nature portfolio

Peer Review File

Neuroblastoma plasticity during metastatic progression stems from the dynamics of an early sympathetic transcriptomic trajectory

Editorial Note: The figure on page 24 of this Peer Review File has been redacted as indicated to remove third-party material where no permission to publish could be obtained.

REVIEWER COMMENTS

Reviewer #2 (Remarks to the Author): Expert in neuroblastoma genomics, functional genomics, in vivo models, metastasis, and scRNAseq

The manuscript `Neuroblastoma heterogeneity and plasticity over disease progression are rooted in the dynamics of an early sympathetic transcriptional trajectory´ by Benjamin Villalard et al investigates how tumor cells adapt during the metastatic sequence in the embryonal tumor neuroblastoma. The study uses an embryonic avian xenograft model and neuroblastoma patient primary tumors and bone marrow metastases and investigates these with single cell RNA-sequencing approaches and microscopy. Published single cell and bulk RNA-sequencing data of patient tumors and normal sympathoadrenal development are used to infer cell states and evaluate clinical significance of cell state associated transcriptomic signatures. The most novel and interesting finding of the study is the inference of the metastatic route and the adaptation of tumor cells to their microenvironments along these routes. Given the poor outcome of patients with neuroblastoma, this is an important and timely study. I have however several concerns regarding limitations in the experimental setup, data interpretation and in part novelty of the biological findings of this study, that need to be addressed.
Major:
1. The avian embryonic microenvironment may not be representative of the human one in order to recapitulate neuroblastoma metastatic processes. Specifically, it is not clear whether and to which extent signals from the tumor to the microenvironment and vice versa are conserved.
2. Related to this, in the introduction on sympathoadrenal development (lines 81-92) it should be clarified to which species the authors are referring to, since there are species-specific differences (e.g. between mouse, human and avian, see e.g. doi: 10.1016/j.ccell.2021.04.009).
3. Human tumor cells are injected into the developing embryo, however, genetic insults most likely happen in different progenitor states in a sequential manner and not at the same time and at different rates. Tumor cell dissemination and metastasis may happen in parallel to the acquisition of genetic insults and this is not addressed in this study. This is a limitation.
4. The authors state in the introduction "…how NB cells adapt to the different microenvironments of the primary tumor sites, metastatic routes and secondary foci remains obscure". As the study is focusing on neuroblastoma primary tumor and bone marrow metastasis in human and animal models it is advised to describe seminal work that has already addressed the questions above (e.g. DOI: 10.1038/s41467-023-38239-5; doi: 10.1038/s41467-023-39210-0 and their own work) in the introduction accordingly and make clear, which specific biological aspects are addressed and novel in their study.
5. The informative content of figure 1i is limited, as quantitative information (e.g. enrichment score) and statistical validity (adj p-value) is missing. Also, the gene sets used are not indicated in the figure or legend.
6. It should be made clear throughout the manuscript, which datasets, figures and results refer to human and which one to mouse or avian xenograft models.
7. Data presented in figure 2f and g are not convincing, since the gene sets found to be specific/unique to c0, c1 and c2 do not show a strong correlation with cell types derived from physiological sympathoadrenal development. Explanation of the color coding in Figure 2f is missing.
8. Showing gene expression on UMAPs only, such as in figure 2c is not sufficient – statistical analysis is required to demonstrate differential expression in specific clusters.

9. For statistical analysis presented in figure 3e-k, how were confounding factors/co-variates modeled for survival analysis and COX regression? In the multivariate analysis, INSS stage was not considered a confounding factor. Therefore, no clear interpretation of the data is possible.
10. Deconvolution of bulk RNA-seq data as presented in Figure 3f is highly questionable, since current deconvolution algorithms cannot appropriately resolve rare cell types and therefore will be biased towards the predominant cell type.
11. The finding that “SA-lineage-related NB states are maintained across the metastatic dissemination” is not novel. The same applies to the statement that the fraction of neuroblast/sympathoblast-like cells and SCPs correlates with outcome and MYCN amplification state (doi: 10.1038/s41588-021-00806-1).
12. Enrichment of bone marrow tumor cells was done using a marker panel. It is not clear which of the markers were used for selection of tumor cells and the gating strategy is missing. Generally, this might introduce a bias, if the markers used, favor specific cell phenotypes, such as sympathoblast-like cells (e.g. in case of GD2 and CD56/NCAM).
13. The authors state “Interestingly for 6 out of 7 patient samples, the fraction of, comSNPCs-like and SNPCs-like cells was increased in the BM as compared to the matched PT, suggesting enhanced plasticity and pathological regulations of transcriptomic dynamics in favor of progenitor-like states in the bone marrow niche.” As the low number of samples does not allow a statistically valid conclusion, this is considered an overinterpretation.
14. I tis not clear how genetic clonal evolution (“relating the clonal evolution of NB cells into the embryo to their physical path” as presented in figure 5 and extended data figure 7) in the avian model can be reconstructed, since cell lines are injected and traced until embryonic day 14, which does not provide much time for genetic evolution. As in extended data figure 7a -c the x-axis label is missing, it is not clear how many variants have been detected and information is missing how variants were classified or which filters were applied.
15. Regarding the analysis of genes associated metastatic neuroblastoma cells presented in figure 7, one major bottle neck is starting with a gene set that is derived from only one cell line xenografted in avian embryo. It is at this point also not clear in how far the avian xenograft and human primary tumor and bone marrow metastasis counterparts resemble each other. Qualitative measures are presented in the form of inference and mapping, but quantitative measures are missing. Furthermore, current algorithms “force” mapping between different datasets and neglect the possibility that transcriptional profiles are not so similar after all. Metastasis steps in between are not accessible in humans. This might limit substantially the interpretability of data.
Minor:
1. The wording is at times not clear and I recommend Englisch proof-reading. For example, the authors state in the abstract that `…, Neuroblastoma (NB) plasticity and heterogeneity remain largely misunderstood´. This would imply that we currently have a wrong understanding of neuroblastoma metastasis, however in my opinion, current understanding is incomplete and studies on this topic are sparse.
2. Please revise “Pediatric cancers are highly heterogeneous diseases that, still, reflect their emergence from developing tissues. Half of them originate from embryonic cell lineages, thus at stages of organ formation and maturation, with ongoing lineage differentiation trajectories.” The term “ongoing lineage differentiation trajectory” is odd.
3. It needs clarification in how far figure 6i and j differ.
4. Please revise “It came out from these studies that two successive neural crest-derived progenitor

states contribute to SA cell contingents…”.
5. The color coding of figure 1j and l should be explained in the figure. Showing the same labels of 11, c2 and c3 on the x and y axis of figure 1k is misleading. This should be revised.

Reviewer #3 (Remarks to the Author): Expert in brain cancers and 3D imaging

Reviewer #

Title: Neuroblastoma heterogeneity and plasticity over disease progression are rooted in the dynamics of an early sympathetic transcriptional trajectory.

Benjamin Villalard and colleagues have produced a very interesting manuscript combining an avian model, neuroblastoma (NB) patient samples and a re-analysis of previously published data.

The authors found that NB cells recapitulate the disease by adopting states aligned to the SNPCs-to-neuroblast differentiation branch and that the primary tumour site conditions their dissemination path. They then analyse the gene expression dynamics throughout the metastatic process using transcriptomic data from paired primary tumour/bone-marrow NB patient samples and identify a list of candidate genes upon bone marrow involvement that are associated with NB growth dependency, validating the relevance of our multimodal approach. Finally, the authors propose these genes as a set of therapeutic candidate genes whose expression is consistent with an active contribution to the growth of bone marrow metastases.

To reach these conclusions, the authors used light-sheet microscopy for the avian model and single-cell RNA expression approaches from isolated chicken embryo and patients NB cells.

The authors asked some challenging questions, focusing on understanding the underlying process of the NG cell: "Consequently, whether NB transcriptomic states across disease progression are still influenced by the dynamics of the SA lineage and how NB cells adapt to the different microenvironments of primary tumour sites, metastatic routes and secondary foci remains unclear."

I think the authors try to draw new conclusions about the clonal evolution of NB cells and develop interesting analyses for genetic validation in avian models and patients. However, it is important to consolidate their conclusions with more robust validations by more experiments for different states/conditions to better characterise the results shown in the manuscript. Below are some details that I suggest to the authors to improve the results in the manuscript.

Major points

I believe that light sheet microscopy is a great approach to analyse the NB cell proliferation in the avian model at early stages.

Fig1. a-n.

-In method section, I found the number of NG cells engrafted in the avian embryo at 2,500 fluorescent NB cells at the neural crest level. Did you try to engraft fewer NG cells and analyse the time course and the primary foci?

-I cannot find the number of embryos used at E0, E5 and E14 for the imaging data and for the genetic findings. Please include this in the text or methods (specifically the number of embryos used to reach these conclusions).

- The authors mentioned that they used the human stage 4 neuroblastoma IGR-N91 and SHEP cells. The two different migration routes and the time course of E0, E5 and E14 could be altered with different NG cell stages. Did the authors use human NG cells of stage 3 or 2? This should be clarified in the methods section.

The primary tumour foci are not quantified (ADR and SG) and this is important to analyse early study migrations in the avian model (calculate the number of cells per volume). Are there any correlations between the two different routes of migration to other areas of the embryo and the number of cells in the primary tumours in ADR and SG? The 3D images from the light sheet made this possible. The images could be improved.

-If I understand correctly, the avian model has two foci before E5, ADR and SG. How is it that these two foci form independently in the avian embryo with a single injection? I found this sentence, but I did not find a reference at the end of the sentence.

“At E5, NB cells exclusively formed tumours within the developing SG and ADR, that lie in proximity, as previously documented”.

- These two major routes (AOR and PN) described in the images are interesting at an early stage, but these have not been quantified to identify which route is more prominent in the E14 embryo to obtain a picture of the main route to BM stage.

-Fig1. d and e show NB cells spreading on nerves. In these images it is not clear whether it is a single cell or a group of cells. Also, in this picture if these cells are really using the nerve for migrations or could this also be random localisation? It would be important to include a video or pictures of the areas with a group of cells migrating using the nerves from light sheet microscope.

-The NG cells localised in the nerves differ from those localised in the aorta in size, morphology or specific markers used in the genetic analysis. It is a really important point to describe if there are any important adaptations besides the genetic differences detected in the paper.

-I did not find a 3D video of the avian embryo at E0, E5 and E14 stages from the light sheet microscope. Addition of these videos would add value to the manuscript.

-In the manuscript it is assumed that before the E5 there is no metastatic onset. How was this conclusion reached? Was the metastatic onset time frame the same for all the embryos that were profiled? The variability and aggressivity of this NG cells stage 4 can produce a different time of the metastasis onset.

-Fig1. Extended Data Fig1. The authors mentioned that α-TOP2A and α -LMNB1 are two important markers for the cluster C2 and E14. Have you tried to identify these two markers that appear in the NG cells on the nerves using light sheet microscope?

Fig2. a, b, c, some of these analyses that confirm the atlas data could be in the supplementary figures because this is the combination of the previous results from the two previous papers published.

-Fig2g. “From this projection dataset, we built a joint heatmap of expression for c0/c1/c2 top markers in both SA and NB cells. The associated hierarchical clustering pointed at tight transcriptional similarities between c2 and SNPCs, c0 and comSNPCs, and c1 and neuroblasts (Nbts/Late Nbts). These relationships were further confirmed by looking at the expression profile of key markers of SA identities into NB c0/c1/c2 clusters. Notably, SCPs emblematic markers -ERBB3, SOX10, PLP1- were all negative (Extended Data Fig. 2d)”.

-Fig4g. ‘‘UMAP plot of IGR-N91::GFP NB cells colored and labelled by cell location at each step of dissemination in E14 avian embryos -257 cells from sympathetic ganglia tumors (SG), 261 cells from adrenal tumors (ADR), respectively 84 and 25 disseminating cells along peripheral nerves (PN) and the aorta (AOR), and 31 cells in the bone marrow (BM).”

-Looking at the three clusters (c0, c1, c2) from Fig. 2g, there is a significant overlap between them in the heatmap. Also, none of the clusters match the SCP-like cells. Do you think that the number of cells from the avian model is too small to draw these conclusions because at E14 avian embryos you analyze only 25 cells disseminating cells along the peripheral nerves (PN)?

- Could the authors please clarify which signatures were used to annotate the scRNA-seq datasets (avian and patient-derived)? A table of top gene markers for each cluster for all datasets would be helpful.

-Fig4. h. ‘‘BM secondary site defined specific adaptations related to a given microenvironment, namely: neuronal-related features at the SG level (NRXN1, GFRA3, NPY, VIM); response to nutrients and extracellular signals at the ADR level (VFG, LDHA, PTN, IGFBP2); cell adhesion at AOR level (OBSCN, GFRA1, ITGA1, CNTN1); neuronal migration at PN level (NRCAM, ERBB4, NTRK2, PLXNB2) and gliogenesis at the BM level (NFAT5, LAMB1, PLEC).’’

Have you tried using NRCAM, ERBB4, NTRK2, PLXNB2 markers in the avian embryo to confirm the expression of neuronal migration at the PN level?

-Fig5. The confocal images from the avian model are clearer compared to Fig1, about the possible PN migration, but the authors only use GPF as a marker to detect them. It should be useful to use NRCAM or ERBB4 or NTRK2 or PLXNB2 as a marker to identify these cells to confirm the genetic profile in combination with GFP and α-NF160.

‘‘These parsimony tree characteristics suggested that primary tumors located in the sympathetic chain or in the adrenal medulla both disseminated via the peripheral nerves while the aortic path preferentially concerned tumors of the adrenal medulla.’’

-In the avian model, the AOR and Sg, the primary foci, are in close proximity to each other. How did the authors assume that these foci are completely independent or are both in constant interaction between NB cells migrating in and out for each focus?

-In Fig6. a the authors mention that variant allele frequencies and scRNA-seq data were used to determine the trajectories. It's not clear to this reviewer how this analysis was performed (especially the integration of mutational and transcriptomic data). Trajectory analysis can also be performed using scRNA-seq data alone, could the authors please clarify why variant allele frequencies were also used?

-Fig7 f. In this figure, 25 genes were found to be harbor NB dependency for cell growth. It would be important to confirm the high expression of the top genes in the BM using patient tissues.

Minor points

-Some acronyms including in the figure Fig2 could be clarified in the legends to help the readers.

Reviewer #4 (Remarks to the Author): Expert in scRNA-seq, lineage analysis, cell plasticity, cancer evolution and intratumour heterogeneity

Comments to the authors:

In the manuscript by Villalard et al entitled “Neuroblastoma heterogeneity and plasticity over disease progression are rooted in the dynamics of an early sympathetic transcriptional trajectory” the authors attempt to bring light into the process of metastatic dissemination in Neuroblastoma. Unfortunately, and it is the opinion of this reviewer, the manuscript does not go sufficiently far onto it and promises too much when only correlations are suggested by the data.

Major comments:

1- One of my major concerns stems from the avian and cellular models used. If I correctly follow the manuscript, the authors engraft ~2500 NB human cells (fully-fledged, please see below for comments on the cell line) in the avian embryo which by the nature of the method will not be all engrafted. So, which is the % of cells that are engrafted? Where do the rest of the cells go? How many cells the avian embryo have at E2? Is it possible that what the authors suggest as metastatic dissemination is just growth from the cells that did not engraft? The authors suggest that the grafts form dense primary tumour masses… How dense? Which is the cellularity? Is the tumour mass infiltrated by other cell types? How many embryos were grafted? How efficient and how reproducible the engraftment is? If it is not efficient… how did the authors chose which ones to follow, and which one should not be followed? Is it possible to follow a single embryo through time after engraftment rather than taking different embryos at different times to verify dissemination? All the above must be addressed to strengthen the claims made by the authors.
2- The NB cell line (NB Stage 4). If we are to assume that NB is anchored in developmental processes, it is the opinion of this reviewer, that a fully-fledged, evolved (in a different organism) and quite probably selected by the environment (of a different organism) would not make a great avatar to study the influence of the developmental process as the NB cell have already experienced all of that. What does it look like the avian engraftment with NB cells from other stages? Wouldn't it be better to test different stages to infer behaviour rather than going all the way with stage 4?
3- The authors argue that NB cells exclusively form tumours within the developing SG and ADR… is this a biological consequence or an artefact of proximity on the engraftment? Numbers are needed here. Occurrence of one over the other. Once again, this could contribute to the dissemination path taken and must be addressed.
4- I find the comment in line 142 "… hence mimicking typical features of metastatic NB in patients…" rather strong. It is just an observation. Numbers are needed. How many times did this happen? Is it reproducible? Is there organ/localization bias? Is there some sort of temporal hierarchy?
5- The authors argue that they take samples from "… 5 physical sites representing different steps of the disease progression…" but then they only mention 3 samples (E0, E5 and E14) that were analysed by scRNA-seq. What happened with all the physical sites? Were the samples pooled? Were some anatomical sites removed from the analysis? Where all the anatomical sites evaluated independently? I find this part of the manuscript quite perplexing as, it is quite probable and there is a lot of literature supporting this concept, each anatomical site would present a different microenvironment. Thus, the NB cells will be embedded in different environments which will affect the NB cells biology (cell-to-cell interactions, signalling, extracellular matrix components, among others) and thus should be analysed independently.
6- Regarding the scRNA-Seq in the avian/NB cells model. What does it mean high depth scRNA-Seq? How many transcripts were detected? How many reads per cell? How many transcripts were fully reconstructed? This information must be included in the manuscript.
7- Also, regarding the scRNA-Seq in the avian/NB cells model, this reviewer finds that the number of cells analysed by scRNA-Seq is way too low to draw any conclusion. Allow me to explain… ~1000 cells were analysed in total in the first avian/NB cells model… in 2 different platforms… with 3 different samples each… including 5 anatomical sites. So how many cells per condition are really there? What about

replicates technical and biological? The authors mention replicates… How many avian embryos were processed? If it is only 2… well the numbers are really low to draw any conclusion.
8- Moreover, regarding the sample at time E0, which essentially are cells in cell culture analysed by scRNA-Seq. Why is this sample in the analysis at all? Cells grown in plastic display massive changes in gene expression when grown in an in-vivo model and this has been documented multiple times. This reviewer agrees that E0 sample must be analysed but I'm not so sure about the value of integrating this sample in the clustering of the avian/NB samples. It could be completely misleading.
9- The authors argue that the E0 samples display a "… homogenous transcriptomic profile…" . I can clearly see 3 clusters in Fig 1l. As the authors are aware, clustering is highly dependent on the math employed. I'm pretty sure that this data deserves a further look and I encourage the authors to revise their data here.
10- This reviewer is not sure whether a Pathway analysis with such a low number of cells is reliable. Replicates and more cells need to be assessed to strengthen the concept. Also, Fig 1i does not display a scale bar.
11- The authors mention "transcriptional state" all over the manuscript. However, the authors do not know whether the observed changes are a consequence of changes in active transcription or steady state RNA levels. I suggest the author to rephrase every "transcriptional state" for "gene expression".
12- Similarly, the "predictive" transcription factor analysis, unless leveraged by measuring "active transcription" and not steady state RNA levels is not relevant.
13- The authors state in line 224 that "… the regression of cell cycle genes… did not interfere with transcriptional similarities…". How is that the cell cycle regression does not interfere? I do not think "interfere" is appropriate here.
14- In line 230, the authors mention: "… by analysing RNA velocity…" please rephrase this as the way the phrase is built it looks like the authors are analysing a speed related behaviour of RNA and not using a computational pipeline.
15- In Figure 2e, the transcription factor profile, I do not see differences that would support the discrimination between late NBts and NBts or between comSNPCs and SNPCs.
16- In line 244, the authors state: "… we depleted cell cycle related genes…". What does the authors mean by depleted? Was the data removed? Was it regressed? If it was regressed… how was it regressed?
17- Regarding the construction of the Atlas, additional information must accompany the manuscript. For instance: How many cells were sequenced per patient sample? How deep were they sequenced?
18- Regarding the integrated Atlas, this reviewer may be mistaken but, I do not think that pooling together datasets from public repositories could be the core of a manuscript. This is standard practice now. I suggest the authors to send all this data to Supplementary material.
19- It would be interesting to see how the single cell data cluster based on localization (i.e only adrenal gland) in addition to all the patients together. Interesting insights could be learnt.
20- In line 275, the authors explain that some patient samples have a low number of cells… How the low number of cells is now relevant and not at the beginning of the manuscript?
21- In line 311, the authors argue that they use "… emblematic markers..."… which markers?
22- In line 316. The authors argue that they've used "… a panel of cell surface markers…"… which markers? How was this controlled? How were the markers validated? Please include data.
23- In line 318, the authors mention that they have "sub-sampled" for deeper analysis. Sub-sampled? Based on which criteria, why and how? This sounds a little too arbitrary to this reviewer.
24- Regarding the genetic analysis derived from the single cell data obtained from the NB cells in the

avian model… The data clearly shows that NB cells are not genetically homogenous… does the genetic divergence happen within the experiment? Or the NB cells used are already genetically heterogeneous? The distinction is fundamental as it could explain differential dissemination, engraftment efficiency, among other features. I suggest the authors to have a look at the data from E0 (cells in culture) to extract this information and to include it in the manuscript. Also, this may take a while but if the genetic alterations are already there, it would be interesting to generate clones of the NB cells used and repeat the experiments here presented.
25- In line with comment 24… How genetically stable is the NB cell line used for the avian experiments? Is the cell line diverging genetically all the time? Is it rather stable?
26- In Line 376 paragraph the authors argue "…Confocal analysis…allowed to document and confirm the occurrence of the physical transitions predicted by genetic variant analysis…" This statement is not correct. Unless you can pinpoint the mutations on-site, which cannot be done by confocal microscopy, this is an overstatement and must be re-phrased and toned down.
27- In Line 406 the authors argue: "… we selected all genes showing significant upregulation… to focus on frequent, and thus druggable events…". What does the authors mean here? There is hardly any correlation between upregulation of transcripts with druggable targets. Modulation of transcripts does not mean at all that those molecules are druggable. Please revise.
28- Regarding the "useful gene set". I do not agree with the conclusions here drawn and I find the entire section overstated. I think it would potentially strengthen the manuscript if the authors would use the listed NB cells lines and repeat the avian graft experiments to actually verify their hypothesis.

Minor comments

1- I do not think the abstract reflects the findings which are mainly based on correlations. The distinction must be made evident. I suggest the authors to tone it down.
2- I suggest the authors to re-shuffle and simplify the introduction. Please revise sentences such as: "… The specific origin triggers the formation…" I do not think that the specific origin is triggering anything. Perhaps "contributes" would be a better option.
3- Please make the nomenclature uniform across the Methods section. Sometimes HH… sometimes E(N).
4- In the Method section the authors sate that the different NB cells lines used are grown in different cell culture conditions (medium) which will undoubtfully alter the metabolism of the cell lines analysed and thus will results in altered gene expression. Please comment.
5- In Line 688 the authors state that IGR-N91::GFP cells were harvested before cell engraftment using a fluorescence stereomicroscope… How was this done with cells in culture growing in a plate?
6- Please check for typos. For instance, in Line 713 the pipeline CutAdapt is spelled CutApapt
7- In Line 723 the authors state: "… These parameters were adapted for each sequenced conditions due to handling bias…". Please state which parameters and how was the criteria chosen?
8- In Line 752 the authors state: "… A set of tools developed in Seurat was applied…". Please describe which set of tools and how the parameters were chosen?
9- Legends are too convoluted and are not sufficiently developed to follow all the figures. Please revise.
10- Unless I'm totally lost here. Could the authors explain what is the difference between Figure 3f and Extended Data Figure 5a?
11- In Extended Figure 2 b clear differences can be observed between the samples obtained at different WPC. Is this biology or a technical artefact? Please comment.

12- Similarly in Extended Data Figure 2e a clear cell cycle bias can be observed between the different samples. Please comment. Perhaps this differential cell cycle feature could be exploited.

Reviewer #5 (Remarks to the Author): Expert in cancer avian embryonic models

The Authors have written an extremely interesting and innovative paper, which increased our knowledge on the pathogenesis and disease progression of neuroblastoma.

Point-by-point answer to the reviewers

We thank very much the reviewers for their constructive comments. We fully understand that these important points needed to be addressed. In our revisions, we conducted a series of novel experiments and analyses, and modified the manuscript to provide answers to these points. Please find below the detailed answers to the points raised by the referees.

Reviewer #2: Expert in neuroblastoma genomics, functional genomics, in vivo models, metastasis, and scRNAseq

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The manuscript `Neuroblastoma heterogeneity and plasticity over disease progression are rooted in the dynamics of an early sympathetic transcriptional trajectory´ by Benjamin Villalard et al investigates how tumor cells adapt during the metastatic sequence in the embryonal tumor neuroblastoma. The study uses an embryonic avian xenograft model and neuroblastoma patient primary tumors and bone marrow metastases and investigates these with single cell RNA-sequencing approaches and microscopy. Published single cell and bulk RNA-sequencing data of patient tumors and normal sympathoadrenal development are used to infer cell states and evaluate clinical significance of cell state associated transcriptomic signatures. The most novel and interesting finding of the study is the inference of the metastatic route and the adaptation of tumor cells to their microenvironments along these routes. Given the poor outcome of patients with neuroblastoma, this is an important and timely study. I have however several concerns regarding limitations in the experimental setup, data interpretation and in part novelty of the biological findings of this study, that need to be addressed.

Major:

1. The avian embryonic microenvironment may not be representative of the human one in order to recapitulate neuroblastoma metastatic processes. Specifically, it is not clear whether and to which extent signals from the tumor to the microenvironment and vice versa are conserved.

We fully agree that animal models cannot entirely recapitulate human disease. Nevertheless, the nature of the questions our study addresses requires on one hand the whole context of an embryo, and on the other hand a model suited for human NB cells. None of other existing models bring conditions meeting this dual need. Several features make the avian embryo a model relevant to study early events of NB disease.

First, it has been widely utilized has an organism model to study vertebrate sympathetic chain morphogenesis, demonstrating strong conservation across vertebrates (Saito et al, 2012; Shtukmaster et al, 2013; Kasemeier-Kulesa et al, 2015; Holzmann et al, 2015). Moreover, birds bare two adrenal glands lying on top of the kidney, with distinct medulla and cortical compartments that closely resemble that of mammals, while fishes have very different organization for example (Capaldo, 2023). Our previous work with the avian embryo model provided evidence that human NB cells establish relevant communications with the embryonic host cells. For example, analysis of the transcriptome of NB tumours formed in the avian embryo showed significant changing of signaling pathways, when compared to that of equivalent cells prior to grafting (in culture). These molecular changes were relevant as they were related to biological processes of cell migration, adhesion, cytoskeletal modifications, fully aligned with the metastatic behaviour manifested by NB cells in the model. Notably, we found these changes significantly distinguished stage 4 NB from other stages in large patient cohorts (Delloye-Bourgeois et al, 2017; Ben Amar et al, 2022). This way, by crossing our findings with the avian embryo model and data from the clinic as we did it in the present work, we expect to assess and validate the relevance of the information.

Second, molecular signaling acting during development to orient cell migration are highly conserved across vertebrate species. As examples, we showed that the mouse and chick secretomes of sympathetic ganglia produced equivalent effects on NB cells which demonstrates NB cells perceive molecules secreted by the developing sympatho-adrenal tissues whatever they are of chick or mouse origin (Ben Amar et al, 2022).

Thus, despite not recapitulating all aspects of the disease, which we fully agree and are aware of, we nevertheless strongly believe that the avian embryo model opens an exceptional window of investigations onto primitive events, that can reveal crucial events remaining inscrutable in human and other animal models.

2. Related to this, in the introduction on sympathoadrenal development (lines 81-92) it should be clarified to which species the authors are referring to, since there are species-specific differences (e.g. between mouse, human and avian, see e.g. doi: 10.1016/j.ccell.2021.04.009).
We fully agree with the reviewer on this point and we have now specified to which species we are referring to regarding information on sympathoadrenal development.

3. Human tumor cells are injected into the developing embryo, however, genetic insults most likely happen in different progenitor states in a sequential manner and not at the same time and at different rates. Tumor cell dissemination and metastasis may happen in parallel to the acquisition of genetic insults and this is not addressed in this study. This is a limitation.
We fully agree with these statements. Our model was designed to recapitulate neither the oncogenic cascade occurring in healthy cells and leading to malignancy nor the heterogeneity of transformed cells/states among patients. Rather, by grafting NB cells back to the neural crest domain, we aimed to reveal the states that these cells manifest in a context mimicking that of disease emergence and their potential proximity to those manifested by the physiological lineage of origin. Our analysis of patient cohorts shows that the identified states are manifested by NB cells of primary tumors and bone marrow metastases of many patients, whatever the nature of the genomic alterations and accumulated mutations during metastatic progression. This indicates that these states allow the expression of all molecular heterogeneity features of NBs including those acquired during disease progression in the patients. Nevertheless, we also found that NB cells dynamically adapt their transcriptome to their microenvironment, still staying in these states. Thus, we truly agree that different mutational insults affecting NB cells could result in transcriptional specificities driving functional differences, but are not necessarily associated to changes of transcriptional states. This is now discussed in line 744.

4. The authors state in the introduction "…how NB cells adapt to the different microenvironments of the primary tumor sites, metastatic routes and secondary foci remains obscure". As the study is focusing on neuroblastoma primary tumor and bone marrow metastasis in human and animal models it is advised to describe seminal work that has already addressed the questions above (e.g. DOI: 10.1038/s41467-023-38239-5; doi: 10.1038/s41467-023-39210-0 and their own work) in the introduction accordingly and make clear, which specific biological aspects are addressed and novel in their study.
We understand this concern and have expanded the description of the background in the Introduction, in particular regarding the two studies mentioned by the referee. We also better introduce the questions addressed by our study. However, we would like to emphasize here that our aims and conclusions are very different from those studies:
- In doi: 10.1038/s41467-023-38239-5, the authors address the genetic and epigenetic mechanisms underlying the plasticity of mesenchymal to adrenergic interconversion of NB cells as an opportunistic manifestation of malignant features, not necessarily stemming from properties inherited from the cells of origin. Thus, studies are focused on primary tumors, and do not experimentally address the effect of this interconversion property during metastasis or in metastatic foci.
- To our understanding, in doi: 10.1038/s41467-023-39210-0, the study aims to compare the single-cell transcriptome of paired primary tumors/bone marrow metastases, as a powerful approach to characterize the molecular communication of NB cells with components of the bone marrow microenvironment (in particular monocytes in this study). However, it does not address the transcriptional and physical dynamics of NB cells progressing towards metastasis, as the analyzed samples analyzed are already established metastases. Our approach brings the intermediate step of dissemination missing in patients, allowing to discriminate among genes expressed by metastatic cells

those that functionally direct bone marrow involvement. Although both studies report on the transcriptome of NB cells, our specific focus on the context of embryogenesis and relationships with the sympatho-adrenal embryonic lineage led to an in-depth comparison of physiological and malignant states that goes beyond analyses produced to date.

5. The informative content of **figure** 1i is limited, as quantitative information (e.g. enrichment score) and statistical validity (adj p-value) is missing. Also, the gene sets used are not indicated in the figure or legend.
We apologize for the lack of clarity of panel 1i, that aimed at showing the major types of transcriptomic modifications occurring in tumors that develop into an embryonic organism. Quantitative information was indeed lacking to this approach; we now have added the mean enrichment score and p-value for each pathway / gene ontology process. Moreover, panel 1i has been placed in Supp Fig. 1e. Instead, as the initial approach alone could introduce a bias towards large gene ontology processes, we have extracted top biological processes (GO terms) differentially represented between E14 and E5 and between E5 and E0 stages in NB cells. This analysis is now shown in Fig 1i.

6. It should be made clear throughout the manuscript, which datasets, figures and results refer to human and which one to mouse or avian xenograft models.
We now have specified where relevant the specie / type of model to which we refer to (core manuscript and figure legends).

7. Data presented in figure 2f and g are not convincing, since the gene sets found to be specific/unique to c0, c1 and c2 do not show a strong correlation with cell types derived from physiological sympathoadrenal development. Explanation of the color coding in Figure 2f is missing.
Figure 2g was proposed in the first version of the article to complete the mapping analysis of NB cells on the SA lineage atlas (Figure 2f) and to highlight the high proximity between c0/c1/c2 pathological states and SA transcriptional states, by showing the level of expression of top genes of physiological clusters in NB cells. As these developmental markers are more expressed in physiological cell populations than in transformed NB cells, we agree that the heatmap doesn't look very convincing. Thus, we have replaced this panel with another type of analysis: we have extracted the lists of genes that allowed to map NB cell clusters close to specific clusters of the SA lineage in Fig. 2f. We have crossed these gene lists with the exhaustive list of marker genes for c0, c1 and c2 clusters (DEG analysis). The resulting signatures (NB-c0, NB-c1 and NB-c2) were scored in each cell population of the SA lineage, showing a clearer correlation between c0 and SNPCs; c1 and committed-SNPCs and c0 and late Neuroblasts (heatmap now shown in Fig. 2g).
We added explanation of the color coding that was missing.

8. Showing gene expression on UMAPs only, such as in figure 2c is not sufficient – statistical analysis is required to demonstrate differential expression in specific clusters.
We fully agree with this remark. In most principal figures, we have chosen to show gene expression on UMAPs to show the expression pattern of genes of interest and to reveal their heterogeneous expression within the cell clusters studied. However, statistical analysis was systematically performed and values are provided in Source Data F2, sheet c.

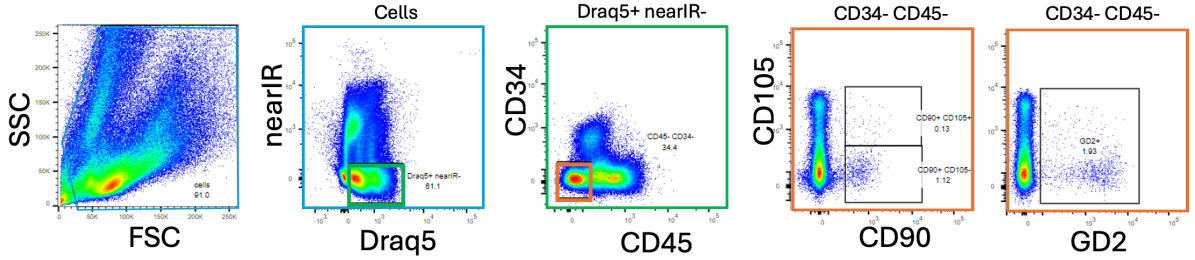
9. For statistical analysis presented in figure 3e-k, how were confounding factors/co-variates modeled for survival analysis and COX regression? In the multivariate analysis, INSS stage was not considered a confounding factor. Therefore, no clear interpretation of the data is possible.
We sincerely apologize as there is an error in Fig3e-K legend. INSS stage was indeed considered a confounding factor, but this information was missing from the legend. This has now been corrected. Confounding factors were corrected for the COX regression analysis in Fig. 3h.

10. Deconvolution of bulk RNA-seq data as presented in Figure 3f is highly questionable, since current deconvolution algorithms cannot appropriately resolve rare cell types and therefore will be biased towards the predominant cell type.
We are in complete agreement with the reviewer's concern about deconvolution algorithms. In the scope of this study, rare and highly transitory cell types (SNPCs, comSNPCs) apply to the physiological

SA atlas and were described from single cell RNASeq data. We used deconvolution of bulk RNAseq data of NB tumors only, in which these physiological-like states were found to be predominant. This strategy was guided by the results we obtained in single cell RNAseq data from NB tumors (Fig.3d-e), showing, among other things, that SNPCs-like and comSNPC-like states are not rare populations in NB tumors and are detected without any ambiguity.

11. The finding that "SA-lineage-related NB states are maintained across the metastatic dissemination" is not novel. The same applies to the statement that the fraction of neuroblast/sympathoblast-like cells and SCPs correlates with outcome and MYCN amplification state (doi: 10.1038/s41588-021-00806-1).
The referee may refer to Fetahu et al., 2023 (doi.org/10.1038/s41467-023-39210-0). We agree that the presence of SA-lineage related states in NB bone marrow metastasis and in primary tumors was demonstrated in this study. However, our point here was to state that the presence of a continuum of SA-lineage-related states remained among NB cells all along the dynamics of metastatic dissemination which, to our knowledge, has not been studied before. We apologize for the lack of clarity and have now reworded this statement. We nevertheless believe that our close examination of physiological states and transitions of the lineage of origin allows further refining the proximities with NB cells.
Regarding the second statement, we are not sure to which sentence the reviewer refers to. We fully agree that it was already described that the fraction of late versus cycling neuroblast-like states in NB tumors had an inverse impact on patient outcome (doi.org/10.1038/s41588-021-00806-1). Our statement regarding the impact of the relative fractions of Nbt/comSNPC/SNPC-like states on patient outcome aimed at highlighting that the comSNPC-like state, which is a cycling population but more engaged in the differentiation path as compared to SNPC-like cells, is associated with the worse impact on disease outcome.

12. Enrichment of bone marrow tumor cells was done using a marker panel. It is not clear which of the markers were used for selection of tumor cells and the gating strategy is missing. Generally, this might introduce a bias, if the markers used, favor specific cell phenotypes, such as sympathoblast-like cells (e.g. in case of GD2 and CD56/NCAM).
We apologize for the lack of clarity regarding the sorting strategy for patient BM and PT samples. The gating strategy was now more deeply described in the method sections, and is the following:



Draq^{+}/NearIR^{-} cells (green box) were selected, then CD45^{-}/CD34^{-} cells (orange box) and finally two parallel selections allowed to sort CD90^{+}/GD2^{+}, CD90^{+}/GD2^{-}, CD90^{-}/GD2^{+} cells.
We added an additional marker next to GD2 as GD2-low or -negative NB cells do exist, also in treatment naïve samples, as already reported in other studies (Terzic et al., 2018; Lazic et al., 2020; Pilgrim et al., 2023). We could previously show that by using CD90 as an additional marker, NB tumor cells were successfully enriched, as shown by FACS-sorting and subsequent qPCR for NB markers (Hochheuser et al, Cancers, 2020).

13. The authors state "Interestingly for 6 out of 7 patient samples, the fraction of, comSNPCs-like and SNPCs-like cells was increased in the BM as compared to the matched PT, suggesting enhanced plasticity and pathological regulations of transcriptomic dynamics in favor of progenitor-like states in the bone marrow niche." As the low number of samples does not allow a statistically valid conclusion, this is considered an overinterpretation.
We agree that this interpretation is not based on a statistical analysis and is rather hypothetic. We have deleted this statement from the results section and have now discussed this hypothesis in the discussion section.

14. I tis not clear how genetic clonal evolution ("relating the clonal evolution of NB cells into the embryo to their physical path" as presented in figure 5 and extended data figure 7) in the avian model can be reconstructed, since cell lines are injected and traced until embryonic day 14, which does not provide much time for genetic evolution.
As in extended data figure 7a -c the x-axis label is missing, it is not clear how many variants have been detected and information is missing how variants were classified or which filters were applied.
We thank the referee for these comments and we apologize for the lack of details and the missing information regarding the analysis of genetic clonal evolution. The missing labels on the x-axis have been added so that the number and the type of variants analyzed is now understandable.
First, these experiments were performed on IGR-N91 cell line – and not on primary cell lines or patient samples- engrafted in the avian embryo, and we could document, in this context, that IGR-N91 cells were actively cycling whatever the embryonic stage or the anatomical site (Supp Fig.2f). Second, we analyzed genetic variants from Smart-Seq2 data only, as this technique allows a very high depth sequencing (Supp. Fig. 1a and Source Data F1, sheet h) and thus ensures a high coverage of newly acquired genetic variants. Consequently, twelve days after their implantation in avian embryos (from E2 to E14), we could extract a median of 740 informative genetic variants per IGR-N91 cell. The filters applied to extract and analyze these variants is now better described in the methods section.
To be more precise regarding the methodology, starting from SNPs and indel informations extracted with GATK protocol, only variants affecting genes expressed in more than 99% of sequenced cells were taken into account. Maftool package (v2.17) was used to summarize variants informations. DENDRO (v0.1.1) was applied to filter out variants with variants allele frequency less than 0.05 (too rare) or greater 0.95 (too common). Genetic divergence matrix was built to compute hierarchical clustering for the parsimony tree, depicting the evolution relationship between subclones. This information has been added to the method section.

15. Regarding the analysis of genes associated metastatic neuroblastoma cells presented in figure 7, one major bottle neck is starting with a gene set that is derived from only one cell line xenografted in avian embryo. It is at this point also not clear in how far the avian xenograft and human primary tumor and bone marrow metastasis counterparts resemble each other. Qualitative measures are presented in the form of inference and mapping, but quantitative measures are missing. Furthermore, current algorithms "force" mapping between different datasets and neglect the possibility that transcriptional profiles are not so similar after all. Metastasis steps in between are not accessible in humans. This might limit substantially the interpretability of data.
We agree with the referee that the gene extraction strategy starts with data from one cell line, which could be restrictive if the objective was to propose an exhaustive signature of genes associated with neuroblastoma metastasis. Our aim here was rather to propose a unique approach to extract genes involved in metastasis to the bone marrow, with a full picture of their dynamic regulation all along the metastatic process, which as the referee highlights, is neither accessible from humans, nor from other neuroblastoma experimental models.
We now have documented with quantitative analyses the proximities of primary tumors/bone marrow metastasis between the avian xenograft model and NB patients. First, we have extracted differentially expressed genes in NB cells between primary tumors and bone marrow metastasis in each context -ie: in the avian xenograft model and in the 7 patients-. This led to 4 gene signatures: avian primary tumor (Avian PT), avian bone marrow metastasis (avian BM), patient primary tumor (patient PT) and patient bone marrow metastasis (patient BM), which composition is given in Source Data F7, sheet a. We have scored these 4 different signatures in each context: avian (ADR, SG, AOR, PN, BM) and patient (PT, BM). This analysis, now shown in Fig. 7b, demonstrates that the expression level of BM signatures is highest in the corresponding anatomical site -ie; both in patient BM and in avian BM-, whatever the context -ie, avian or human. The corollary is also true for PT signatures.
However, the content of each gene signature (that contain the top 100 genes of the DEG) reveals context specificities (only 10% of genes in common between avian and human BM signatures; 19% of genes in PT signatures). We thus wondered whether the expression of these context-specific signatures was correlated or not in NB cells from avian and patient contexts. We thus performed a second analysis that aimed at measuring the correlation between each gene signature expression in both contexts. This analysis shown in Supp. Fig. 9a reveals a systematic and significant correlation between avian and patient gene signature expression in a given anatomical site. Thus, even if top genes can be different in the avian experimental model and in patients, this quantitative analysis suggests that similar biological mechanisms are involved in BM metastasis in both contexts.

Minor:

1. The wording is at times not clear and I recommend English proof-reading. For example, the authors state in the abstract that `…, Neuroblastoma (NB) plasticity and heterogeneity remain largely misunderstood´. This would imply that we currently have a wrong understanding of neuroblastoma metastasis, however in my opinion, current understanding is incomplete and studies on this topic are sparse.
We have reread the manuscript and hope to have improved the wording. In particular, we have changed the sentence underlined by the referee, as we fully agree with his/her opinion.

2. Please revise "Pediatric cancers are highly heterogeneous diseases that, still, reflect their emergence from developing tissues. Half of them originate from embryonic cell lineages, thus at stages of organ formation and maturation, with ongoing lineage differentiation trajectories." The term "ongoing lineage differentiation trajectory" is odd.
This has been modified accordingly.

3. It needs clarification in how far figure 6i and j differ.
We apologize as the titles of figures 6i and 6j were missing: they concern gene expression regulation during dissemination from sympathetic tumors (6i) and adrenal tumors (6j).

4. Please revise "It came out from these studies that two successive neural crest-derived progenitor states contribute to SA cell contingents…".
The sentence has been revised accordingly.

5. The color coding of figure 1j and l should be explained in the figure. Showing the same labels of c1, c2 and c3 on the x and y axis of figure 1k is misleading. This should be revised.
We agree with the referee that using the same labels was misleading. We have added information on the figure about color coding and have reorganized figure 1 and Supp. Fig. 1 to avoid any misunderstanding.

Reviewer #3: Expert in brain cancers and 3D imaging

Title: Neuroblastoma heterogeneity and plasticity over disease progression are rooted in the dynamics of an early sympathetic transcriptional trajectory.

Benjamin Villalard and colleagues have produced a very interesting manuscript combining an avian model, neuroblastoma (NB) patient samples and a re-analysis of previously published data.

The authors found that NB cells recapitulate the disease by adopting states aligned to the SNPCs-to-neuroblast differentiation branch and that the primary tumour site conditions their dissemination path. They then analyse the gene expression dynamics throughout the metastatic process using transcriptomic data from paired primary tumour/bone-marrow NB patient samples and identify a list of candidate genes upon bone marrow involvement that are associated with NB growth dependency, validating the relevance of our multimodal approach. Finally, the authors propose these genes as a set of therapeutic candidate genes whose expression is consistent with an active contribution to the growth of bone marrow metastases.

To reach these conclusions, the authors used light-sheet microscopy for the avian model and single-cell RNA expression approaches from isolated chicken embryo and patients NB cells.

The authors asked some challenging questions, focusing on understanding the underlying process of the NG cell: "Consequently, whether NB transcriptomic states across disease progression are still

influenced by the dynamics of the SA lineage and how NB cells adapt to the different microenvironments of primary tumour sites, metastatic routes and secondary foci remains unclear."

I think the authors try to draw new conclusions about the clonal evolution of NB cells and develop interesting analyses for genetic validation in avian models and patients. However, it is important to consolidate their conclusions with more robust validations by more experiments for different states/conditions to better characterise the results shown in the manuscript. Below are some details that I suggest to the authors to improve the results in the manuscript.

Major points

I believe that light sheet microscopy is a great approach to analyse the NB cell proliferation in the avian model at early stages.

Fig1. a-n.

-In method section, I found the number of NG cells engrafted in the avian embryo at 2,500 fluorescent NB cells at the neural crest level. Did you try to engraft fewer NG cells and analyse the time course and the primary foci?
In our initial work, we found that such range of cell numbers allowed good engraftment and tumor formation. We reported rate of successful tumor formation of 100% for IGR-N91-, 88% of SH-SY5Y- and 89% of SHEP-grafted embryos (Delloye-Bourgeois et al, 2017). However, working with patient tumours, we faced heterogeneity of sample sizes and had to reduce the number of engrafted cells sometimes below 1000. We observed that despite being in much lower density, NB cells could find their way to the SA territories, manifesting general behaviors that were close to those observed with NB cell lines (Delloye-Bourgeois et al, 2017).

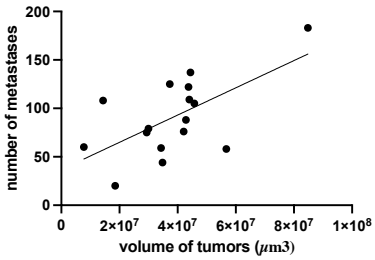
-I cannot find the number of embryos used at E0, E5 and E14 for the imaging data and for the genetic findings. Please include this in the text or methods (specifically the number of embryos used to reach these conclusions).
We apologize as this information was missing. Numbers of embryos used were now added in the figure legends for each type of experiment.

- The authors mentioned that they used the human stage 4 neuroblastoma IGR-N91 and SHEP cells. The two different migration routes and the time course of E0, E5 and E14 could be altered with different NG cell stages. Did the authors use human NG cells of stage 3 or 2? This should be clarified in the methods section.
NB cells from localized stages hardly grow in culture and the great majority of available NB cell lines were derived from metastatic stages, most of them from bone marrow (Thiele, 1998). In our first study reporting the setting of the avian embryo model, we could engraft a primary cell line derived from stage 2 NB and one patient sample from resected localized tumor. In both cases, NB cells formed primary tumours within the sympathico-adrenal derivatives and strikingly no metastases (Delloye-Bourgeois et al, 2017). In contrast, NB cells from both cell lines and patient samples formed primary tumours and metastases via dissemination along peripheral nerves and dorsal aorta (Delloye-Bourgeois et al, 2017; Ben Amar et al, 2022). However, we fully agree that heterogeneity between NBs (patient tumours and cell lines) could be manifested through preferential dorsal aorta versus nerve migration routes. Heterogeneity could also be reflected in the dynamics of migration, escape from primary tumor and metastatic progression. We already documented that during their migration from the site of implantation towards the sympatho-adrenal territories, NB cells moved either as small masses (IGR-N91 cells) or forming chains of migrating cells (SH-SY5Y and SHEP cells) (Delloye-Bourgeois et al, 2017). For the purpose of the present study, we adapted the time course to the characteristics of IGR-N91 cell line, knowing that NB cells engage into dissemination from E5.

- The primary tumour foci are not quantified (ADR and SG) and this is important to analyze early study migrations in the avian model (calculate the number of cells per volume). Are there any correlations between the two different routes of migration to other areas of the embryo and the number of cells in

the primary tumours in ADR and SG? The 3D images from the light sheet made this possible. The images could be improved.

We examined if the size of the primary tumours correlates with the amplitude of the metastatic process, by measuring the volume of primary tumours and quantifying the number of metastatic foci from light sheet imaging of whole embryos at E9, an intermediate time point during which NB cells are actively disseminating. We found weak correlation (R^2=0.3850), suggesting that the propensity of cells to detach from the primary mass and to engage into dissemination does not depend on the tumour size. We provide here the analysis and thought it is not necessary to include it in the manuscript.



-If I understand correctly, the avian model has two foci before E5, ADR and SG. How is it that these two foci form independently in the avian embryo with a single injection? I found this sentence, but I did not find a reference at the end of the sentence.

"At E5, NB cells exclusively formed tumours within the developing SG and ADR, that lie in proximity, as previously documented".

We apologize and understand from this comment that our text suffers from insufficient information. We confirm that a single injection can drive NB cells to form a tumour both in the adrenal medulla and a sympathetic ganglion. We interpret this in light of cell of origin. The sympathetic ganglia and the adrenal medulla both arise from the SA-neural crest. Beyond, the developmental trajectory of this branch of the trunk neural crest is complex and not entirely elucidated (Gonzalez Malagon and Liu, 2022). The majority of sympathetic neurons and chromaffin cells (composing the adrenal medulla) is acknowledged to arise from different progenitors, sympathetic neurons from the first wave of NCCs, chromaffin cells arising secondly from NCC-derived Schwann cell precursors (Furlan et al, 2017). A minority of cells of both organs (estimated as approximatively 5 to 10%) may have common progenitors. From our scRNAseq data, we know that following their implantation, NB cells migrating to reach the SA derivatives express a transcriptional state that matches that of a specific migrating SA-NCC progenitor (SNPC). We think this state enables the targeting of both adrenal and sympathetic territories at the abdominal level, which at these early stages, lie close to each other. This is now discussed in the article, line 714.

The sentence referred to our previous studies with the model (Delloye-Bourgeois et al, 2017; Akkermans et al, 2022; Ben Amar et al, 2022). We added the references to the text, line 144.

- These two major routes (AOR and PN) described in the images are interesting at an early stage, but these have not been quantified to identify which route is more prominent in the E14 embryo to obtain a picture of the main route to BM stage.

The images were taken from E14 embryos, thus effectively meaning that these are disseminating NB cells on their way to distal sites (bones, bone marrow). In our previous work, we analyzed the metastatic onset at earlier stages, both for cell lines and patient samples engrafted in avian embryos. Specifically for the IGR-N91 cell line that we use in the present study, we found that cells start to escape from the primary tumour at about E5, and migrate along nerves and dorsal aorta. We quantified the dissemination process at E9 and found (for the IGR-N91 cell line) that 31% of grafted embryos presented NB cells disseminating along aorta and 25% of them had NB cells disseminating along nerves (Delloye-Bourgeois et al, 2017). Similar data for other NB cell lines and stage 4 patient samples were provided in Delloye-Bourgeois et al (2017).

In the present study, thanks to our results from combined genetic/transcriptional single cell data, we have performed additional computational analysis to quantify the respective propensity of cells migrating along nerves or aorta to colonize the bone marrow. We found that both types of migration path have similar abilities to drive NB cells to the bone marrow with 45% and 55% of cells detected in the BM related to a nerve and to an aortic path respectively. This analysis has been included in Supp. Fig 8h.

-Fig1. d and e show NB cells spreading on nerves. In these images it is not clear whether it is a single cell or a group of cells. Also, in this picture if these cells are really using the nerve for migrations or could this also be random localisation? It would be important to include a video or pictures of the areas with a group of cells migrating using the nerves from light sheet microscope.
We performed additional immunolabeling of markers like Neurofilament and human-specific cell adhesion molecule L1CAM to better illustrate NB cell migration along nerves (now in Supp. Fig. 8j). Observation of the labeling in confocal microscopy shows close apposition of NB cells to nerves. We also provide examples with PLXNB2 and ERBB4 labeling, where groups of migrating cells along nervous tracts are detected (now in Fig. 4i). We also now included lightsheet movie of NB cells migrating on nerves.
From these observations, we confirm that cells are seen migrating individually or as group of cells.

-The NG cells localised in the nerves differ from those localised in the aorta in size, morphology or specific markers used in the genetic analysis. It is a really important point to describe if there are any important adaptations besides the genetic differences detected in the paper.
We agree that these are very interesting aspects to study. Our illustration showing gfp+/L1CAM^{+} NB cell with a bipolar morphology migrating on NF^{+} axons (Supp. Fig. 8j), supports NB cell morphological remodeling, adapted to the migration mode. Consistently, we performed additional analysis of the pathways and processes that distinguish the transcriptional programs of cells migrating on the aorta or the nerves, suggesting specific adaptations for each of the paths (now shown in Fig. 4k). We feel further addressing these aspects goes beyond the scope of the present work, that focuses on the transcriptional states and adaptations of NB cells over disease progression. Indeed, properly characterizing adaptations of NB cell morphologies according to the migration mode and identifying with functional assays the key molecular signaling controlling these adaptations is planned as the next step.

-I did not find a 3D video of the avian embryo at E0, E5 and E14 stages from the light sheet microscope. Addition of these videos would add value to the manuscript.
We repeated graft experiments and reprocessed 3D acquisitions to now provide several 3D videos for all stages, including 3D movie showing NB cells on nerve (Supp. Movies 1-10). E0 corresponds to cells in culture so this time point is not included in lightsheet analyses.

-In the manuscript it is assumed that before the E5 there is no metastatic onset. How was this conclusion reached? Was the metastatic onset time frame the same for all the embryos that were profiled? The variability and aggressivity of this NG cells stage 4 can produce a different time of the metastasis onset.
This time frame was defined in our previous work (Delloye-Bourgeois et al, 2017 and Ben Amar et al, 2022), and based on the behavior of several stage 4 cell lines and patient samples from different stages. We more deeply characterized the timing of metastatic onset for IGR-N91 cell line (Ben Amar et al, 2022) and we did observe that E5 time point was a well fit. We quantified the number of buds detaching from primary tumours. Within 10 embryos, all presented between 10 and 20 buds. Such budding pattern was not observed at E4 (representing 2 days post-implantation). From these observations we deduced that the metastatic onset might start between E4 and E5, with this NB cell line. We fully agree with the referee that this precisely applies to the IGR-N91 cell lines but not necessarily to all NB cell lines. However, for all types of samples and cell lines, we could establish in previous studies the systematic presence of NB cells disseminated at distance from SA tumors as early as stage E9 (Delloye-Bourgeois et al, 2017). We now make it clear in the results section (line 188) that this time point is related to IGR-N91 cell line.

-Fig1. Extended Data Fig1. The authors mentioned that α-TOP2A and α -LMNB1 are two important markers for the cluster C2 and E14. Have you tried to identify these two markers that appear in the NG cells on the nerves using light sheet microscope?
To address this question, we performed immunolabeling to detect TOP2A and LMNB1 together with Neurofilament on slices from E14 grafted embryos. Wholemount immunofluorescence protocols for these two antibodies were not convincing, thus we did not use lightsheet microscopy here. Using confocal microscopy, we observed that NB cells migrating along nerves express heterogeneous levels of both these state markers, as expected from our transcriptional data (presence of the c2 cluster at E14, coexisting with c0 and c1 clusters). These experiments are now illustrated in Fig. 4h and Supp. Fig. 7k.

-Fig2. a, b, c, some of these analyses that confirm the atlas data could be in the supplementary figures because this is the combination of the previous results from the two previous papers published.
We agree that atlas data results from the combination of the previous data from two published papers. However, this combination allows to highlight novel types of clusters (SNPCs, comSNPCs), which a key result of our article. For this reason, we wish to keep these data in the main figures, as the interpretation of the following results may be difficult to follow.

-Fig2g. “From this projection dataset, we built a joint heatmap of expression for c0/c1/c2 top markers in both SA and NB cells. The associated hierarchical clustering pointed at tight transcriptional similarities between c2 and SNPCs, c0 and comSNPCs, and c1 and neuroblasts (Nbts/Late Nbts). These relationships were further confirmed by looking at the expression profile of key markers of SA identities into NB c0/c1/c2 clusters. Notably, SCPs emblematic markers -ERBB3, SOX10, PLP1- were all negative (Extended Data Fig. 2d)”.
-Fig4g. “UMAP plot of IGR-N91::GFP NB cells colored and labelled by cell location at each step of dissemination in E14 avian embryos -257 cells from sympathetic ganglia tumors (SG), 261 cells from adrenal tumors (ADR), respectively 84 and 25 disseminating cells along peripheral nerves (PN) and the aorta (AOR), and 31 cells in the bone marrow (BM).”
-Looking at the three clusters (c0, c1, c2) from Fig. 2g, there is a significant overlap between them in the heatmap. Also, none of the clusters match the SCP-like cells. Do you think that the number of cells from the avian model is too small to draw these conclusions because at E14 avian embryos you analyze only 25 cells disseminating cells along the peripheral nerves (PN)?
We fully agree with the referee that the presence of NB cells along peripheral nerves could rely on transcriptomic programs shared with SCPs. We didn’t identify any match between NB cells -migrating or not along the nerves- and SCPs transcriptional states. For NB cells migrating on nerves, we agree that the low number of collected cells could explain, that statistically, none of these cells shows an SCPs-like phenotype. However, our conclusions here relate to c0/c1/c2 clusters that are (1) not specific of a given location, (2) and thus concern higher numbers of cells. This conclusion is also strengthened by our mapping results of patient samples on the SA atlas, that do not highlight any transcriptional match with the SCP cluster, despite the very high number of cells for most of the samples (N=41 patient samples, Supp. Fig 5).

- Could the authors please clarify which signatures were used to annotate the scRNA-seq datasets (avian and patient-derived)? A table of top gene markers for each cluster for all datasets would be helpful.
We apologize as these data were not properly cited in the manuscript. All signatures and markers used to annotate scRNAseq datasets are given in Source Data F1 (sheet l) and F2 (sheet c). We now have more clearly mentioned these source data in the manuscript.

-Fig4. h. “BM secondary site defined specific adaptations related to a given microenvironment, namely: neuronal-related features at the SG level (NRXN1, GFRA3, NPY, VIM); response to nutrients and extracellular signals at the ADR level (VFG, LDHA, PTN, IGFBP2); cell adhesion at AOR level (OBSCN, GFRA1, ITGA1, CNTN1); neuronal migration at PN level (NRCAM, ERBB4, NTRK2, PLXNB2) and gliogenesis at the BM level (NFAT5, LAMB1, PLEC).”Have you tried using NRCAM, ERBB4, NTRK2, PLXNB2 markers in the avian embryo to confirm the expression of neuronal migration at the PN level?
We performed additional experiments to answer this question. Immunolabeling of NRCAM, ERBB4, NTRK2, PLXNB2 were achieved, together with that of Neurofilament or β3-tubulin in E14 embryo sections. We successfully detected ERBB4 and PLXNB2 in NB cells migrating on nerves, while in the same embryos, both proteins showed lower expression levels in primary tumors. We tested two different anti-NrCAM antibodies and two anti-NTRK2 antibodies, with different fixation protocols but failed to obtain satisfying detection.
These immunolabeling of ERBB4 and PLXNB2 have been included in Fig. 4j.

-Fig5. The confocal images from the avian model are clearer compared to Fig1, about the possible PN migration, but the authors only use GPF as a marker to detect them. It should be useful to use NRCAM or ERBB4 or NTRK2 or PLXNB2 as a marker to identify these cells to confirm the genetic profile in combination with GFP and □-NF160.
Our immunolabeling data with PLXNB2 and ERBB4 (Fig. 4j) as well as those with L1CAM (Supp. Fig. 8j) now illustrate NB cells migrating on neurofilaments and axons.

‘‘These parsimony tree characteristics suggested that primary tumors located in the sympathetic chain or in the adrenal medulla both disseminated via the peripheral nerves while the aortic path preferentially concerned tumors of the adrenal medulla.’’
-In the avian model, the AOR and Sg, the primary foci, are in close proximity to each other. How did the authors assume that these foci are completely independent or are both in constant interaction between NB cells migrating in and out for each focus?
We agree with this interesting comment of the referee. None of our experiments exclude exchanges between primary tumour sites to occur. Answers could only be given using videomicroscopy, which requires complex settings to achieve it in living avian embryos. Nevertheless, would such changes occur, what our bioinformatic analysis tells is that the preferential path of NB cells exiting sympathetic tumour is the nerve, while NB cells from adrenal tumour takes both nerve and dorsal aorta paths. This would remain the case for NB cells that moved from adjacent sympathetic ganglion to adrenal medulla and vice versa. Of note, the dissection procedure at E14 allows to unambiguously separate tumors located in the SG and tumors located in the ADR or in the AOR.

-In Fig6.a the authors mention that variant allele frequencies and scRNA-seq data were used to determine the trajectories. It's not clear to this reviewer how this analysis was performed (especially the integration of mutational and transcriptomic data). Trajectory analysis can also be performed using scRNA-seq data alone, could the authors please clarify why variant allele frequencies were also used?
We apologize for the lack of clarity regarding the method and the justification to use genetic data combined with transcriptional ones. The methodology is now more precisely described in the method sections. In our case, trajectory analysis to depict the series of phenotypical adaptations occurring across the metastatic process was not feasible with transcriptional data only. Indeed, NB cells did not cluster according to their physical position in the metastatic cascade but rather according to their SA-related phenotypical state, both in the avian model and in patient samples (Fig 4c and 4f). Integrating mutational / SNP data allowed to trace the physical path taken by NB cells that escape from SG and ADR tumors. And then from this genetic-based trajectory analysis we could extract the transcriptional programs and their evolution across the metastatic path.

-Fig7 f. In this figure, 25 genes were found to be harbor NB dependency for cell growth. It would be important to confirm the high expression of the top genes in the BM using patient tissues.
We now have included an analysis of the expression level of these 25 genes in matched PT/BM patient samples. We calculated the ratio of expression for each gene in bone marrow versus primary tumors (shown in Fig. 7f). This analysis confirms that for 100% of the genes, the mean ratio of expression is in favor of the BM.

Minor points

-Some acronyms including in the figure Fig2 could be clarified in the legends to help the readers.
We have added some explanations regarding the acronyms in particular in Fig2 legend.

Reviewer #4: Expert in scRNA-seq, lineage analysis, cell plasticity, cancer evolution and intratumour heterogeneity

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Comments to the authors:

In the manuscript by Villalard et al entitled “Neuroblastoma heterogeneity and plasticity over disease progression are rooted in the dynamics of an early sympathetic transcriptional trajectory” the authors attempt to bring light into the process of metastatic dissemination in Neuroblastoma. Unfortunately, and it is the opinion of this reviewer, the manuscript does not go sufficiently far onto it and promises too much when only correlations are suggested by the data.

Major comments:

1- One of my major concerns stems from the avian and cellular models used. If I correctly follow the manuscript, the authors engraft ~2500 NB human cells (fully-fledged, please see below for comments on the cell line) in the avian embryo which by the nature of the method will not be all engrafted. So, which is the % of cells that are engrafted? Where do the rest of the cells go? How many cells the avian embryo have at E2? Is it possible that what the authors suggest as metastatic dissemination is just growth from the cells that did not engraft?
We understand the referee's concern about the engraftment method as we have not re-exposed here the data that validated the xenograft model of metastatic NB in the avian embryo (doi: 10.1016/j.ccell.2017.09.006.; doi: 10.1038/s41467-022-30237-3.) The method is based on the microinjection of a highly concentrated cell preparation, which allows to engraft a patch of cells (and not a suspension). Thus, in itself, this method precludes the "loss" of cells outside the implantation site. In cases where the grafting procedure damages the neural tube, or the injection is made outside the target site, the embryo is systematically excluded from the experiment (5 to 10% of cases). We also have direct evidence for considering that "metastatic dissemination" is not due to cells that didn't engraft, from. Our data from engraftment of patient samples/cell lines showed that metastatic foci were observed for those graded as stage 4/M and not for those from localized stages showed (doi: 10.1016/j.ccell.2017.09.006.).

The authors suggest that the grafts form dense primary tumour masses…
How dense? Which is the cellularity? Is the tumour mass infiltrated by other cell types?
Again, these « dense primary tumor masses" were described in previous work and the term was re-used here without re-showing the founding data. The term « dense » refers to the fact that at E5, NB cells are embedded in HNK1$^+$ tissues, forming a tightly cohesive structure (see confocal analysis of immunofluorescent labeling in Fig. 1m and 1n and Supp. Fig1f-k for illustration). As measured as the embryo develops, the primary tumour mass grows within a stroma that complexifies with axon and vascular networks, as shown at E14 in Fig1b-b' and Supp. Fig.8i-k'.

How many embryos were grafted?
We apologize as this key information was missing from qualitative/imaging data. We have now added the number of embryos analyzed at each time point, and for each cell line in the figure legends.

How efficient and how reproducible the engraftment is? If it is not efficient… how did the authors chose which ones to follow, and which one should not be followed?
As explained above, the graft method is 100% efficient. No embryos are excluded from the experiments as the intake rate is 100%, so we do not "select" the embryos that will be followed. The only cases where embryos are excluded is at the graft time, in cases where the grafting procedure damages the neural tube, or the injection is made outside the target site (5 to 10% of embryos).

Is it possible to follow a single embryo through time after engraftment rather than taking different embryos at different times to verify dissemination? All the above must be addressed to strengthen the claims made by the authors.
Unfortunately, live imaging with current technologies (biphoton microscopy in particular) would not allow to precisely monitor NB cells that migrate as small groups of cells or even individually in depth of tissues that are opaque. This makes it not possible to conduct longitudinal imaging in single embryos and this is the reason why we use light sheet imaging / confocal imaging on groups of embryos at different times.

2- The NB cell line (NB Stage 4). If we are to assume that NB is anchored in developmental processes, it is the opinion of this reviewer, that a fully-fledged, evolved (in a different organism) and quite probably selected by the environment (of a different organism) would not make a great avatar to study the influence of the developmental process as the NB cell have already experienced all of that. What does it look like the avian engraftment with NB cells from other stages? Wouldn't it be better to test different stages to infer behaviour rather than going all the way with stage 4?
We fully agree that these are crucial aspects of the avian model, which we mainly addressed in Delloye-Bourgeois et al, Cancer Cell 2017 (doi: 10.1016/j.ccell.2017.09.006.). Indeed, we did exactly what the

reviewer suggested: we compared the behavior of localized NB samples (primary cell lines and patient biopsies) with that of stage 4/M NB samples after their transplantation into series of avian embryos. While the formation of adrenal or sympathetic tumors was observed with similar efficiency for both types of samples, the presence of metastatic NB foci was strictly limited to stage 4/M samples. Thus, remarkably, although having already experienced the influence of the developmental process in patients, NB cells can replay the sequence when returned to the embryonic context of emergence, manifesting specific behaviors correlating with localized versus metastatic forms. These findings demonstrate that human NB cells establish relevant communication with the avian embryonic host cells. As an example, we have shown that mouse and chick sympathetic ganglia secretomes have equivalent effects on NB cells, demonstrating that NB cells perceive molecules secreted by developing sympatho-adrenal tissues, regardless of whether they are of chick or mouse origin (Ben Amar et al, 2022). Indeed, the avian embryo is a well-recognized model to study the morphogenesis of the vertebrate sympathetic chain, showing strong conservation across vertebrates (Saito et al, 2012; Shtukmaster et al, 2013; Kasemeier-Kulesa et al, 2015; Holzmann et al, 2015). The avian adrenal gland is also well representative of that of mammals (Capaldo, 2023).

3- The authors argue that NB cells exclusively form tumours within the developing SG and ADR… is this a biological consequence or an artefact of proximity on the engraftment?
The formation of primary tumours in the sympathetic ganglia and adrenal medulla is not an artefact. Both structures arise from the sympathico-adrenal (SA) lineage of the neural crest that delaminate from the dorsal edge of the neural tube to migrate ventrally and arrest close to the dorsal aorta. The engraftment site (the dorsal roof of the neural tube) and primary tumour sites are thus physically distinct and far distant from each other. By implanting NB cells in the pre-migratory neural crest domain, we induce NB cells to recapitulate the ventral migration of the physiological cells committed to give rise to the SA derivatives as reported in previous studies (Delloye-Bourgeois et al, Cancer 2017; Akkermans et al., Cell 2022).

Numbers are needed here. Occurrence of one over the other. Once again, this could contribute to the dissemination path taken and must be addressed.
Number of embryos have now been added in the figure legends, with the occurrence of phenotypes shown on representative images. This particular type of analysis was not included as a result panel in this article as this analysis was already described in previous work.
As an example, see Figure S1, Delloye-Bourgeois et al, Cancer Cell 2017:

[Figure S1 from "Delloye-Bourgeois, C. et al. Microenvironment-Driven Shift of Cohesion/Detachment Balance within Tumors Induces a Switch toward Metastasis in Neuroblastoma. Cancer Cell 32, 427-443.e8 (2017)." has been redacted.]

4- I find the comment in line 142 "… hence mimicking typical features of metastatic NB in patients…" rather strong. It is just an observation. Numbers are needed. How many times did this happen? Is it reproducible? Is there organ/localization bias? Is there some sort of temporal hierarchy?
We observed that colonization of bones and bone marrow by IGR-N91 cells is a recurrent pattern. It was found in 100% of analyzed E14 embryos grafted with IGR-N91::GFP cells and imaged with 3D lightsheet confocal microscopy. Number of embryos have now been added in the figure legends, with the occurrence of phenotypes shown on representative images. Bone and bone marrow involvement were not observed at E9 while cells disseminating on nerves / aorta were present at this time point (Delloye-Bourgeois et al., 2017), illustrating the temporal hierarchy of NB dissemination paths in the developing embryo.

5- The authors argue that they take samples from "… 5 physical sites representing different steps of the disease progression…" but then they only mention 3 samples (E0, E5 and E14) that were analysed by scRNA-seq.

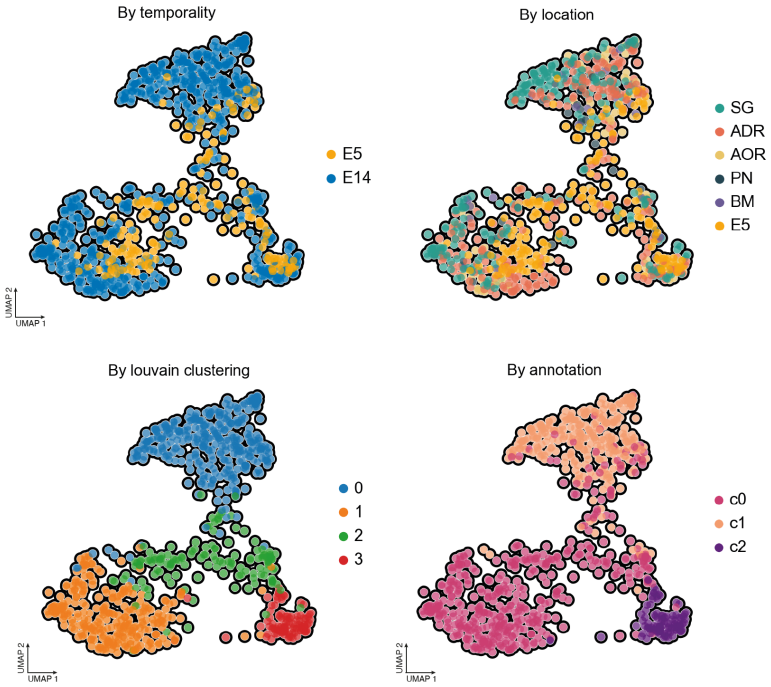
What happened with all the physical sites? Were the samples pooled?
Were some anatomical sites removed from the analysis?
Where all the anatomical sites evaluated independently?
I find this part of the manuscript quite perplexing as, it is quite probable and there is a lot of literature supporting this concept, each anatomical site would present a different microenvironment. Thus, the NB cells will be embedded in different environments which will affect the NB cells biology (cell-to-cell interactions, signalling, extracellular matrix components, among others) and thus should be analysed independently.
We apologize for the confusion and now clarified the text, as it seems that there is a confusion between temporality (E0, E5, E14) and physical locations (SG, ADR, PN, AOR, BM).
Effectively in terms of time points, we harvested the cells at 3 stages: prior to their grafting (E0), at E5 and E14. In terms of "steps of the disease progression" we harvested NB cells at E14: (i) from primary tumors (ADR and SG), (ii) from peripheral nerves and (iii) from bone marrow. Thus, overall, for the scRNAseq sequencing, we had 7 conditions: E0 (representing cultured NB cells), E5 primary tumors, E14 primary ADR tumors, E14 primary SG tumors, E14 Peripheral nerve NB cells, E14 Aorta NB cells and E14 bone marrow metastases.
We confirm that our approach was designed to assess the influence of the different microenvironments to which NB cells are exposed when they form tumors in the different SA derivatives, during their dissemination and within the bone marrow. In some cases, bioinformatic analyses were conducted on NB cells pooled together whatever the physical site of their harvesting or timepoint. This aim was to depict the range of transcriptional programs they express and to determine if these states discriminate the physical site or the stage. In other bioinformatic analyses, we compared the transcriptome of NB cells from the different physical sites to extract differentially expressed genes reflecting the adaptations of NB cells to the different microenvironments.

6- Regarding the scRNA-Seq in the avian/NB cells model. What does it mean high depth scRNA-Seq? How many transcripts were detected? How many reads per cell? How many transcripts were fully reconstructed? This information must be included in the manuscript.
We used the Smart-Seq2 and SeqWell technologies allowing "high depth" full length sequencing of transcripts, as opposed to drop-seq approaches (10x sequencing or similar technologies with 3'-counting). We apologize as the information regarding the number of transcripts and reads per cell was indeed given in the Source data F1 (sheet h), but the latter was not properly cited in the manuscript. This is now corrected. We also added violin plots for each sequencing method in Supp. Fig. 1a (nCounts and nFeatures).

7- Also, regarding the scRNA-Seq in the avian/NB cells model, this reviewer finds that the number of cells analysed by scRNA-Seq is way too low to draw any conclusion.
Allow me to explain… ~1000 cells were analysed in total in the first avian/NB cells model… in 2 different platforms… with 3 different samples each… including 5 anatomical sites. So how many cells per condition are really there? What about replicates technical and biological? The authors mention replicates… How many avian embryos were processed? If it is only 2… well the numbers are really low to draw any conclusion.
First, we understand the concern of the referee regarding the "low" number of cells used in single cell RNAseq data. We would like to emphasize two points: (1) SmartSeq/SeqWell techniques do not apply to very high numbers of cells, as their field of applications is not related to the identification of rare cell identities but rather relate to the questions of close transitional states that need very high depth sequencing to be clearly distinguished (see for example Furlan et al, Science 2017); (2) in the present article, cells are collected by manual microdissection from avian embryos. To ensure that cells are properly harvested from specific embryonic tissues (SG, ADR, PN, AOR, BM) we selected cells that we could unambiguously isolate from these 5 locations. Together with the sorting step to eliminate avian cells, this explains the low number of cells finally analyzed in scRNASeq.
Second, we apologize for the lack of clarity regarding the number of cells and embryos. We now have added in the figure legends the number of embryos used at each step, and the exact number of cells

sequenced for each physical location. At least 4 embryos were pooled for each sequencing experiment, and for each condition, and the lower number of cells that we had was for the nerve localization (25 cells from 4 embryos, which was the most difficult area to dissect out from E14 embryos).

8- Moreover, regarding the sample at time E0, which essentially are cells in cell culture analysed by scRNA-Seq. Why is this sample in the analysis at all?
Cells grown in plastic display massive changes in gene expression when grown in an in-vivo model and this has been documented multiple times. This reviewer agrees that E0 sample must be analysed but I'm not so sure about the value of integrating this sample in the clustering of the avian/NB samples. It could be completely misleading.
We fully agree with the referee's comment about cells grown in plastic. We decided to integrate the E0 sample to the analysis as a starting point to document the transcriptional changes occurring in NB cells upon exposure to the embryonic tissues. While this condition is definitely not a perfect « t0 », it was necessary to analyze this condition to address changes occurring in the avian embryo. Moreover, we could verify that E0 cells did not cluster a part from E5/E14 cells (avian conditions) (Fig. 1h and novel Supp. Fig. 1c). We also repeated our clustering analysis without E0 cells -only with E5 and E14 cells harvested from avian embryos- and observed a very moderate impact on clustering. As shown below, the clustering remains independent of cell location, but rather relies on c0, c1 and c2 SA-related phenotypes.



9- The authors argue that the E0 samples display a "… homogenous transcriptomic profile…" . I can clearly see 3 clusters in Fig 1l. As the authors are aware, clustering is highly dependent on the math employed. I'm pretty sure that this data deserves a further look and I encourage the authors to revise their data here.
Again, we fully agree that the clustering is highly dependent on the math employed. Here, we used the same resolution and clustering parameters for E0, E5 and E14 conditions. This method led to a single cluster at E0 (2 at E5, 3 at E14), which is why we depicted a "homogeneous transcriptomic profile". We have modified the sentence to explain that this homogeneity is to be considered in comparison with E5 and E14 cells.

10- This reviewer is not sure whether a Pathway analysis with such a low number of cells is reliable. Replicates and more cells need to be assessed to strengthen the concept. Also, Fig 1i does not display a scale bar.
Pathway analyses are based on low numbers of cells but again, based on SMARTseq2/SeqWell data, that allow a resolution/depth close to bulk RNASeq, in single cells. we now have added the mean enrichment score and p-value for each pathway / gene ontology process. However, we modified Fig. 1i

to be able to show statistics on pathway analysis, which were missing in the first version of the figure. Former panel 1i has been placed in Supp Fig. 1e with the scale bar. Instead, as the initial approach alone could introduce a bias towards large gene ontology processes, we have extracted top biological processe (GO terms) differentially represented between E14 and E5 and between E5 and E0 stages in NB cells.

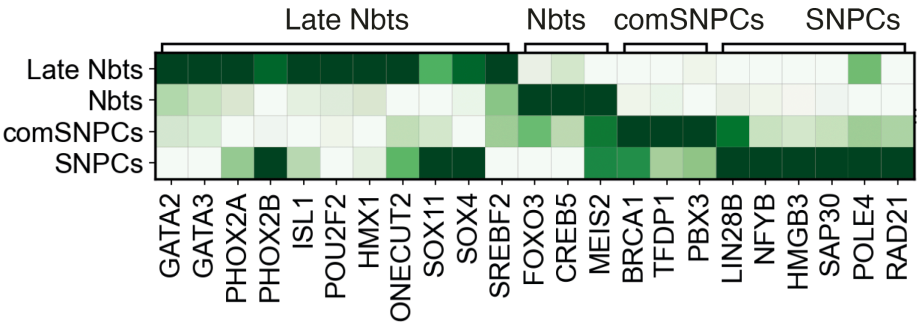
11- The authors mention "transcriptional state" all over the manuscript. However, the authors do not know whether the observed changes are a consequence of changes in active transcription or steady state RNA levels. I suggest the author to rephrase every "transcriptional state" for "gene expression".
To our knowledge, and as exemplified in multiple studies the term "transcriptional state" doesn't necessarily apply to active transcription especially when using the term "state" to relate a cell population to transitory / bridge embryonic cell populations (see for example Jansky et al., 2021; Kastriti et al; 2022). To avoid any confusion, we have now defined the term "transcriptional state" in the manuscript, as we believe that "gene expression" is rather vague to define a group of cells showing similar transcriptional programs.

12- Similarly, the "predictive" transcription factor analysis, unless leveraged by measuring "active transcription" and not steady state RNA levels is not relevant.
The predictive analysis of transcription factors activity was made with the SCENIC pipeline (Aibar et al., Nat methods 2017; Van de Sande et al., Nat Protoc., 2020). We agree that measuring active transcription would be a more direct and satisfying approach to analyze transcription factor activities. However, the SCENIC pipeline has been designed and validated for this type of data, and extensively used in published studies, especially those dedicated to differentiation trajectories with a continuum of transcriptional programs (again see for example Jansky et al., 2021; Kastriti et al; 2022). Here we inferred transcription factor activity in each single cell and could extract groups of transcription factors which predicted activity matches with physiological cell states of the SA lineage.

13- The authors state in line 224 that "… the regression of cell cycle genes… did not interfere with transcriptional similarities…". How is that the cell cycle regression does not interfere? I do not think "interfere" is appropriate here.
We agree that the term "interfere" was not appropriate here. Our objective here was to say that upon regression of cell cycle genes, transcriptional similarities between NB cell states and SA states remained similar, suggesting that the expression of cell cycle genes do not govern by itself transcriptional similarities between NB and physiological cell states.

14- In line 230, the authors mention: "… by analysing RNA velocity…" please rephrase this as the way the phrase is built it looks like the authors are analysing a speed related behaviour of RNA and not using a computational pipeline.
We modified the sentence to remove this ambiguity.

15- In Figure 2e, the transcription factor profile, I do not see differences that would support the discrimination between late NBts and NBts or between comSNPCs and SNPCs.
First, we would like to mention that there was an error in Fig. 2e that has now been corrected: the transcription factor SREBF2 concerns the "late Nbts" cluster (and not the "Nbts" cluster).
Second, although the transcription factors chosen allow us to distinguish between cell populations, we can easily appreciate from Fig. 2e that SNPCs are more closely linked to Nbts than SCPs are (as also suggested by other previous studies, such as Jansky et al., 2021). As we presented a heatmap of the full SA lineage, the scaling visually attenuates differences in predicted transcription factor activity between closer cell states. Nevertheless, if we restrict the analysis to the SNPCs to late Nbts differentiation axis, differences become clearer:



16- In line 244, the authors state: "… we depleted cell cycle related genes…". What does the authors mean by depleted? Was the data removed? Was it regressed? If it was regressed…how was it regressed?
In this case, genes related to the cell cycle (GO_CellCycle_0007049) were removed (and not regressed) from gene signatures. This has now been precised in the method section.

17- Regarding the construction of the Atlas, additional information must accompany the manuscript. For instance: How many cells were sequenced per patient sample? How deep were they sequenced?
These data were indeed given in Source Data of Supp. Fig. 4 but these were not cited properly. We now refer to these source data (Source Data F3, sheet a) in the figure legends. To complete this point, we also now have included in Supp. Fig. 4a nFeatures and nCounts for all samples of the atlas.

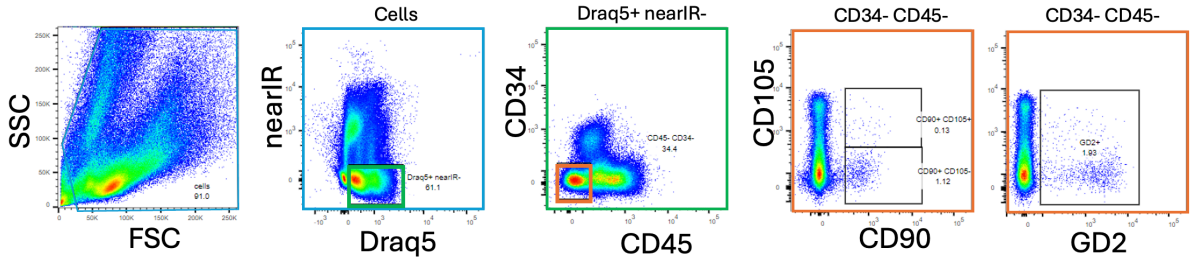
18- Regarding the integrated Atlas, this reviewer may be mistaken but, I do not think that pooling together datasets from public repositories could be the core of a manuscript. This is standard practice now. I suggest the authors to send all this data to Supplementary material.
We are not sure whether the reviewer refers to the SA lineage atlas or to patient data.
We agree that the SA lineage atlas results from the combination of the previous data from two published papers. However, this combination allows to highlight novel types of clusters (SNPCs, comSNPCs), which a key result of our article. For this reason, we wish to keep these data in the main figures, as the interpretation of the following results may be difficult to follow.
Regarding the NB atlas, we included 14 samples (matched PT/BM from 7 patients) that were not from public repositories but from novel data. We have presented in main Fig. 3 what we think are minimal data to follow the identification of SA-lineage related states in this atlas. If required by the referee, we could send Fig. 4a in Supp. Fig. 4.

19- It would be interesting to see how the single cell data cluster based on localization (i.e only adrenal gland) in addition to all the patients together. Interesting insights could be learnt.
We exploited the clinical data to perform a clustering based on tumor localization (adrenal gland only, versus other locations). We could extract the fraction of SNPCs-, comSNPCs- and Nbts-like cells in each type of tumor localization. While we could see some differences in between tumor localizations, we systematically observed the same type of hierarchy: a predominant fraction of Nbts-like cells, an intermediate fraction of comSNPCs-like cells and a minority fraction of SNPCs-like cells.
These data are presented in Supp. Fig. 4c,d.

20- In line 275, the authors explain that some patient samples have a low number of cells… How the low number of cells is now relevant and not at the beginning of the manuscript?
This consideration relates to the sequencing method. In line 275, we refer to a 10X sequencing approach with nFeatures and nCounts that are respectively 4.9 and 251.7 times lower than with high depth sequencing approaches mentioned at the beginning of the manuscript. Thus, in the case of patient samples, samples with very low number of cells with much lower quality and sensitivity could be biased on major types of genes, such as cell cycle genes.

21- In line 311, the authors argue that they use "… emblematic markers..."… which markers?
We apologize for the lack of details; these markers were detailed in Supp. Fig. 7b and 7f, but we may have not cited them explicitly. This is now corrected in the manuscript.

22- In line 316. The authors argue that they've used "… a panel of cell surface markers…"… which markers? How was this controlled? How were the markers validated? Please include data.
We apologize for the lack of clarity regarding the markers and gating strategy used to sort NB tumor cells from BM patient samples. These details are now given in the methods section and were based on previous experiments of co-authors and on literature.
The gating strategy used and is the following:

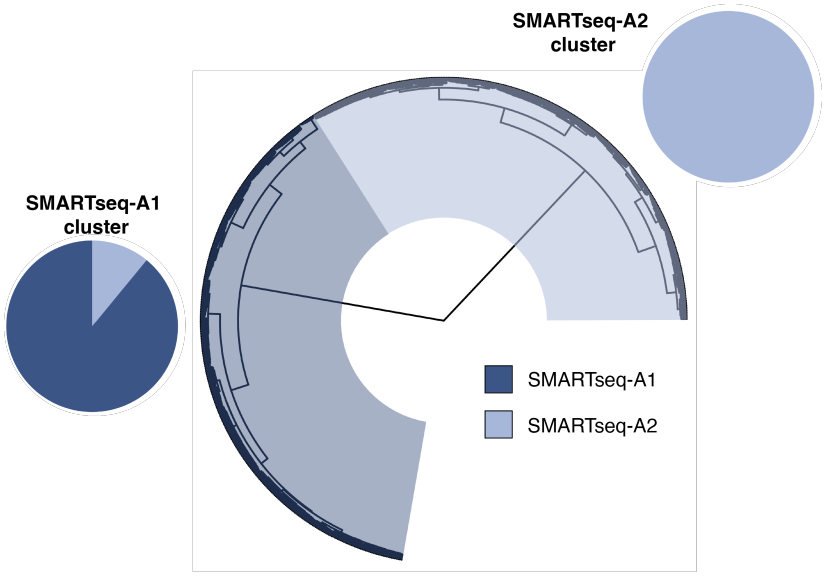


Draq^{+}/NearIR^{-} cells (green box) were selected, then CD45^{-}/CD34^{-} cells (orange box) and finally two parallel selections allowed to sort CD90^{+}/GD2^{+}, CD90^{+}/GD2^{-}, CD90^{-}/GD2^{+} cells.
We added an additional marker next to GD2 as GD2-low or -negative NB cells do exist, also in treatment naïve samples, as already reported in other studies (Terzic et al., 2018; Lazic et al., 2020; Pilgrim et al., 2023). We could previously show that by using CD90 as an additional marker, NB tumor cells were successfully enriched, as shown by FACS-sorting and subsequent qPCR for NB markers (Hochheuser et al, Cancers, 2020).
Complementary to these markers used for the sorting, other markers were assessed to validate the gating strategy such as CD3 (T Lymphocytes), CD56/CD81 (tumor markers), CD13/CD73/CD105/CD146/CD271 (mesenchymal markers).

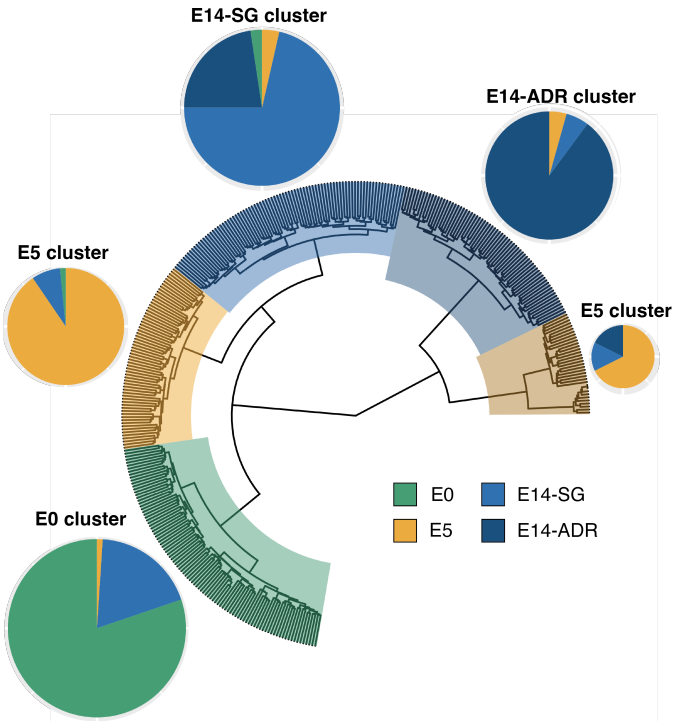
23- In line 318, the authors mention that they have "sub-sampled" for deeper analysis. Sub-sampled? Based on which criteria, why and how? This sounds a little too arbitrary to this reviewer.
We apologize for the lack of clarity but would like to emphasize here that the sub-sampling was based on precise criteria and was not arbitrary. NB cells were « sub-samples », meaning that we focused on NB tumor cells, based on the absence of expression of non-tumoral cell population markers (listed in the method section) and Copy Number Variations (CNVs) counts. The methodology is now better described in the method section and filtering steps are shown in Supp. Fig 7a-h.

24- Regarding the genetic analysis derived from the single cell data obtained from the NB cells in the avian model… The data clearly shows that NB cells are not genetically homogenous… does the genetic divergence happen within the experiment? Or the NB cells used are already genetically heterogeneous? The distinction is fundamental as it could explain differential dissemination, engraftment efficiency, among other features. I suggest the authors to have a look at the data from E0 (cells in culture) to extract this information and to include it in the manuscript. Also, this may take a while but if the genetic alterations are already there, it would be interesting to generate clones of the NB cells used and repeat the experiments here presented.
25- In line with comment 24… How genetically stable is the NB cell line used for the avian experiments? Is the cell line diverging genetically all the time? Is it rather stable?
We thank the referee for these interesting points, and performed additional analysis to address the question of genetic heterogeneity and kinetics of divergence that are raised in points 24 & 25.

Concerning the genetic stability of the IGR-N91 cell line, we extracted genetic variants from our two experimental SMARTSeq2 studies, carried out six months apart, and then analyzed their genetic proximities. We observed a rapid genetic evolution of the cell line over this short period, as the dendrogram analysis allows to almost perfectly separate IGR-N91 cells from each experiment, based on genetic variants (now shown as a histogram in Supp. Fig. 8a).



Next, we assessed whether clonal genetic heterogeneity at E0 (in cell culture) could influence the physical localization and behavior of cells once engrafted in avian embryos. By analyzing genetic proximities in a dendrogram with E0, E5 and E14 NB and building a dendrogram, cells were indeed mostly grouped according to their "temporal origin" (E0/E5/E14). In addition, we also verified in this analysis that E14 cells clustered in two branches, each highlighting the position of cells within SG or ADR tumors (now shown as a histogram in Ext. Data Fig. 8b). We believe that together these data support the fact that the kinetics of IGR-N91 cell line genetic evolution is relevant to trace the metastatic dissemination paths of NB cells. This is in accordance with the molecular clock concept, based on the fact that neutral SNPs accumulate linearly over time, and can therefore be used as tools to time spatio-temporal genetic evolution of cells. Interestingly, we could also document in the manuscript that even if IGR-N91 rapidly evolve genetically, the extracted variants (SNPs) converge on the same genetic regions in cells confronted to the avian embryonic microenvironement (Supp. Fig. 8e).



26- In Line 376 paragraph the authors argue "…Confocal analysis…allowed to document and confirm the occurrence of the physical transitions predicted by genetic variant analysis…" This statement is not

correct. Unless you can pinpoint the mutations on-site, which cannot be done by confocal microscopy, this is an overstatement and must be re-phrased and toned down.
We modified this statement accordingly.

27- In Line 406 the authors argue: "… we selected all genes showing significant upregulation… to focus on frequent, and thus druggable events…". What does the authors mean here? There is hardly any correlation between upregulation of transcripts with druggable targets. Modulation of transcripts does not mean at all that those molecules are druggable. Please revise.
We revised this sentence as we agree that the formulation was confusing.

28- Regarding the "useful gene set". I do not agree with the conclusions here drawn and I find the entire section overstated. I think it would potentially strengthen the manuscript if the authors would use the listed NB cells lines and repeat the avian graft experiments to actually verify their hypothesis.
We revised this part to tone down our conclusions. However, we would like to precise here that the final gene set -considered as of potential interest for the clinics- indeed relies on functional experiments performed in 18 NB cell lines. This genome-wide CRISPR loss-of-function screening (DepMap, https://depmap.org/portal) established the growth dependency of these metastatic cell lines on the expression of the 25 genes. While we agree that grafting these 18 listed cell lines, modified with CRISPR for each of the 25 genes, would allow to verify the clinical relevance of this gene set, we think that such heavy experiments are not reasonable to perform in the scope of this study, and we have planned in next studies to analyze in depth some of these gene candidates.

Minor comments

1-I do not think the abstract reflects the findings which are mainly based on correlations. The distinction must be made evident. I suggest the authors to tone it down.
We are not sure here to which type of "correlations" the reviewer refers too.
Most of our findings are based on differential gene expression analyses which are not based on correlative statistics. Mapping analyses are indeed associated with correlative data (SingleR), but were completed with gene enrichment analyses (not correlative) with patient data / avian data projection on the SA lineage atlas without any correlative bias. Similarly, genetic tracing experiments and dynamic trajectories were not based on correlations but on enrichment analyses.

2- I suggest the authors to re-shuffle and simplify the introduction. Please revise sentences such as: "… The specific origin triggers the formation…" I do not think that the specific origin is triggering anything. Perhaps "contributes" would be a better option.
We modified this sentence accordingly and tried to simplify the introduction.

3- Please make the nomenclature uniform across the Methods section. Sometimes HH… sometimes E(N).
We have homogenized the nomenclature accordingly.

4- In the Method section the authors sate that the different NB cells lines used are grown in different cell culture conditions (medium) which will undoubtfully alter the metabolism of the cell lines analysed and thus will results in altered gene expression. Please comment.
Cell lines were each cultivated in the medium recommended to maintain them in culture. While we agree that the culture conditions may have an impact on cell metabolism, we kept the same culture conditions for each cell line in every experiment presented here.

5- In Line 688 the authors state that IGR-N91::GFP cells were harvested before cell engraftment using a fluorescence stereomicroscope… How was this done with cells in culture growing in a plate?
The exact sentence to which the reviewer refers to is: "IGR-N91::GFP cells were harvested before engraftment by trypsination and from chick embryos using a fluorescence stereomicroscope"
We apologize for this confusion. Before engraftment, cells were detached from the culture dish by tryspination. This was corrected in the manuscript. The stereomicroscope only applies to cells harvested from avian embryos.

6- Please check for typos. For instance, in Line 713 the pipeline CutAdapt is spelled CutApapt

The typo has been corrected.

7- In Line 723 the authors state: "… These parameters were adapted for each sequenced conditions due to handling bias…". Please state which parameters and how was the criteria chosen?
Seurat package (v4.0.1) was used to compute the quality control metrics based on the number of both distinct and total count genes, and mitochondrial genes to have an overview of the cell viability state. Dispersion of each paramater was vizualized to remove cells exhibitting extensive variability compared to the overall dispersion for each sequenced condition. This has now been better explained in the method section.

8- In Line 752 the authors state: "… A set of tools developed in Seurat was applied…". Please describe which set of tools and how the parameters were chosen?
A set of tools developed in Seurat was applied to find anchors and project query labels onto a UMAP structure of the reference. Tools consisted in determining sets of shared anchors between a reference and a query object with FindTransferAnchors, in order to classify the query cells based on reference data using TransfertData function. Next, the number of neighbors to use for finding anchors was adjusted according to each dataset because the choice of K is well known to depend on the quality of input data quality (noise / number of cells / sequencing depth), ie: low k values can have high variance, but low bias, and high k values may lead to high bias and lower variance. The choice of k crucially depends on the input data as data with more outliers or noise will likely behave better with higher values of k. This has now been better explained in the method section.

9- Legends are too convoluted and are not sufficiently developed to follow all the figures. Please revise.
We have tried to developed the legends, but faced space constraints. We hope that these adjustments meet the referee expectations.

10- Unless I'm totally lost here. Could the authors explain what is the difference between Figure 3f and Extended Data Figure 5a?
Figure 3f and Supp. Fig. 5a concern the same type of analysis but on two different patient cohorts.

11- In Extended Figure 2 b clear differences can be observed between the samples obtained at different WPC. Is this biology or a technical artefact? Please comment.
We do not have any precise argument to state that the differences observed are linked to biology or technical artefact. However, the sequencing data of the SA-lineage were analyzed from two published studies (Jansky et al., 2021 and Kameneva et al., 2021) that do not highlight any specific technical variations from one WPC to another.

12- Similarly in Extended Data Figure 2e a clear cell cycle bias can be observed between the different samples. Please comment. Perhaps this differential cell cycle feature could be exploited.
The differences observed in cell cycle phases (now Supp. Fig. 2f) concern the different cell populations rather than the samples. While we agree that these data could be exploited, we decided not to explore these aspects in the scope of this study, except than to take into account the weight of these key genes in our bioinformatics analyses.

Reviewer #5: Expert in cancer avian embryonic models

The Authors have written an extremely interesting and innovative paper, which increased our knowledge on the pathogenesis and disease progression of neuroblastoma.
We thank the referee for his/her very kind feedback on our work.

Reviewer #3 (Remarks to the Author):

Dear authors,

Thank you for answering my questions and trying to address my concerns. I have also seen all the replies from reviewers number 2 and 4.

After analysing the 3D movies using light sheet microcopy that the authors have included, I found it very complicated to confirm that before E4-E5 there are no NG cells scaping or spreading around the focus in embryo E5, I can see many of them around the focus at this stage. The other important point to highlight is the independence of the ADR and SG foci. In these movies I cannot see the two independent foci in before E14.

These two important points were also strongly mentioned by another reviewer #2 and #4 with different expertise in cancer biology.
In my final opinion, the avian embryo approach has some weaknesses to accept the conclusions reached by the authors in the manuscript, and it is a main part of the manuscript. Unfortunately, I don't recommend to accepting the manuscript at this time.

Reviewer #4 (Remarks to the Author):

Comments to the Authors:

I thank the Authors for revising their manuscript and addressing most of this reviewer comments. However, I find the manuscript very difficult to follow in certain passages and I've got lost checking some of the figures. I've gathered my thoughts about it all for the authors to see below:

1- In line 167, the authors mention for the first time E0 whilst the nature of E0 is not described but later on.
2- In line 181, the authors mention "… two major biological functions…". However, unless I have completely missed it, I cannot find data anywhere that actually uses the terms "… developmental cell cycle…" or "… development of the sympatho-adrenal lineage…". Please check.
3- In line 198, the authors mention "… differentiation axes that fuel SA tissues…" I'm not sure "fuel" is the right word here.
4- In line 217, the authors mention "In-silico analysis… confirmed the progenitor status…". It is the opinion of this reviewer that the in-silico analysis described in that particular part of the manuscript is at best "predictive". Therefore, it cannot "confirm".
5- I may repeat myself here with my initial revision but, the term "transcriptional" alludes to mechanisms of control related to "transcription" and using this wording suggests that transcription is the sole determinant of steady state RNA levels. However, steady state RNA levels are controlled by multiple mechanisms independent of "transcription" (e.g miRNA regulation, RNA decay, splicing, among others). I

suggest once again the authors to replace “transcriptional” for “transcriptome” or something alike as to keep open the possibility that “transcriptome states” are not only driven/established/maintained by transcriptional events.
6- In line 234, the authors mention “… SCPs emblematic markers… were all negative…”. Please develop/present an explanation or hypothesis. Could it be an artefact based on the avian model?
7- In page 241, the author mention “… performing a computational prediction of RNA velocity.”. Rather a “… computational inference of temporal dynamics using scRNA-Seq (RNA velocity).” If I may.
8- In page 255, I suggest the authors to re-phrase: “depleted”.
9- In page 330, the authors mention their strategy to enrich the different compartments analysed. However, no data is shown in the manuscript. As far as I understand, it is mandatory (Nature Communications rules) to include in the manuscripts the actual data from the sorting as supplementary material.
10- Starting in line 368, the authors depict their combined analysis of genetic and transcriptome data. Unfortunately, I find the figures for this section, especially figure S8, completely confusing and incomplete. For instance… why S8 panel d only shows SMART-Seq A1? Seems quite arbitrary. In panel e… what is 309 samples? Is it cells? From which experiment? Also… I’m not sure what is the message of figure S8 panel a and b. I suggest the authors to re-shuffle most of the figures for the section to make them auto explicative (label, samples, etc). Also, the figures do not necessarily follow the text. Please have a look.
11- Why Figure 5a only display data for 309 cells? Is this a subset defined by the genetic analysis? If so… what does the clustering of the remaining cells look like? I suspect that alleviating the clustering conditions additional lineages could also be extracted and extra insights obtained.
12- In line 426, the authors mention “… pipeline of genes…”. Please rephrase.
13- In line 442, the authors mention “… in more than 5 out 7 patients…”. I’m not sure I understand the sentence.
14- In line 474. Please rephrase “… before disease diagnosis…”
15- In line 481, the authors mention “… our analysis of older grafted embryos…” Older? What does it mean?
16- In line 483, the authors mention “Close analyses…” Please develop “close” or re-phrase.
17- In line 561, the authors mention “… targeting could drive… preceding bone marrow involvement.” Involvement in which sense? Please develop.
18- In line 567, The authors mention “… pro-tumoral dialogs cell-cell dialogs…” Some words seem to be missing here.

Additional comments

One of the reviewers suggested to include the distinction between avian and human within the overall manuscript… I find this comment quite on point and I think the authors should revise their manuscript once again to fulfil this caveat including text and figures.

Legends to figures are too convoluted. Although I agree with the fact that the authors have limited space, the legends should be informative and the figures auto explicative. Too hard to follow in some instances.

Point-by-point answer to the reviewers

We thank the reviewers for these additional comments. Please find below the detailed answers to the additional points raised.

Reviewer #2

(Remarks to the Author): I appreciate the thorough response to my comments as now provided by the authors and new analyses and clarifications that have been included in the revised manuscript. I would like to suggest to the authors to consider the following point :

about the avian embryonic microenvironment and human tumor cells, I agree in principle with the response by the authors, but would like to suggest to discuss the advantages and limitations of the avian model in the manuscript in more depth, specifically regarding cross-species compatibility of microenvironmental cues to tumor cells.

We agree that this is important to note. So, in the discussion we added the following text line 623: "Moreover, inter-specie differences also have to be taken into consideration, and human specificities may exist that could be translated into additional molecular signaling mediating cancer cell-environment communications lacking in the avian embryo model".

Reviewer #3

(Remarks to the Author): Dear authors, Thank you for answering my questions and trying to address my concerns. I have also seen all th replies from reviewers number 2 and 4.

After analysing the 3D movies using light sheet microcopy that the authors have included, I found it very complicated to confirm that **before E4-E5 there are no NG** cells scaping or spreading around the focus in embryo E5, I can see many of them around the focus at this stage.

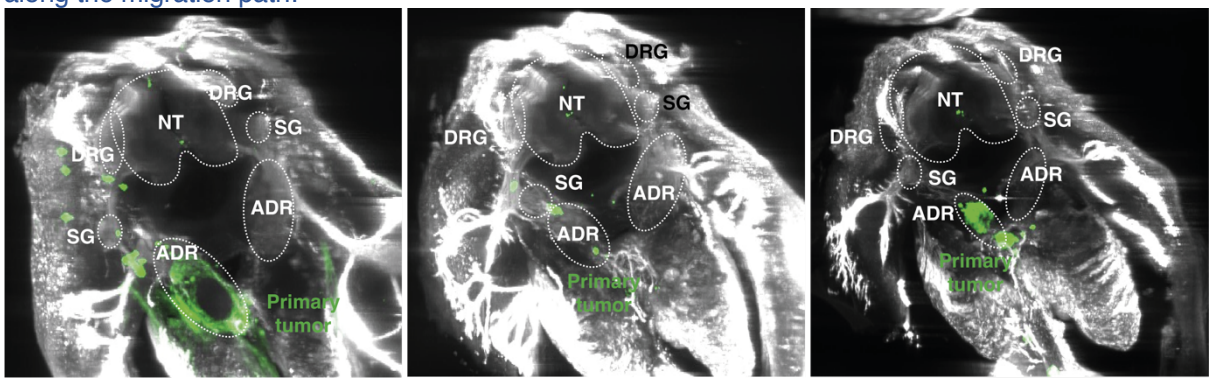
We fully understand this comment and agree that the 3D movie brings confusion on the topography of the tumor foci. We apologize for the lack of information to explain the pattern of tumor foci.

The presence of several foci indeed results from several processes:

-first, implanted NB cells do not all migrate at the same speed. Consequently, it is frequent to find cells along the ventrally-oriented sympatho-adrenal path. In our previous work (Delloye-Bourgeois et al, 2017), we mapped the physiological migration path through time course imaging of HNK1+ neural crest cells. IGR-N91 cells migrate in individual small clusters that occupy this path, that appears to be quite large along the medio-lateral axis.

-second, in some cases there are residual cells at the injection site.

To illustrate this, we made optical transverse sections of 3D movies of IGR-N91 cells showing the cells along the migration path:



-third, with ongoing tissue growing, the area occupied by cancer cells settling in the sympatho-adrenal territories is broader than that of the initial injection site. There are also antero-posterior movements of migrating IGR cells that can lead to colonization of 2 adjacent ganglia.
-fourth in the clinic, beyond the sympathetic ganglia and the adrenal medulla, other sympathico-adrenal-derived structures can be affected by NB. These are the periarterial sympathetic network (including the thoracic aortic, abdominal aortic and celiac plexus), the aorticorenal ganglia, the superior and inferior mesenteric plexus, the superior hypogastric plexus, and the iliac plexus (Brisse et al, 2017). We already observed that NB cells can colonize primordia of these structures after implantation in avian embryos (Akkermans et al, 2022).
Thus, IGR-N91 NB cells engrafted in the pre-migratory neural crest domain do not randomly migrate within the embryonic tissues but take the sympatho-adrenal path leading to the sympatho-adrenal derivatives.

The other important point to highlight is **the independence of the ADR and SG foci**. In these movies I cannot see the two independent foci in before E14.
We apologize if we were unclear in the manuscript on this point but we fully agree with the referee. At E5 in transverse sections, the two derivatives can be distinguished but with our method of manual microdissection we could not harvest separately tumors in the sympathetic ganglia and tumor in the adrenal medulla. This is why we have a single sample at E5.

These two important points were also strongly mentioned by another reviewer #2 and #4 with different expertise in cancer biology. In my final opinion, the avian embryo approach has some weaknesses to accept the conclusions reached by the authors in the manuscript, and it is a main part of the manuscript. Unfortunately, I don't recommend to accepting the manuscript at this time.
We respectfully would like to emphasize here that our approach consisting in correlating data from the avian embryo model with data from the clinic precisely aimed at deciphering whether the avian embryo model would be strong enough, despite not being a human embryo or fetus, to allow identification of signaling pathways relevant to the disease. We chose to focus on bone marrow involvement, a main metastatic site considered to play prominent contribution in disease progression and relapse. To our point of view, strong validation of our approach comes with the list of 25 genes that we found specifically active during bone marrow involvement in the model and also up-regulated in bone marrow samples when compared to their paired primary tumor samples. We found Midkine among these genes, recently reported from patient tumors analyses to mediate NB cells/bone marrow immune microenvironment communications (Fetahu et al, 2023). Importantly, this 25 genes list is a subset of a broader 65 genes list refined based on already reported requirement for NB cells growth (DepMap, https://depmap.org/portal). Thus, almost 40% of the predicted genes already match genes with demonstrated functional contribution to NB cell growth. We believe these findings fully supports the overall relevance of our integrative approach and allow proposing that these genes contribute to the formation of metastatic foci in the bone marrow. Functions of remaining ones still have to be studied, which will be the scope of future studies.

Reviewer #4

(Remarks to the Author): Comments to the Authors: I thank the Authors for revising their manuscript and addressing most of this reviewer comments. However, I find the manuscript very difficult to follow in certain passages and I've got lost checking some of the figures. I've gathered my thoughts about it all for the authors to see below:

1- In line 167, the authors mention for the first time E0 whilst the nature of E0 is not described but later on.
We apologize for this error, which we have corrected accordingly.

2- In line 181, the authors mention "… two major biological functions…". However, unless I have completely missed it, I cannot find data anywhere that actually uses the terms "… developmental cell cycle…" or "… development of the sympatho-adrenal lineage…". Please check.
We agree that the expression "major biological functions" was confusing, as these functions do not formally relate to a given nomenclature. We have modified the text accordingly and have added references to clarify our interpretation.

3- In line 198, the authors mention "… differentiation axes that fuel SA tissues…" I'm not sure "fuel" is the right word here.
We have replaced "fuel" by "give rise to".

4- In line 217, the authors mention "In-silico analysis… confirmed the progenitor status…". It is the opinion of this reviewer that the in-silico analysis described in that particular part of the manuscript is at best "predictive". Therefore, it cannot "confirm".
We have replaced "confirmed" by "predicted".

5- I may repeat myself here with my initial revision but, the term "transcriptional" alludes to mechanisms of control related to "transcription" and using this wording suggests that transcription is the sole determinant of steady state RNA levels. However, steady state RNA levels are controlled by multiple mechanisms independent of "transcription" (e.g miRNA regulation, RNA decay, splicing, among others). I suggest once again the authors to replace "transcriptional" for "transcriptome" or something alike as to keep open the possibility that "transcriptome states" are not only driven/established/maintained by transcriptional events.
We understand the referee's concern and have replaced "transcriptional state" by "transcriptome states" in the manuscript.

6- In line 234, the authors mention "… SCPs emblematic markers… were all negative…". Please develop/present an explanation or hypothesis. Could it be an artefact based on the avian model?
The contribution of SCPs to neuroblastoma initiation is indeed actively debated, but has never been formally demonstrated (Kastriti, 2022; Hanemaaijer, 2021, Ponzoni, 2022). While we cannot exclude that the absence of expression of SCP markers in NB cells was related to our experimental set up, we nevertheless reached similar conclusions with the 41 patient samples that we analyzed (Supp Fig. 4e-f and Supp Fig.5). These results lead us to hypothesize that either NB do not originate from SCPs (which we did not experimentally assess in our study), or that independently from their cell of origin, they preferentially manifest SNPCs/Nbts-related states of the lineage, for reasons that could be related to oncogenic events occurring later in the malignant process and disease progression like mutational, epigenetic, NB/microenvironmental crosstalk. We have discussed this in more detail in lines 577-580.

7- In page 241, the author mention "… performing a computational prediction of RNA velocity.". Rather a "… computational inference of temporal dynamics using scRNA-Seq (RNA velocity)." If I may.
We agree with the referee's concern and have rephrased the sentence accordingly.

8- In page 255, I suggest the authors to re-phrase: "depleted".
We have replaced "depleted" by "removed".

9- In page 330, the authors mention their strategy to enrich the different compartments analysed. However, no data is shown in the manuscript. As far as I understand, it is mandatory (Nature Communications rules) to include in the manuscripts the actual data from the sorting as supplementary material.
We apologize as we thought that describing the sorting strategy in the methods and the number of cells actually sorted for each sample (Source Data Supp Fig 7) was sufficient. We now have provided a Supplementary material file including a figure of the gating strategy that was used for the 7 patient bone marrow samples with an infiltration at diagnosis comprised between 0.4 % – 70 % (Supplementary table 1). For these samples only, an enrichment of tumor cells was performed based on a panel of cell surface markers (described in the Methods section) leading to a final fraction of tumor cells comprised between 40% and 74% (Source Data Supp Fig 7). A total of 6,693 tumor cells from both PTs and BMs was further sub-sampled for deeper analysis.

10- Starting in line 368, the authors depict their combined analysis of genetic and transcriptome data. Unfortunately, I find the figures for this section, especially figure S8, completely confusing and incomplete. For instance… why S8 panel d only shows SMART-Seq A1? Seems quite arbitrary.
We apologize for the lack of clarity regarding the combined analysis of genetic and transcriptome data. As stated in the manuscript, the IGR-N91 cells used here are actively cycling whatever the embryonic

stage or the anatomical site (Supp Fig. 2f and related Source Data). Therefore, IGR-N91 cells acquire genetic modifications which allow to almost perfectly distinguish cells (1) between two independent series of scRNA-Seq datasets (SMARTseq-A1 and SMARTseq-A2, shown in Supp Fig 8a) and (2) between time points in a given scRNA-Seq dataset (E0, E5, E14 from SMARTseq-A2 dataset, shown in Supp Fig8b).
This is not an arbitrary selection of one dataset over another, but rather the fact that the rapid genetic evolution precludes the possibility to pool together the two independent SMARTseq datasets to extract informative genetic variants along NB metastatic trajectories. We thus focused the rest of the analyses on SMARTseq-A1 dataset, covering each physical location of NB cells along their metastatic paths. Of note, we analyzed genetic variants from Smart-Seq2 data only, as this technique allows a very high depth sequencing (Supp Fig. 1a and related Source Data) and thus ensures a high coverage of newly acquired genetic variants. Consequently, twelve days after their implantation in avian embryos (from E2 to E14), we could extract a median of 740 informative genetic variants per IGR-N91 cell. The filters applied to extract and analyze these variants is now better described in the methods section.

In panel e… what is 309 samples? Is it cells? From which experiment?
We apologize as the word “sample” was confusing; we indeed referred here to 309 cells from the SMARTseq-A1 experiment. We have modified the figure accordingly.

Also… I’m not sure what is the message of figure S8 panel a and b. I suggest the authors to re-shuffle most of the figures for the section to make them auto explicative (label, samples, etc). Also, the figures do not necessarily follow the text. Please have a look.
We now have added some explanations in the manuscript and extended the legends of the figures related to the combined analysis of genetic and transcriptomic data to make them auto explicative.

11- Why Figure 5a only display data for 309 cells? Is this a subset defined by the genetic analysis? If so… what does the clustering of the remaining cells look like? I suspect that alleviating the clustering conditions additional lineages could also be extracted and extra insights obtained.
As explained in the previous concern, the 309 cells are not a subset defined by the genetic analysis but correspond to the complete SMARTseq-A1 dataset. We fully agree that having more cells would help gaining in precision although in our case, pooling two independent experiments is not possible as the IGR-N91 cell line rapidly evolves genetically.

12- In line 426, the authors mention “… pipeline of genes…”. Please rephrase.
We have replaced “pipeline” by “list”.

13- In line 442, the authors mention “… in more than 5 out 7 patients…”. I’m not sure I understand the sentence.
Our intention here was to explain that we selected the genes whose expression was higher in the BM than in the PT for at least 5 patients (out of 7). We now have rephrased the sentence.

14- In line 474. Please rephrase “… before disease diagnosis…”.
We have rephrased the sentence accordingly.

15- In line 481, the authors mention “… our analysis of older grafted embryos…” Older? What does it mean?
We meant that in this study the model was adapted to avian embryos at developmental stages later than in our previous studies. The sentence has been rephrased accordingly.

16- In line 483, the authors mention “Close analyses…” Please develop “close” or re-phrase.
We have replaced “close” by “fine”.

17- In line 561, the authors mention “… targeting could drive… preceding bone marrow involvement.” Involvement in which sense? Please develop.
We have replaced “involvement” by “invasion”.

18- In line 567, The authors mention "… pro-tumoral dialogs cell-cell dialogs…" Some words seem to be missing here.
We apologize for this incorrect sentence; it has now been rephrased.

Additional comments :

One of the reviewers suggested to include the distinction between avian and human within the overall manuscript… I find this comment quite on point and I think the authors should revise their manuscript once again to fulfil this caveat including text and figures.
We have revised our manuscript and have tried to add this information in both the text and figures.

Legends to figures are too convoluted. Although I agree with the fact that the authors have limited space, the legends should be informative and the figures auto explicative. Too hard to follow in some instances.
We have revised the legends and have added as much as possible information to make them auto-explicative.

REVIEWERS' COMMENTS

Reviewer #3 (Remarks to the Author):

Reviewer #3
Dear authors,

Thank you for answering my questions and for clarifying the three points I've mentioned as important in discussing the early avian model.

“After analysing the 3D movies using light sheet microscopy that the authors have included, I found it very complicated to confirm that before E4-E5 there are no NG cells scaping or spreading around the focus in embryo E5, I can see many of them around the focus at this stage.” my previous text on this point.

The authors explain very well my question and the problem and challenge of injecting IGR-N91 cells and their migration in the sympatho-adrenal pathway. I think it will be great to include in the discussion this point of possible variability in the genetic profile when the cells are harvested at E5.

‘‘We apologize if we were unclear in the manuscript on this point but we fully agree with the referee. At E5 in transverse sections, the two derivatives can be distinguished but with our method of manual microdissection we could not harvest separately tumors in the sympathetic ganglia and tumor in the adrenal medulla. This is why we have a single sample at E5.’’ From the authors

This is a similar point to highlight where the authors explain very well the same possible problem in E5 microdissection. I think, clarify this point in the discussion to see the possible challenge in E5 of the early development of the primary tumor.

‘‘We respectfully would like to emphasize here that our approach consisting in correlating data from the avian embryo model with data from the clinic precisely aimed at deciphering whether the avian embryo model would be strong enough, despite not being a human embryo or fetus, to allow identification of signaling pathways relevant to the disease. We chose to focus on bone marrow involvement, a main metastatic site considered to play prominent contribution in disease progression and relapse. To our point of view, strong validation of our approach comes with the list of 25 genes that we found specifically active during bone marrow involvement in the model and also up-regulated in bone marrow samples when compared to their paired primary tumor samples. We found Midkine among these genes, recently reported from patient tumors analyses to mediate NB cells/bone marrow immune microenvironment communications (Fetahu et al, 2023). Importantly, this 25 genes list is a subset of a broader 65 genes list refined based on already reported requirement for NB cells growth (DepMap,https://depmap.org/portal). Thus, almost 40% of the predicted genes already match genes with demonstrated functional contribution to NB cell growth. We believe these findings fully supports the overall relevance of our integrative approach and allow proposing that these genes contribute to the formation of metastatic foci in the bone marrow. Functions of remaining ones still have to be studied, which will be the scope of future studies.’’ From the authors

On this point, I agree with the authors that they explained to me and the final list of 25 to 65 genes and I think it is relevant for the correlation data between human and avian model. My main point is the injection and the specificity at the E5 time point. For the final conclusions I agree with the authors, but I think the avian model could be improved in the long term to be used in the future. I believe that the conclusions of the manuscript are relevant to neuroblastoma pathology and agree that the manuscript could be useful for a broad readership for the journal Nature communication. However, I would like the authors to mention the limitation of the avian model

Reviewer #4 (Remarks to the Author):

Comments to the Authors

I thank the Authors for revising their manuscript. However, and based on their rebuttal letter, given that SMART-Seq A1 and SMART-Seq A2 datasets are considered replicates, I think it would strengthen the claims made within the manuscript that a comparative analysis would be displayed (The authors have already presented something on those lines in a previous revision, but it was not included in the manuscript). Especially for figure 5 and Supplementary Figure 8. I do not see any reason not to display both analysis in parallel. In turn, reproducibility could also be evaluated regarding clonal divergence and the mutational landscape that could bring additional insights into the process. Please consider that this query could not have been posed in an earlier revision due to lack of clarity about how the experiment/analysis was performed.

Perhaps the main message of the manuscript will not change by including this additional data analyses, but it would help alleviate concerns about the reproducibility and robustness of the avian model and its interaction with the NB cells throughout the process of dissemination.

Also, unless I'm completely lost in my understanding of the data displayed in Figure 5 and Supplementary Figure 8, isn't the analysis suggesting that a certain genetic landscape preferentially "disseminate" to different physical targets (SG, ADR, AOR, PN subclones)? Please develop.

I also suggest the authors to include a clear explanation within the main manuscript describing the number of avian embryos grafted (including the number of cells grafted, efficiency of grafting, everything related to the grafting itself. I do not think that citing previous research should suffice here) to complement the data presented. So far, all this information is scattered all over the manuscript and, it is the opinion of this reviewer, that needs to be collated together for the reader to understand in one go the experimental design and potential caveats that may stem from it.

Moreover, it is the opinion of this reviewer, that the authors should develop/describe the caveats of the model in a robust "complete" way (not sufficient to cite previous research). Examples of caveats may include the following… if 2500 cells are grafted at t0 per embryo (looking at Figure 1h legend; E5, 22 embryos in 5 independent experiments and E14 15 embryos in 5 independent experiments… In this

context, what is an independent experiment?), then approximately 92500 cells were grafted in total (not even considering cell division here) and only 998 NB cells were analysed across all conditions described... It begs the question... Why? Although the authors suggest that this is due to technical limitations of the well-based single cell analysis, there is no description of whatever happens to all the remaining cells? Are they lost during sample enrichment? Do most of the cells die in the embryo? Do they die during retrieval? Following the same line of thought, how many NB cells are actually recovered/sequenced from a single embryo (If theoretical homogenous distribution of cells from the 998 analysed is assumed, then ~27 cells per embryo were recovered/sequenced)? Is the molecular profile between embryos somehow reproducible? As the authors state, in their dynamic system the cells are disseminating at a different pace… wouldn't this observation/interpretation suggest that each embryo may display an entirely different dissemination landscape? How was this controlled before committing an embryo to single cell analysis? Please develop.

Point-to-point answers to the reviewers

Reviewer's Comments:

Reviewer #3 (Remarks to the Author)

Reviewer #3

Dear authors,

Thank you for answering my questions and for clarifying the three points I've mentioned as important in discussing the early avian model.

"After analysing the 3D movies using light sheet microscopy that the authors have included, I found it very complicated to confirm that before E4-E5 there are no NG cells scaping or spreading around the focus in embryo E5, I can see many of them around the focus at this stage." my previous text on this point.

The authors explain very well my question and the problem and challenge of injecting IGR-N91 cells and their migration in the sympatho-adrenal pathway. I think it will be great to include in the discussion this point of possible variability in the genetic profile when the cells are harvested at E5.
We now included the following paragraph in the discussion, line 584 to clarify these aspects:
"Additional levels of heterogeneity among IGR-N91 cells exist that we did not study. For example, following NB cell grafting, we observed a continuum of migrating cells spanning the sympatho-adrenal path. Cells at the rear did not reach the target at the first time (E5) of our tumor microdissections. These cells may manifest some transcriptional differences that we did not capture by focusing on primary tumor sites. Moreover, at E5, sympathetic and adrenal derivatives already emerge as physically distinct territories. However, for technical feasibility, NB cells were collected indifferently of their sympathetic or adrenal location. Thus, we may have missed some early location-specific transcriptional features."

''We apologize if we were unclear in the manuscript on this point but we fully agree with the referee. At E5 in transverse sections, the two derivatives can be distinguished but with our method of manual microdissection we could not harvest separately tumors in the sympathetic ganglia and tumor in the adrenal medulla. This is why we have a single sample at E5.'' From the authors

This is a similar point to highlight where the authors explain very well the same possible problem in E5 microdissection. I think, clarify this point in the discussion to see the possible challenge in E5 of the early development of the primary tumor.
This is now clarified in the added paragraph.

''We respectfully would like to emphasize here that our approach consisting in correlating data from the avian embryo model with data from the clinic precisely aimed at deciphering whether the avian embryo model would be strong enough, despite not being a human embryo or fetus, to allow identification of signaling pathways relevant to the disease. We chose to focus on bone marrow involvement, a main metastatic site considered to play prominent contribution in disease progression and relapse. To our point of view, strong validation of our approach comes with the list of 25 genes that we found specifically active during bone marrow involvement in the model and also up-regulated in bone marrow samples when compared to their paired primary tumor samples. We found Midkine among these genes, recently reported from patient tumors analyses to mediate NB cells/bone marrow immune microenvironment communications (Fetahu et al, 2023). Importantly, this 25 genes list is a subset of a broader 65 genes list refined based on already reported requirement for NB cells growth (DepMap). Thus, almost 40% of the predicted genes already match genes with demonstrated functional contribution to NB cell growth. We believe these findings fully supports the overall relevance of our integrative approach and allow

proposing that these genes contribute to the formation of metastatic foci in the bone marrow. Functions of remaining ones still have to be studied, which will be the scope of future studies.'' From the authors

On this point, I agree with the authors that they explained to me and the final list of 25 to 65 genes and I think it is relevant for the correlation data between human and avian model. My main point is the injection and the specificity at the E5 time point. For the final conclusions I agree with the authors, but I think the avian model could be improved in the long term to be used in the future. I believe that the conclusions of the manuscript are relevant to neuroblastoma pathology and agree that the manuscript could be useful for a broad readership for the journal Nature communication. However, I would like the authors to mention the limitation of the avian model

In the discussion, with our paragraph following the one that already discussed inter-specie differences, we believe that the limitations of the model have now been made clear to the reader.

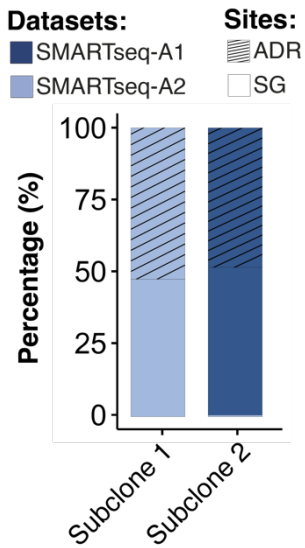
Reviewer #4 (Remarks to the Author)

Comments to the Authors

I thank the Authors for revising their manuscript. However, and based on their rebuttal letter, given that SMART-Seq A1 and SMART-Seq A2 datasets are considered replicates, I think it would strengthen the claims made within the manuscript that a comparative analysis would be displayed (The authors have already presented something on those lines in a previous revision, but it was not included in the manuscript). Especially for figure 5 and Supplementary Figure 8. I do not see any reason not to display both analysis in parallel. In turn, reproducibility could also be evaluated regarding clonal divergence and the mutational landscape that could bring additional insights into the process. Please consider that this query could not have been posed in an earlier revision due to lack of clarity about how the experiment/analysis was performed.

Perhaps the main message of the manuscript will not change by including this additional data analyses, but it would help alleviate concerns about the reproducibility and robustness of the avian model and its interaction with the NB cells throughout the process of dissemination.

We agree that analyzing in parallel two experimental replicates of sequencing experiments could strengthen the manuscript, although not changing our main message. However, we apologize if it was not clear enough, but our single cell RNASeq datasets are not adapted to such a comparative analysis, as SMARTseq-A1 and -A2 datasets are only partially overlapping, and do not constitute strict replicates.



The SMARTseq-A1 dataset aimed to cover the physical locations of NB cells over the metastatic process and was performed on NB cells harvested from E14 embryos (SG, ADR, PN, AOR, BM). The SMARTseq-A2 dataset aimed to characterize the temporal evolution of NB cell transcriptomes and was performed on NB cells at E0, at E5 (sympatho-adrenal tumors) and at E14 (SG, ADR). We now state this more clearly both in the main text and in the methods, rather than in the figure legends only.

We presented a comparative analysis of genetic variants in a previous revision that is indeed shown in Supp. Fig. 8a as a bar plot, rather than as a dendrogram. As suggested, we have now added this dendrogram in Supp. Fig. 8b. As SMARTseq-A1 and -A2 experiments were conducted with separate batches of IGR-N91::GFP cells (six months apart, with more than 10 passages in cell culture in between), we could show that genetic modifications acquired by NB cells over time (and cell division) allow to cluster NB cells according to each data set. We would like to precise here that we didn't use the same batches of IGR-N91 cells for SMARTseq-A1 and -A2 experiments precisely because we wished to ensure that the behavior of NB cells during the metastatic-like dissemination in the avian

embryo was not dependent on a specific initial genetic profile. To follow on reviewer #4 suggestion, as ADR and SG conditions overlap in between SMARTseq-A1 and -A2 datasets, we have now performed a comparison of genetic variants in NB cells at E14, in SG and in ADR tumors. We observe that NB cells first perfectly cluster according to the dataset of origin (as shown in previous Supp Fig. 8a, now replaced by this novel analysis), and then by physical location (as shown in Supp. Fig 8c for SMARTseq-A2, and Supp. Fig. 8h). Together these analyses confirm that the use of genetic variant information constitutes a convenient tracking approach.
Regarding reproducibility between our datasets, we also would like to highlight here that both SMARTseq-A1 and A2 datasets allow to show that NB cell clonal evolution is sufficiently rapid and informative to track NB cells having an ADR or an SG origin in the avian model (as these are the two experimental conditions replicated in SMARTseq-A1 and A2 experiments).

Also, unless I'm completely lost in my understanding of the data displayed in Figure 5 and Supplementary Figure 8, isn't the analysis suggesting that a certain genetic landscape preferentially "disseminate" to different physical targets (SG, ADR, AOR, PN subclones)? Please develop.
Our data indeed show that genetic variants are rapidly acquired by NB cells, with a temporality that make genetic variants sufficiently informative to trace NB cells across the metastatic process in the avian embryo. We did not observe any precise genetic pattern preferentially enriched at each stage of metastatic dissemination (Supp Fig. 8f). This is in line with the molecular clock concept, based on the fact that neutral SNPs accumulate linearly over time, are transmitted to daughter cells, and can therefore be used as tools to time spatiotemporal genetic evolution of cells. Based on this principle, cells migrating via the nerves or the aorta share the same variants with the primary tumor site from which they arise and acquire novel variants, allowing to trace the physical path of NB cell metastatic dissemination. This finding corroborates observations made by Korber et al (2023), who identified a similar mutational profile between the primary tumor and secondary sites.
Instead of considering genetic information as a driver of metastatic dissemination, we used it to track NB cells over time and physical location (space). However, we fully agree that certain genetic variants may be "functionally" associated with different physical targets, which is a very interesting question, yet beyond the scope of our study. We now have discussed it the manuscript.

I also suggest the authors to include a clear explanation within the main manuscript describing the number of avian embryos grafted (including the number of cells grafted, efficiency of grafting, everything related to the grafting itself. I do not think that citing previous research should suffice here) to complement the data presented. So far, all this information is scattered all over the manuscript and, it is the opinion of this reviewer, that needs to be collated together for the reader to understand in one go the experimental design and potential caveats that may stem from it.
We apologize if the information regarding the grafting method were not sufficiently clear. We have collated all these information in specific method sections and have added some precisions regarding the grafting and the dissection steps. In particular, we have better explained the way we standardize these two crucial steps of the process (controlling the number of grafted cells, embryo survival; standardizing the dissection by systematically collecting the same bones /nerves/ portion of the aorta).
Related to this, we also now better discuss that our standardized approach allows to describe key routes of metastatic dissemination from NB primary tumors to the bone marrow (NB main metastatic site), but does not exhaustively cover potential alternative metastatic paths and/or other final metastatic sites that are well known to occur in NB patients.

Moreover, it is the opinion of this reviewer, that the authors should develop/describe the caveats of the model in a robust "complete" way (not sufficient to cite previous research). Examples of caveats may include the following… if 2500 cells are grafted at t0 per embryo (looking at Figure 1h legend; E5, 22 embryos in 5 independent experiments and E14 15 embryos in 5 independent experiments… In this context, what is an independent experiment?
then approximately 92500 cells were grafted in total (not even considering cell division here) and only 998 NB cells were analysed across all conditions described... It begs the question... Why?
Although the authors suggest that this is due to technical limitations of the well-based single cell analysis, there is no description of whatever happens to all the remaining cells? Are they lost during sample enrichment? Do most of the cells die in the embryo? Do they die during retrieval?

By independent experiment we mean a grafting experiment that exploits the exact same batch of NB cells (same culture dish) and the same batch of embryonated eggs, incubated at the same time. We now have better explained this in the method section dedicated to the graft technique.
Regarding the number of cells that were analyzed by single cell RNASeq, the 999 NB cells analyzed are only a fraction of all cells that are indeed present and viable in embryos. Both SMARTseq and SeqWell approaches are designed for relatively small number of cells per experimental condition (sequencing in 96-well plates and cost). As already mentioned in our first point-to-point answers, this order of magnitude in the number of analyzed cells is typically that recommended and published for high-depth plate sequencing approaches.
Our strategy here was to sequence a maximum of 96 cells per condition (5 conditions: ADR, SG, AOR, PN, BM for SMARTseq-A1 for example). Moreover, cells were collected by manual microdissection from avian embryos. To ensure that cells were properly harvested from specific embryonic tissues (SG, ADR, PN, AOR, BM) we selected cells that we could unambiguously isolate from these 5 locations, thus leaving some cells which location was unclear. We also cannot exclude that a few GFP^+ cells were lost during the sorting step, especially as remaining doublets and cell aggregates were discarded upon sorting, to plate single cells only.
With this approach, we could fill in complete 96-well plates with ADR, SG and AOR cells (and unsorted cells for these conditions, containing the supposedly remaining “92500” grafted cells, were discarded here). PN and BM foci were more discrete and also more difficult to harvest by manual microdissection. Thus, we sorted the entire samples but we did not obtain the targeted 96 cells, which explains the lower number of analyzed cells for these two conditions.

Following the same line of thought, how many NB cells are actually recovered/sequenced from a single embryo (If theoretical homogenous distribution of cells from the 998 analysed is assumed, then ~27 cells per embryo were recovered/sequenced)? Is the molecular profile between embryos somehow reproducible? As the authors state, in their dynamic system the cells are disseminating at a different pace… wouldn’t this observation/interpretation suggest that each embryo may display an entirely different dissemination landscape? How was this controlled before committing an embryo to single cell analysis? Please develop.
We apologize if our explanations on the embryo procedure before performing single cell analyses were not sufficient. The number of cells retrieved from the different tissue sites, especially in the BM and in the PN, were too low to proceed to sorting embryo by embryo. For each independent experiment, we pooled the dissected cells/tissues from embryos per anatomical localization to reach a sufficient number of cells, before the sorting step. Thus, we could not formally determine the exact number of cells recovered per embryo, nor whether the molecular profile between embryos is similar. Moreover, embryos used for imaging approaches that document the localization of NB cells are necessarily distinct from embryos dissected to proceed to single cell RNASeq as the approaches are mutually exclusive. Therefore, we cannot correlate a given molecular profile with a specific dissemination landscape. However, both 3D light sheet and confocal imaging approaches allowed to show that, in the avian model of NB, embryos engrafted with a given cell line show stereotypic patterns of dissemination, as mentioned in the figure legends where each dissemination phenotype per embryo was quantified, especially at E14.