migration in scratch assays and invasion in transwell invasion assays in SCC cells (**Fig.3N and New Supplementary Fig.8I,J**).

Together, these functional assays directly demonstrate that CBX3 promotes cell migration and invasion while restraining cell proliferation, thereby supporting the conclusions stated in the manuscript. To improve clarity and address the reviewer's concern, we have reorganized the relevant Results sections and figure presentations to explicitly highlight these functional data.

Point-by-point Response to Reviewers' comments

Authors' comments:

- We thank all the reviewers for their analysis of our manuscript and for the constructive suggestions.
- For an easier assessment by the editor, the changes in the main text with respect to the first submission are highlighted in blue.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed my comments thoroughly and satisfactorily. I have no further concerns and recommend acceptance of this manuscript.

Reviewer #2 (Remarks to the Author):

The authors have clarified the figures particularly Figure 1a to more accurately represent their new and novel screening technique addressing that concern. Similar corrections have also been addressed in subsequent figures. The grammatical errors have been corrected and the revised manuscript is much improved.

A issue on K6a/b use as exclusively a marker of activation of cellular stress. The authors have tried to address my concern experimentally by generating a new K6b-GFP promoter and exposed cells to multiple physiologically relevant stressors showing through flow cytometry analysis that K6 is upregulated. These experiments show that stress induces K6b expression. It does not show that K6b expression is exclusively linked to stress as keratinocytes in sweat glands in humans and other non stress conditions can and may express K6a and k6b (simply google searching and investigating webtools to search for k6a/b expression in scRNA-seq data sets reveals this). Consequently, I'm not convinced that K6 expression exclusively marks stress conditions but rather different states of keratinocytes differentiation, including stress. The fact that K6 expression is not exclusive to stress does not invalidate the manuscripts premise but k6 expression outside of stress should not be ignored. To address my concern (no new experiments are needed) the authors can add this critical discussion point to their introduction and in their discussion as a caveat and/or alternative interpretation to their study. This would greatly enhance the manuscript.

We thank the reviewer for this important point. As requested, we have added a clarifying sentence to the Discussion. More broadly, this limitation is inherent to genetic screens relying on single-gene readouts, as no single gene can be considered an exclusive marker of a specific cellular state. However, based on the data provided in the revised manuscript, Krt6b promoter activity under our experimental conditions is most consistent with, and likely reflects, a stress-associated response; notably, the validated gene hits are functionally linked to cellular stress pathways. Accordingly, we have included the following sentence in the Discussion: As with genetic screens relying on a single-gene readout, a potential limitation of our approach is that the transcriptional activity of the Krt6b promoter may not be exclusively indicative of stress; however, under culture conditions its induction is most consistent with, and likely reflects, a stress-associated response, despite reports of in vivo expression in other differentiated epithelial cell states."

Reviewer #3 (Remarks to the Author):

In the manuscript "Multilayered control of mRNA delivery to cell protrusions drives localized mini-cytoplasm establishment for stress-induced cell activation," Duval et al. examine mechanisms that regulate epidermal stem cell (EpSC) activation during wound healing and squamous cell carcinoma (SCC). Using a newly developed sequencing approach (ReGenT-seq), the study identifies Kmt5c, Ehmt2, and Cbx3 as epigenetic regulators involved in cell activation and protrusion formation. A combination of genomic and molecular approaches is then used to explore how these regulators contribute to EpSC plasticity.

RNA-seq combined with GSEA analysis suggests that Ehmt2 acts as a negative regulator of cell plasticity, whereas Kmt5c and Cbx3 function as positive regulators. To investigate the role of Cbx3 during wound activation, the authors perform scratch assays in Cbx3 knockdown cells and report reduced migratory capacity, suggesting a role for Cbx3 in cell migration. Expression analysis further shows that Cbx3 is enriched at the migratory front of wounds. In SCC samples, however, Cbx3 appears predominantly cytoplasmic in deeper tumor regions, raising the possibility that it also functions outside the nucleus.

To better understand the molecular activity of Cbx3, the authors perform seCLIP-seq and find that Cbx3 primarily binds mature RNAs at multiple sites. They then explore whether Cbx3 regulates RNA nuclear export during EpSC activation and protrusion formation. Nuclear and cytoplasmic fractions are isolated and analyzed by RNA-

seq to compare RNA localization patterns. Similar analyses in Cbx3 knockdown cells identify gene sets associated with wound healing, extracellular matrix organization, focal adhesion, and organelle establishment. Based on these findings, the authors propose that Cbx3 plays distinct roles in the nucleus and cytoplasm that contribute to cell plasticity and protrusion dynamics during wound repair and SCC.

The manuscript introduces an interesting sequencing strategy and presents several observations suggesting roles for Cbx3 in cell activation and protrusion biology. The dataset is substantial and potentially supports a compelling story. However, the overall narrative would benefit from clearer focus and organization. At present, multiple mechanistic directions are explored in parallel, which makes the central message somewhat difficult to follow.

Major comments

1. The RNA-seq and ChIP-seq analyses are often described in broad terms, which makes it difficult to evaluate the conclusions. For example, the statement that “ChIP-seq peaks with deregulated genes in CBX3-depleted cells revealed only a limited direct role for CBX3 in regulating migration-associated genes” is quite general. It would help to highlight specific genes identified in these analyses and explain how they support the proposed functional model. Providing concrete examples throughout the Results section would make the analysis more convincing and easier to follow.

We thank the reviewer for this helpful suggestion. We have revised the Results section to improve clarity and better guide the reader through the RNA-seq and ChIP-seq analyses, including more explicit descriptions of the relevant figures (Supplementary Fig. 9H; Supplementary Fig. 14C). Given the genome-wide nature of these datasets, we focused our analysis on gene ontology and pathway-level enrichment rather than individual genes, as prioritization at the single-gene level would be sensitive to arbitrary thresholds and would not capture the coordinated and distributed effects of CBX3 on chromatin.
Consistent with this approach, adjusted p-values from GO enrichment analysis using the MSigDB Hallmark gene sets, performed on the overlap between ChIP-seq peaks and genes deregulated in CBX3-depleted cells, indicate that CBX3 plays a modest direct role in regulating migration-associated gene expression, while exerting a stronger influence on other gene sets (Supplementary Fig. 9H). This is consistent with the results of the viability screen (Fig. 1F–H) and its validation (Supplementary Fig. 6D,E). Later in the manuscript, we integrate all omics datasets to investigate the multilayered control exerted by CBX3 across different gene sets. There, we highlight that gene sets related to migration and cell protrusions, such as focal adhesion, are directly regulated by CBX3 both at the chromatin level and through RNA localization within protrusions (Supplementary Fig. 14C). To ensure that this message is clearly conveyed to the reader, we provide a mechanistic model in Fig. 7K.

2. The manuscript explores several possible functions of CBX3, including transcriptional regulation, RNA processing, RNA export, and cytoplasmic roles. While each of these analyses is interesting, the overall narrative becomes somewhat fragmented. A more focused story would strengthen the paper. For example, the manuscript reports that splicing alterations mainly affect genes linked to proliferation and RNA processing rather than migration, yet earlier sections emphasize a role for CBX3 in cell migration. Clarifying this discrepancy—or narrowing the analysis to mechanisms directly linked to migration—would improve the overall coherence of the study.

We thank the reviewer for this insightful comment. We agree that a clearer distinction between the different regulatory layers analyzed in the manuscript is important to avoid the impression of a fragmented narrative. In particular, Figures 2H and 4I address mechanistically distinct aspects of CBX3 function. In Fig. 2H, we performed GO enrichment analysis on differentially expressed genes in CBX3-depleted versus control cells, capturing the combined effects of both direct and indirect transcriptional regulation. These analyses therefore reflect global transcriptional changes, including secondary downstream effects. In contrast, Fig. 4I focuses specifically on post-transcriptional regulation. Here, GO term enrichment was performed on the subset of genes that are both differentially spliced upon CBX3 depletion and directly bound by CBX3 (as identified by eCLIP), thereby defining a more direct layer of RNA processing regulation. The apparent differences in functional outputs highlighted by the reviewer reflect these distinct regulatory layers rather than a contradiction. This is consistent with a model in which CBX3 exerts multilayered control over gene expression, with different molecular functions contributing to distinct biological programs. To address this point, we have revised the Results section to more clearly distinguish these regulatory layers and guide the reader through their interpretation.

In addition, the discussion frequently moves between wound healing and SCC, which can make the flow harder to follow. Structuring the manuscript so that SCC is discussed primarily toward the end may help maintain a clearer narrative.

We thank the reviewer for this suggestion. Upon revisiting the Discussion, we note that wound healing and SCC are often discussed together, as they both involve stress-induced cell activation and the formation of protrusions with shared characteristics. We therefore chose to maintain this integrated presentation to emphasize the

common underlying mechanisms. Nevertheless, to improve clarity and avoid potential confusion, we have revised several sentences in the Discussion to streamline the narrative.

3. It remains somewhat unclear whether nuclear CBX3 activity directly contributes to migration phenotypes.

We note that nuclear CBX3 contributes to migration indirectly through transcriptional regulation of gene programs associated with cell motility, as described in Fig. 2H. This complements its cytoplasmic role in mRNA localization, together supporting migration phenotypes. Please see also response to point 1 Reviewer #3.

The data appear to suggest a stronger role in protrusion formation and focal adhesion dynamics. If this is the central mechanism, the manuscript could benefit from emphasizing these processes more clearly.

We thank the reviewer for this comment. We agree that the role of CBX3 in protrusion formation and focal adhesion dynamics represents a central mechanism of our study. This aspect is already emphasized throughout the manuscript, including in the title, abstract, concluding section of the Introduction, multiple sections of the Results, and the Discussion. To further ensure clarity, we have revised the text to more explicitly highlight these processes where appropriate.

The relevance of the RNA-processing analyses to migration should also be clarified.

We thank the reviewer for this comment. We now have clarified the relevance of the RNA-processing analyses to migration in the revised manuscript. First, we note that CBX3-dependent splicing regulation includes genes associated with cell migration, as shown in Fig. 4I and further clarified in the revised Results section (see response to Reviewer #3, point 2). Second, the link between CBX3 function and migration is further supported by its role in mRNA localization to cell protrusions. Specifically, mRNAs encoding migration-related factors are targeted to protrusions (Fig. 5F,H), supporting a functional connection between RNA localization and migratory behavior. While these findings relate to migration, the final part of Results section focuses primarily on cell protrusion biology, as this represents the central mechanistic framework of the study, as also highlighted by the reviewer in its previous point. To avoid shifting the emphasis away from this core mechanism, we have selectively revised the text to make the links to migration more explicit while maintaining a coherent focus on protrusion maturation.

4. The data analyses are generally thorough, but the Results section would benefit from clearer guidance through the experimental logic. In several places, figure panels, experimental descriptions, and data interpretation are presented somewhat separately. Integrating these elements more tightly would help readers follow how each experiment supports the overall model.

We thank the reviewer for this helpful comment. We agree that some aspects of the Results section were not sufficiently explicit. To improve readability, we have revised selected parts of the Results to better align experimental descriptions with the corresponding data and their interpretation, thereby providing clearer guidance through the experimental logic. In particular, we have amended specific sentences to more explicitly connect the results to the figures, including those related to Fig. 2F, H and Fig. 4I,J, while maintaining the overall structure of the section.