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Full Review

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Marker-based CRISPR screens identify POU2F1 as a regulator of DLL3 and neuroendocrine identity in small cell lung cancer

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THIS REPORT COVERS

- Quality Scorecard
- 1. Novelty & Conceptual Advance
- 2. Strengths & Weaknesses of the Data Package
- 3. Journal Fit & Publication Potential
- Revision Plan

+ 8 supporting sections in the report

REVISION PLAN

Start here. Address the Essential items before submission, then use the Recommended items to strengthen the paper's position.

A. Essential: Narrow the claims: retitle and reword the abstract so that tandem motifs are a hypothesis, "code" and "orientation-specific" are removed or qualified, and "direct" is dropped unless supported. Describe AlphaFold results as predictions.

B. Essential: Strengthen KO validation: quantify Westerns across at least 3 replicates, report editing efficiency, state which sgRNAs were used for RNA-seq, and add a non-targeting control. Reanalyze Fig. 2B using count-based differential-expression analyses that account for the available design factors; because cell line and sampling day are not fully crossed, report cell-line-specific effects and clarify how day and batch were handled rather than relying on pooled Mann-Whitney tests on TPM.

C. Essential: Statistical corrections: use adjusted p-values and an expressed-gene background for Enrichr; rename the DESCARTES analysis; state the GSEA ranking metric and permutations; give leading-edge genes. Add matched random, single-motif and non-tandem null sets for Figs. 5 and 7, with a direct test of tandem versus ASCL1-only enrichment. Replace the pooled t-test in Fig. 6B with a model accounting for seed and model nesting.

D. Essential: Resolve screen specificity: filter the genome-wide hits against common-essential genes, report replicates, sgRNA-level data and QC, and discuss the global machinery hits. Acknowledge that permeabilized staining reports total DLL3.

E. Recommended: Direct versus indirect: POU2F1 KO with ASCL1 re-expression, a shorter time course or acute degradation, and POU2F1 rescue with an sgRNA-resistant cDNA.

F. Recommended: Test the motif claim: luciferase or reporter assays with wild-type, reversed, single-half-site and spacing mutants of the DLL3 promoter element in H209, or endogenous editing; plus input-controlled ChIP-seq, a POU2F1 peak overlap statistic, and co-IP or EMSA with interface mutants.

Figure review coverage: Automated figure analysis produced usable results for all 7 detected figure-bearing pages, but some figures, panels, or analysis fields were only partially captured. This is a review-processing limitation, not evidence of missing or defective figures.

EXTERNAL ASSESSMENT

Quality Scorecard

Originality: 3/5, POU2F1 as a DLL3 and neuroendocrine regulator in SCLC is new, but ASCL1 regulation of DLL3 (Borromeo et al., 2016) and domain-focused CRISPR screening in SCLC (Huang et al., 2018) have precedent, and the tandem-motif "code" is largely inferred.

Importance of research question: 4/5, DLL3 is the target of clinically used therapies, so how its expression is encoded matters for antigen durability.

Claims are well-supported: 2/5, Necessity of POU2F1 for DLL3 mRNA is reasonably shown, but "direct regulation", "cis-regulatory code" and "orientation-specific" cooperativity rest on motif scans, enrichment statistics and AlphaFold predictions.

Methodological soundness: 2/5, The screens are performed in one cell line; ChIP-seq peak calling was performed without input control; RNA-seq sgRNA use is not specified; no rescue or motif-level perturbation is reported; and analyses that pool timepoints do not model timepoint effects.

Clarity of writing: 3/5, The narrative is readable, but some axis labels and legends require correction or clarification, including the Fig. 1 legend reference to a “Serpin B5 reporter screen.”

Value to community: 3/5, The screen datasets and POU2F1 dependency are useful resources; the tandem-motif model is not yet usable.

Prior work contextualization: 3/5, The manuscript situates POU2F1 alongside POU2F3 and ASCL1 literature; the distinction between its findings and broader SCLC lineage-plasticity mechanisms could be clearer.

Overall: The POU2F1-DLL3 dependency is a credible, interesting observation, but the paper is currently at the level of a good field-journal descriptive study. The gap to Cancer Cell is mostly that the headline mechanism, a tandem-motif cis-regulatory code, is supported only by correlative genomics and structure prediction rather than by perturbation of the motif.

1. Novelty & Conceptual Advance

The new finding is that a ubiquitously expressed POU factor, POU2F1, is required for high DLL3 and neuroendocrine gene expression in three SCLC-A lines. The manuscript says POU2F1 has not previously been linked to neuroendocrine lineages. ASCL1 as a DLL3 driver is established (Borromeo et al., 2016 showed ASCL1 regulation of NOTCH-pathway genes including DLL3), so the incremental advance is identification of a candidate collaborator. The pairing with POU2F3-centered work from the same group (Huang et al., 2018) is conceptually appealing, but that is an analogy, not evidence.

The "cis-regulatory code" claim is the main conceptual pitch and the weakest part. The tandem motif is a manually concatenated PWM of MA0785.1 and MA1100.2. Its enrichment for "Lung Neuroendocrine" (Fig. 5B, log10 p ≈ 2.4 for 227 genes) is modest, and the ASCL1-only gene set is already enriched for the same term. A tandem site is therefore not shown to add information beyond ASCL1 motifs alone. I would emphasize the POU2F1-DLL3 dependency and treat the tandem grammar as a hypothesis.

The therapeutic framing (antigen durability under lineage plasticity) is speculative. No experiment tests whether POU2F1 loss or perturbation changes DLL3 surface display or sensitivity to DLL3-directed agents.

2. Strengths & Weaknesses of the Data Package

What is working well

1. Two independent screens (DBD-focused and Brunello) converge on POU2F1 alongside ASCL1 as the positive control (Fig. 1D-E), and the DLL3 antibody is validated with DLL3 KO (Fig. 1A-B).
2. POU2F1 KO reduces DLL3 mRNA in three neuroendocrine lines, with n = 6 replicates per timepoint (Fig. 2B-E), and ASCL1 KO is run in parallel as a benchmark.
3. GSEA with the SCLC-A and fetal lung neuroendocrine signatures is directionally consistent in all three lines (Fig. 3A-F), and CHGA, INSM1, GRP and TRPM8 are among the most dependent genes.
4. Two POU2F1 antibodies give concordant ChIP-seq (374 of 693 peaks shared, Fig. 4A), and both factors occupy the DLL3 promoter (Fig. 4D).
5. Data deposition (GEO) and detailed screen methods are provided.

What needs improvement

1. **Does the screen measure DLL3 regulation or general expression?** Cells are methanol-fixed and permeabilized, so the readout is total DLL3, not surface antigen, yet the therapeutic rationale is surface display. The genome-wide hits (Fig. 1E) include Pol II, Mediator, snRNP and mRNA processing factors, which suggests global expression and fitness effects. The Methods say the DLL3-high gate was insufficiently diverse for the focused screen, so it relied on low versus bulk. I would want: (A) a counter-screen or sort on an unrelated marker to separate DLL3-specific hits from global ones; (B) filtering against common-essential genes; (C) live-cell surface DLL3 flow after POU2F1 KO; (D) sgRNA-level concordance and replicate numbers for the screens. As shown, POU2F1 ranks alongside general machinery in Fig. 1E, so "specific" is not established.
2. **Direct versus indirect regulation.** POU2F1 KO changes DLL3 in 4-6 day RNA-seq, and Fig. 2E labels ASCL1 itself as differentially expressed after POU2F1 KO in H1836. Can the authors exclude that POU2F1 acts through ASCL1 levels or general loss of cell state? The ASCL1 blots in Fig. 2A are not quantified. An ASCL1 re-expression epistasis experiment (recommendation) and early or acute depletion would separate these possibilities.
3. **KO validation and statistics in Fig. 2.** Western blots are “representative of 2 biological replicates,” with no densitometry. RNA-seq displays day 4 and day 6 points together in one box and applies uncorrected Mann-Whitney tests to TPM values; the analysis does not model timepoint effects, and all displayed comparisons are reported as “$p < 0.0001$.” It is unclear whether RNA-seq used

one or both sgRNAs, and no rescue or non-targeting control is reported. Day 4 for H209 versus day 6 for the others limits direct cross-line comparison.

4. **Heterogeneity across lines is glossed over.** In Fig. 3H (H1436), Lung Neuroendocrine ranks fourth behind kidney, corneal, and mesothelial terms, and the SCLC-A GSEA there is weaker than in the other lines (FWER 0.035, Fig. 3B). This variation should be acknowledged when presenting the three-line result. Enrichr analysis of an arbitrary top-250 list, nominal p-value highlighting, and the label "Gene Ontology" for DESCARTES cell-type signatures all need correction. The SCLC-A and fetal neuroendocrine signatures may overlap, so they should not be treated as independent confirmations without reporting their overlap.
5. **ChIP-seq is thin.** MACS2 was run without an input control, with fewer than 700 POU2F1 peaks, and 319 peaks are supported by only one antibody. The ASCL1 enrichment at POU2F1 peaks is qualitative (Fig. 4B); no overlap statistic or reciprocal analysis is given, and the E-box is only the tenth HOMER motif (Fig. 4C). Tandem motifs are shown at three presented loci (DLL3, INSM1, DLL1) in Fig. 4E. This supports co-localization at some sites, not motif-level cooperativity. POU2F1 ChIP specificity (for example in KO cells) is not shown.
6. **The tandem-motif analyses (Figs. 5 and 7) are sequence-based and under-controlled.** The 16-bp motif is hand-built from two JASPAR PWMs, scanned at 1e-7, and genes within 20 kb are tested by Enrichr. Gene sets differ about 50-fold in size (227 versus 11,581), so p-values are not directly comparable. The reversed motif yields 226 genes versus 227, which warrants reporting the overlap between the two gene sets. The absence of Lung Neuroendocrine enrichment in the reversed set is a negative result, not a tested difference. In Fig. 7, I need to see size- and expression-matched random sets, POU2F1-only and ASCL1-only sets, and non-tandem spacing controls. Do the similar ASCL1-KO findings in Fig. S6B-C indicate that tandem-associated genes are also ASCL1-dependent?
7. **AlphaFold 3 is over-interpreted.** The pLDDT comparison (Fig. 6B) applies a Welch's unpaired t-test to nucleotide-level values derived from 20 related models and does not account for model or seed structure. Forward and reversed DNA differ in sequence order, so confidence differences could reflect sequence context. No scrambled, single-motif, or mutant DNA controls are reported. The key TCF12-POU2F1 contacts have PAE values of roughly 7 to 12.7 Å and contact probabilities of 0.29 to 0.65 (Fig. 6D). These predictions require experimental validation. The model also uses TCF12 as the E-protein partner, but the endogenous ASCL1 partner is not established in the SCLC experiments.
8. **Specificity claim.** The abstract claims an SCLC-specific role, yet no non-SCLC or non-neuroendocrine line (or POU2F3-high SCLC) is tested for POU2F1 dependence, and Fig. 1F-G show POU2F1 is uniform across subtypes. Context specificity is inferred, not demonstrated.

3. Journal Fit & Publication Potential

Fit scores are editorial judgments for the current state; please verify current policies on the journals' sites.

Journal	Fit score	Tier	Rationale
Cancer Cell (stated target)	38/100	Target	Editorially, functional motif-level and in vivo or therapeutic evidence would strengthen a mechanistic story that is currently correlative at its headline claim.
Molecular Cancer Research	74/100	Realistic	Editorially, the dataset appears aligned with a molecular cancer biology readership at its present depth.
NAR Cancer	72/100	Fallback	Editorially, the genomics and transcriptional-regulation emphasis may be relevant if the mechanistic claims are calibrated.
Cancer Research	66/100	Alternative	A solid new regulator of a clinically important antigen fits a cancer-focused specialty readership.
Nature Communications	55/100	Reach	Suitable breadth for a screen plus genomics story if claims are tempered.

To move up a tier, the authors need motif-level perturbation (endogenous editing or reporters at DLL3 and INSM1) showing that the tandem arrangement is necessary, and evidence that DLL3 surface display or antigen stability is affected.

4. Mechanistic Depth

For Cancer Cell, showing necessity of POU2F1 for DLL3 is not sufficient. A convincing mechanism would show that POU2F1 directly binds the DLL3 promoter (input-controlled ChIP or CUT&RUN in KO-validated cells), that mutation of the POU half-site or its spacing relative to the E-box reduces activity at DLL3 and INSM1, and that ASCL1 and POU2F1 are interdependent for binding or accessibility. Currently the authors' own hierarchical model (ASCL1 as pioneer, POU2F1 reinforcing) is untested: they have no accessibility or order-of-binding data after either KO.

The structural model is a hypothesis generator. Mutation of the predicted TCF12-POU2F1 basic patch with co-IP or DNA-binding readouts would be the minimum to turn it into mechanism. Without this, the explanation of how a ubiquitous factor gains lineage specificity is untested.

5. Broader Significance & Claim Calibration

The framing is broader than the evidence. The abstract's "previously unrecognized transcriptional logic" and "cis-regulatory code for the lung neuroendocrine cell fate" rest on enrichment of fetal neuroendocrine signatures among 227 genes assigned near 882 genome-wide motif instances and on structure predictions. The extension to normal neuroendocrine contexts is speculative, since no normal neuroendocrine model is tested. The therapeutic implications for DLL3-directed agents are also untested.

What is demonstrated is that POU2F1 loss reduces DLL3 and neuroendocrine gene expression in three SCLC-A lines, and that POU2F1 and ASCL1 co-localize at a subset of sites. Framed that way, the work is a solid specialty contribution. Realistically it sits at a field-journal level now. Reaching Cancer Cell would require functional motif-level evidence and an in vivo or therapeutic link to DLL3 durability.

References

The literature this review draws on. These are the reviewer's sources, not your manuscript's own reference list (which is screened separately in the Reference Integrity Check).

1. Mark D. Borromeo et al. (2016). ASCL1 and NEUROD1 reveal heterogeneity in pulmonary neuroendocrine tumors and regulate distinct genetic programs. doi:10.1016/j.celrep.2016.06.081

2. Yu-Han Huang et al. (2018). POU2F3 is a master regulator of a tuft cell-like variant of small cell lung cancer. doi:10.1101/gad.314815.118

Verification Receipt

What we machine-checked on your submission. These are deterministic checks (not opinions); the detail behind each is in the sections below.

- No retracted, expression-of-concern, or hijacked-journal references were detected among the 67 reference(s) we could resolve and screen.

- 2 claim(s) where a cited source may not fully support the statement as written, please review (detail in Citation Accuracy Analysis below).

Resolved and screened 67 of 68 references (only references that resolve to a bibliographic record can be auto-screened) and 13 citation-claim pair(s). Items marked to verify are signals for your attention, not verdicts.

Reference Integrity Check

We screen your manuscript's own reference list below; the numbers here are your manuscript's own bibliography, not the sources cited by the reviewer.

68 references analyzed | 67 verified | 0 minor mismatch(es) | 0 misattributed | 0 abstract-only | 1 could not confidently match | 0 retracted

Citation recency: 22 refs from last 5 years, 15 from 5-10 years, 31 older than 10 years

Median citation year: 2016

Citation Accuracy Analysis

13 claim-citation pairs checked | 6 Supported | 3 Partially Supported | 0 Not Supported | 4 Unable to Verify *(per cited source)*

These checks compare each claim against the retrieved abstract of each cited source. They are signals to verify against the full papers, not verdicts. A claim supported by several sources together is not flagged here; only claims where no cited source we could read supports them are listed below.

1 of the checks above were rechecked using available open-access full text. Full-text access does not guarantee a conclusive verdict; the outcome counts above include any inconclusive checks.

Claims to verify against your cited sources

[Verify] "In contrast to canonical Delta-like ligands that activate Notch signaling at the cell surface, DLL3 primarily localizes to intracellular compartments and functions as a negative regulator of Notch pathway activity during development6,7."

- The cited source only partially supports this claim as written, consider tightening it or confirming against the full paper.

- **Resolved source:** Divergent functions and distinct localization of the Notch ligands DLL1 and DLL3 in vivo (doi:10.1083/jcb.200702009)

- **What that source's abstract states:** The paper demonstrates that DLL3 localizes to the Golgi apparatus rather than the cell surface (unlike DLL1), and that DLL3 does not activate Notch signaling in their experimental systems. However, the paper explicitly states they do not observe evidence for antagonism or repression of Notch activity by DLL3.

- **Possible tightening:** "In contrast to canonical Delta-like ligands that activate Notch signaling at the cell surface, DLL3 primarily localizes to intracellular compartments (the Golgi apparatus) and does not activate Notch signaling."

- **Resolved source:** The divergent DSL ligand Dll3 does not activate Notch signaling but cell autonomously attenuates signaling induced by other DSL ligands (doi:10.1083/jcb.200503113)

- **What that source's abstract states:** The paper demonstrates that DLL3 does not activate Notch signaling and functions as a dedicated inhibitor of Notch signaling in a cell-autonomous manner, suppressing signaling induced by other DSL ligands.

- **Possible tightening:** "In contrast to canonical Delta-like ligands that activate Notch signaling, DLL3 does not activate Notch signaling but functions as a negative regulator of Notch pathway activity during development, suppressing signaling induced by other DSL ligands."

[Verify] "CRISPR-based loss-of- function screens have been particularly informative in this disease, revealing subtype-specific regulators, lineage vulnerabilities, and chromatin dependencies that are not readily inferred from gene expression or genomic data alone23."

- The cited source only partially supports this claim as written, consider tightening it or confirming against the full paper.

- **Resolved source:** Defining a Cancer Dependency Map (doi:10.1016/j.cell.2017.06.010)

- **What that source's abstract states:** The paper analyzed 501 genome-scale loss-of-function screens across diverse cancer cell lines and identified 769 genes differentially required in subsets of cell lines, developing predictive models for dependencies. However, the paper used RNAi-based screens rather than CRISPR-based screens.

- **Possible tightening:** "Loss-of-function screens have been particularly informative in this disease, revealing subtype-specific regulators and dependencies that are not readily inferred from gene expression data alone."

Summary

2 claim(s) where no cited source we could read fully supports the claim as written, verify each against the full paper(s). Separately, 4 citation-claim pair(s) could not be confirmed from the available checks.

Submission Completeness (Data, Code, Trial Registration)

A reviewer routinely checks whether the data / code are shared and, for clinical trials, whether the study was registered. Missing disclosures are common desk-return reasons. This is a detection check on the manuscript text; it flags a clear gap, not whether a named accession or registration is valid (verify those yourself).

Verdict: Availability and registration disclosures look complete

Data-availability statement: detected | **Deposits detected:** GEO (3)

Scope of this review

This assessment is based on the manuscript text and the figures in the file reviewed. Supplementary data tables were not analyzed as structured data, so any validation, extended results, or statistics reported only in the supplementary materials were not independently checked here. If your supplementary files contain data central to the manuscript's claims (for example, method-validation tables, per-item results, or full statistics), include them with the manuscript for the most complete assessment.