Reviewer’s report

Title: Epithelial Notch signaling is a limiting step for pancreatic carcinogenesis.

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Reviewer: Charles Murtaugh

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This is an interesting, well-written report taking a novel approach to inhibiting Notch signaling in a mouse model of pancreatic cancer initiation. The strategy is to express a dominant-negative Mastermind-like-1 protein (dnMAML - expressed as fusion protein to GFP) from a Cre-dependent ROSA26 knock-in allele, together with the well-studied Kras-G12D conditional mutant allele, using a pancreas-specific Ptf1a-Cre deleter transgene. The resulting mice (KC-DNMAML) should have activated Kras but inhibited Notch signaling, and are compared to mice expressing Kras-G12D alone (KC). The latter mice develop precancerous PanIN lesions by 15 weeks of age (referred to hence as the “early” timepoint), which progress and become more numerous by 26 weeks (“late”). The authors find that PanIN formation is inhibited by dnMAML expression at the early timepoint, but that PanIN numbers begin to catch up at 26 weeks. The PanINs that do form in the context of dnMAML expression are essentially identical to those of KC controls, albeit lower grade due (potentially) to the delay in their development. When the authors stimulate PanIN formation via caerulein-induced acute pancreatitis, PanIN formation is accelerated to near-control levels in KC-DNMAML mice. The authors conclude that Notch signaling “contributes to pancreatic carcinogenesis but it is not absolutely required” (p. 14).

HOWEVER: as the authors themselves note, dnMAML protein becomes excluded from the nucleus in lesions of later-stage KC-DNMAML mice, meaning that it may no longer effectively inhibit Notch. Moreover, there is no evidence for robust Notch target gene repression in established PanINs, even at early stages (the best data on Notch target genes comes from whole-organ RT-qPCR). As a result, the authors are limited in the conclusions they can draw about the role of Notch in established PanINs. This is a technical problem, and there’s nothing the authors can do about it other than acknowledge it more explicitly, which means avoiding statements such as, “once PanINs had formed, they had the expected proliferation and cell survival rate; neither of which was affected by inhibition of Notch signaling” (p. 10). If anything, the results presented here suggest that Notch signaling is completely essential for PanIN formation and maintenance, as the only PanINs that do form are ones that have, via unknown mechanism, overridden dnMAML. Given the controversies in the literature over the role of Notch in pancreatic cancer (which the authors summarize fairly), several points need to be clarified in order for this paper to represent a net gain to the field. These are listed below, in approximate order in which they arise within the manuscript - essentially all that is required, although I do think it is required,
more rigorous and quantitative analysis of GFP expression at various stages of PanIN initiation and progression (Major Compulsory Revisions):

1. p. 11: “GFP staining overlapped with DAPI nuclear staining, suggesting that DNMAML was localized in the nuclear compartment (Figure 2A).” This is a critical point: does GFP ever show up in the nuclei of PanIN cells, indicating that lesions can develop despite potentially successful Notch inhibition? It isn’t clear, in Fig. 2A, whether the GFP staining (which does appear nuclear) actually corresponds to PanIN cells. This could be resolved by co-staining for claudin-18. In addition, at least rough quantitation should be performed to determine the fraction of PanINs containing nuclear GFP staining at the early vs. late timepoints.

2. Related to the above, does the shift from nuclear to cytoplasmic staining from early to late timepoints occur only in PanINs, or in normal acinar cells as well?

3. The Hes1 staining in Fig. 2 is unconvincing - it looks mostly cytoplasmic and background-y. I’m not sure it can support strong conclusions about Notch pathway activity.

4. p. 12: “Thus, the loss of phenotype in KC;DNMAML mice over time coincided with re-activation of Notch signaling.” Exactly right, and this point makes it impossible to sustain the claim that established PanINs do not require ongoing Notch signaling.

5. p. 13: “Notch signaling inhibition did not inhibit Kras downstream effector activity in our system.” Only true IF Notch is really inhibited in PanIN lesions, which the authors have not fully established.

6. p. 14: “Similarly, KC;DNMAML developed PanINs, although they retained acinar clusters 2 and 3 weeks after pancreatitis, compared to KC animals (Figure 4B, C). However, by 4 weeks after pancreatitis the two cohorts were indistinguishable. Thus, the induction of pancreatitis bypassed the requirement for epithelial Notch signaling during the onset of pancreatic carcinogenesis.” Again, this conclusion requires establishing that the PanIN lesions formed post-caerulein exhibit nuclear GFP-dnMAML localization.

Additional minor points for which clarification/correction is required (Minor Essential Revisions):

7. Since Ivan Maillard is an author on the paper, the dnMAML mice shouldn’t be referred to as his “gift.” (p. 5)

8. Not sure what this means (p. 5): “5 animals per cohort (#5 mice/cohort) were aged 15 and 26 weeks before euthanasia.” Is it 5 mice, or #5 mice?

9. p. 6: “For a list of antibodies used, see Supplemental table 1.” I can’t find this table (or Supp Table 2, p. 7) in the submission.

10. p. 8: “In brief, 5 randomly selected, non-overlapping high-power images (20x objective) were taken for each slide. A minimum of 50 total acinar or ductal
clusters was counted from at least three independent animals for each group.” How are “clusters” being defined here? Five 20x fields should contain far more than 50 acinar clusters, is each one really included in the count here?

11. There is a “Cell Culture” section within the Materials and Methods (pp. 8-9) that doesn’t seem to go with any part of the Results.

12. Other groups have reported developmental consequences from mis-expressing dnMAML in the pancreas (Horn et al PNAS 2012, Afelik et al Development 2012) - although it is not necessary for the authors of this manuscript to fully characterize the developing pancreas, they should at least comment on whether or not there are any detectable abnormalities and, if not, speculate on what distinguishes their study from those mentioned above.

13. p. 12, Fig. 2C: “At 15 weeks, we observed a decrease in HES1 in KC-DNMAML samples (n # 3) when compared to KC samples, as predicted.” Not clear if this is statistically significant, given large error bars in KC sample. Across the board, there is a lot of noise in the KC samples of Fig. 2C, undermining confidence in the comparison to KC-DNMAML.

14. Resolution of all figures is rather low - not sure if this is fault of authors or journal reviewing system, but hopefully higher-quality figures will accompany published version.

15. p. 14: Section titled “Assessing Notch activity in the tumor microenvironment” doesn’t contain any data, and should not be in Results section.

16. p. 15: “Additional mouse models that conditionally ablate Notch receptors in the pancreas epithelium demonstrated that Notch2 and not Notch3, is critical for PanIN initiation [17]. This finding deviates from prior studies that showed Notch3 is required for PanIN initiation [10, 11].” These references show that Notch3 is upregulated during PanIN/PDAC development, but none of them actually addresses the function of Notch3.

17. It seems unnecessary to cite both the 2009 and 2013 “Cancer Statistics” papers (refs. 1, 3).

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**

I declare that I have no competing interests.