Author's Point-by-point Response to Reviewers' Comments

[cancers-1746495]
NR2F1, a tumor dormancy marker, is expressed predominantly in cancer-associated fibroblasts and is associated with suppressed breast cancer cell proliferation

Reviewer #1 (General comments):
This manuscript adds evidence that NR2F1 is a dormancy marker. In addition, it shows that NR2F1 is primarily expressed in CAFs, particularly in inflammatory CAFs.

Response:
We would like to thank reviewer #1 for his/her time and effort to review our manuscript. Please find our point-by-point responses below.

Comment 1:
Given that dormant disseminated tumor cells (DTCs) highly express NR2F1 (Fluegel et al. Nat Cell Biol 2017), it is puzzling how NR2F1 in CAFs of the primary tumor would contribute to dormancy of DTCs. It would be nice, if the authors would discuss this important point in more detail.

Response 1:
We totally agree with the reviewer that it is puzzling how NR2F1 expressed in CAFs of the primary breast cancer contribute to the dormancy of DTCs, thus the addition of a discussion on this point in more detail will strengthen this manuscript. Based on our results, NR2F1 expression in primary bulk tumor is associated with several pathways related to dormancy, and NR2F1 is most predominantly expressed in CAFs in the tumor microenvironment. However, we did not prove the underlying mechanism through which CAF-expressed NR2F1 regulates dormancy, and we do not intend to claim a causal relationship. Single-cell sequence data of metastatic tumor cohorts will allow us to investigate whether the expression of NR2F1 in CAFs in the metastatic TME is related to the dormancy of DTCs. We added the following sentences to the discussion section.

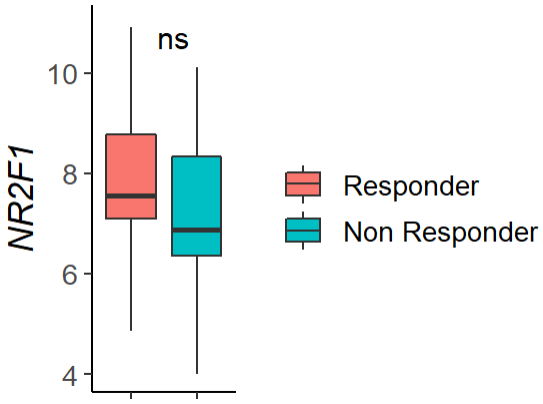
Discussion section (Page 13, line 427):
The current study demonstrated that *NR2F1* expression in the bulk tumor of primary breast cancer is associated with decreased cell proliferation and cancer stem cell-like characteristics. Previous studies mentioned that *NR2F1* was highly expressed in DTCs [67]. We found that NR2F1 is most predominantly expressed in CAFs in the TME of primary breast cancer. However, the findings from our study are not ample to substantiate that CAF-expressed *NR2F1* regulates breast tumor dormancy. Aguirre-Ghiso group recently demonstrated that *NR2F1* agonist treatment induced cancer cell dormancy [21], which raised an expectation that *NR2F1* expression in primary breast cancer may have the possibility to be a biomarker. The novelty of our study is that it is CAFs, not cancer cells, that are the dominant source of *NR2F1* expression in the bulk tumor. To this end, we believe that *NR2F1* expression in the

bulk tumor does not reflect the expression in the cancer cells, thus its value as a biomarker is in doubt. *NR2F1* expression in cancer cells of primary breast cancer was not associated with cancer stem cell-like characteristics at all. In order to prove that CAF-expressed *NR2F1* regulates breast tumor dormancy, one needs to analyze the single-cell sequencing data and show whether the late recurrence in other organs, such as lungs, bones, and brain, other than lymph nodes, is correlated with *NR2F1* expression in the CAFs.

Comment 2:
The authors choose chemotherapy as a treatment option to compare it with NR2F1 levels. It would be interesting to see, how NR2F1 levels would change with endocrine treatment and/or endocrine resistance.

Response 2:
We agree with the reviewer that it would be interesting to see how NR2F1 levels would change with endocrine therapy. However, we do not have access to cohorts that include tumor samples before and after neoadjuvant endocrine therapy at this point. What we do have access to regarding endocrine therapy is a cohort comparing responders and non-responders to neoadjuvant endocrine therapy (GSE145325). We found that NR2F1 expression between responders and non-responders to endocrine therapy was not different. We added this to the results section as follows.

Results section (Page 11, line 346):
There was no difference in NR2F1 expression between responder and non-responder to neoadjuvant endocrine therapy (Supplementary Fig. 9).



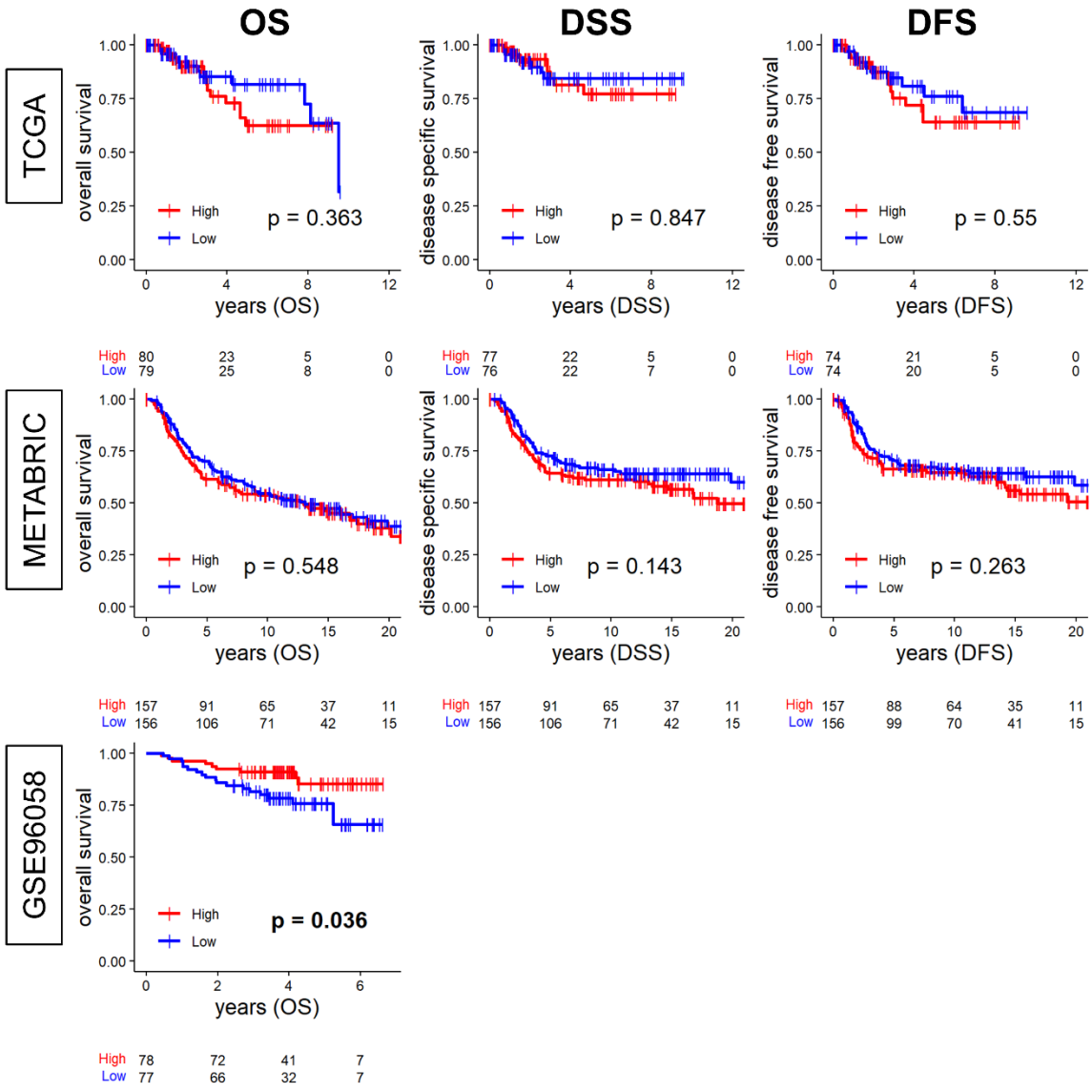
Supplementary Figure 9. Expression of NR2F1 between endocrine therapy responder and non-responder in hormone-positive primary breast cancer. Boxplots showing NR2F1 expression in responder and non-responder to neoadjuvant endocrine therapy in GSE145325. Man-Whitney U test was used for analysis, symbol in the figure means as follows ns: $p > 0.05$.

Comment 3:

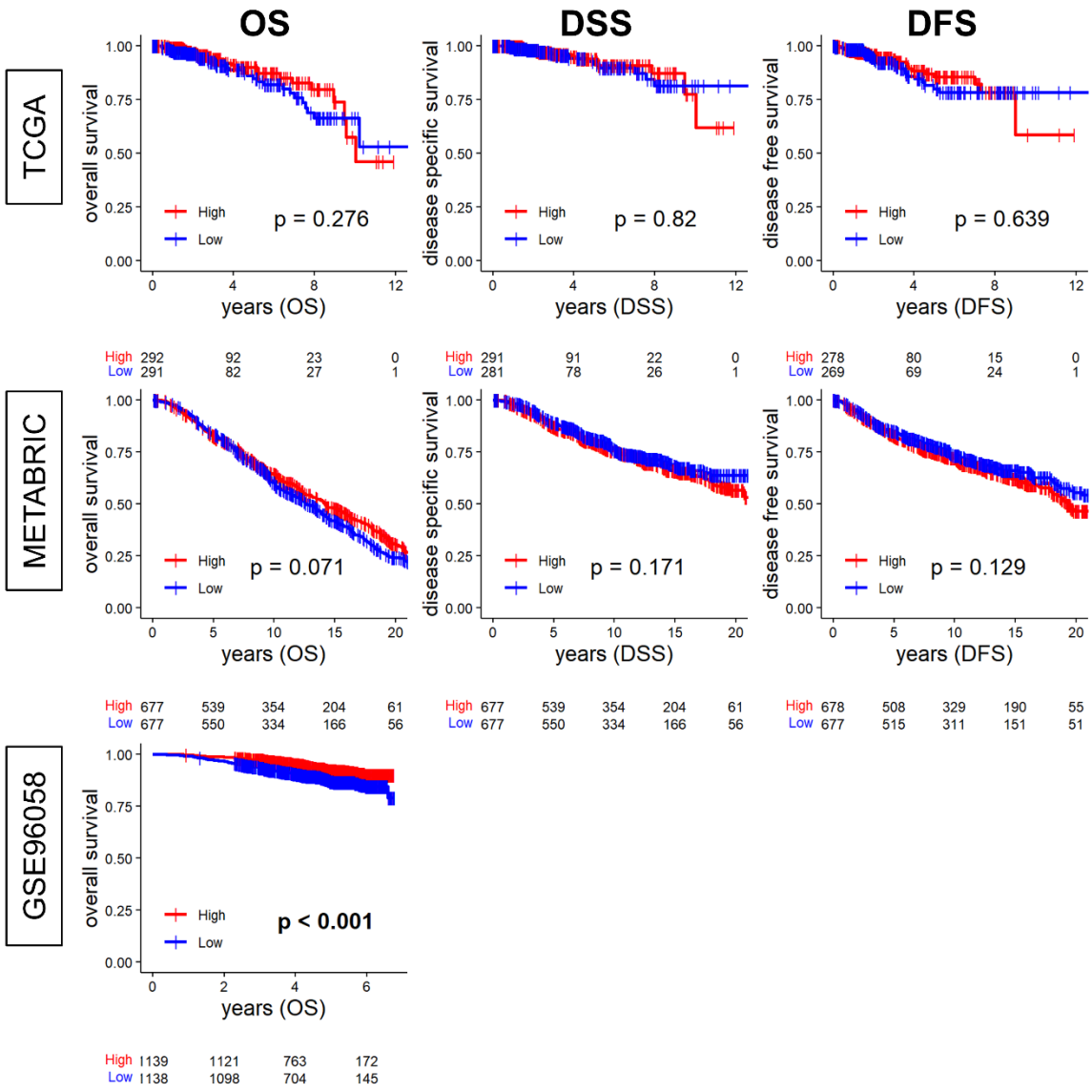
Breast cancer is a heterogenous disease. Different subtypes behave differently in many aspects. It would be great to see some subtype-specific data.

Response 3:
We agree with the reviewer that breast cancer is a heterogenous disease and we should show subtype-specific data. We analyzed survival outcomes and the cell fraction in TME by each immunohistological subtype. We did not observe any validated difference in survival outcomes. Cell fractionation of immune cells and stromal cells showed almost similar trends for the scores such as intratumor heterogeneity, HRD, mutation rate, and neoantigens across all subtypes. The results for each immunohistological subtype of single-cell Cohort 2 are shown in Supplementary Figure 7, and each subtype showed the same trend. We have revised the results section and added supplementary data as follows.

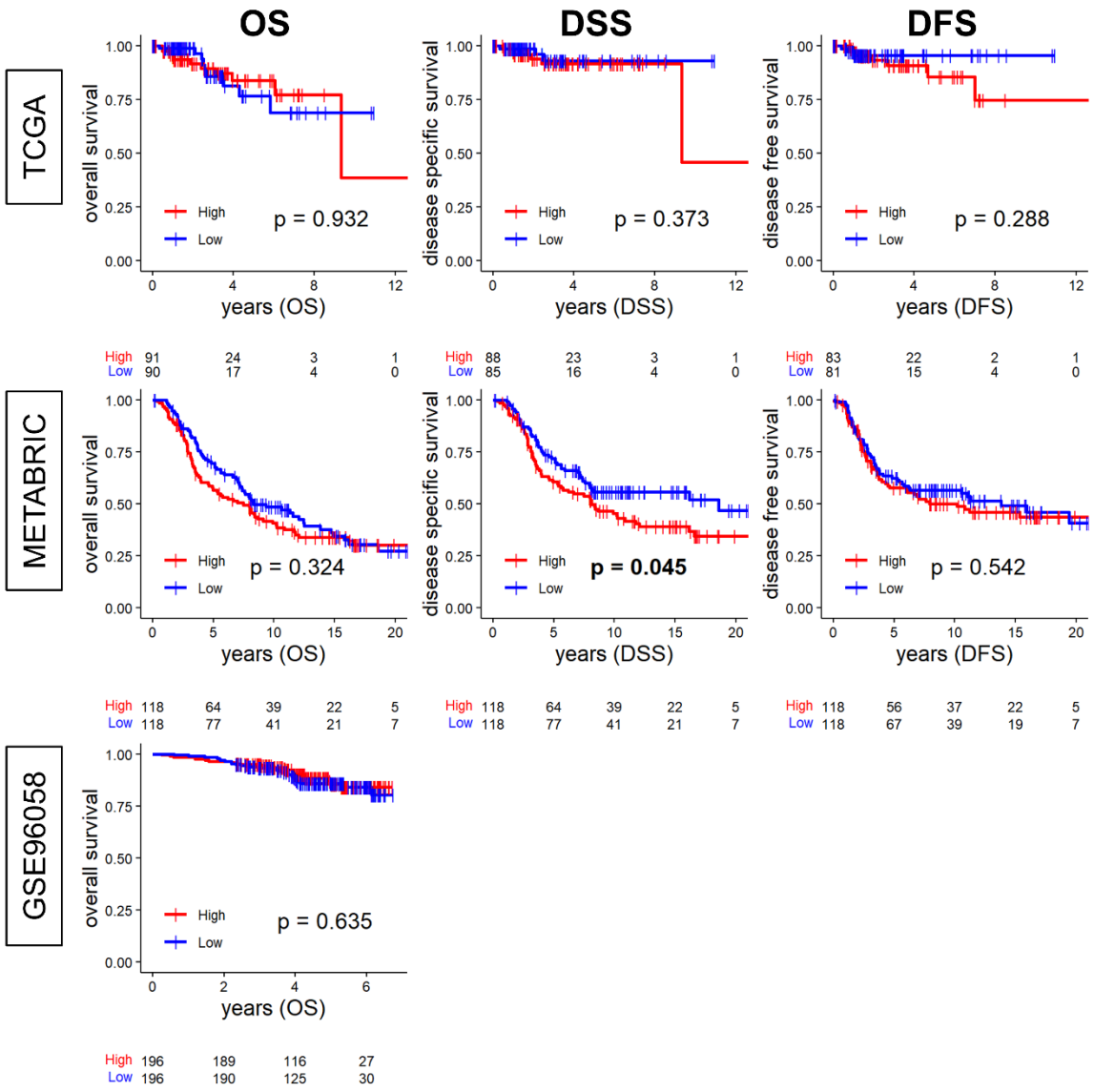
Results section (Page 6, line 215):
Given that breast cancer is a heterogenous disease, it was of interest to investigate the survival outcome by NR2F1 expression of each subtype. The OS of NR2F1 high triple-negative and ER-positive/HER2-negative subtypes were associated with better survival in GSE96058, and DSS of NR2F1 high HER2-positive subtypes was associated with worse survival in METABRIC. But none of these results were validated by the other breast cancer cohorts (Supplementary Figure 1-3).



Supplementary Figure 1. Survival analysis between high and low *NR2F1* group in triple-negative breast cancer. Kaplan-Meier curves of overall survival (OS), disease-specific survival (DSS), and disease-free survival (DFS) based on the high and low *NR2F1* expression in triple-negative breast cancer of three large cohorts. Triple-negative subtype was determined by immunohistochemistry. Log-rank test was used for the analysis, and significant p values are shown in bold. $p < 0.05$ was considered significant.

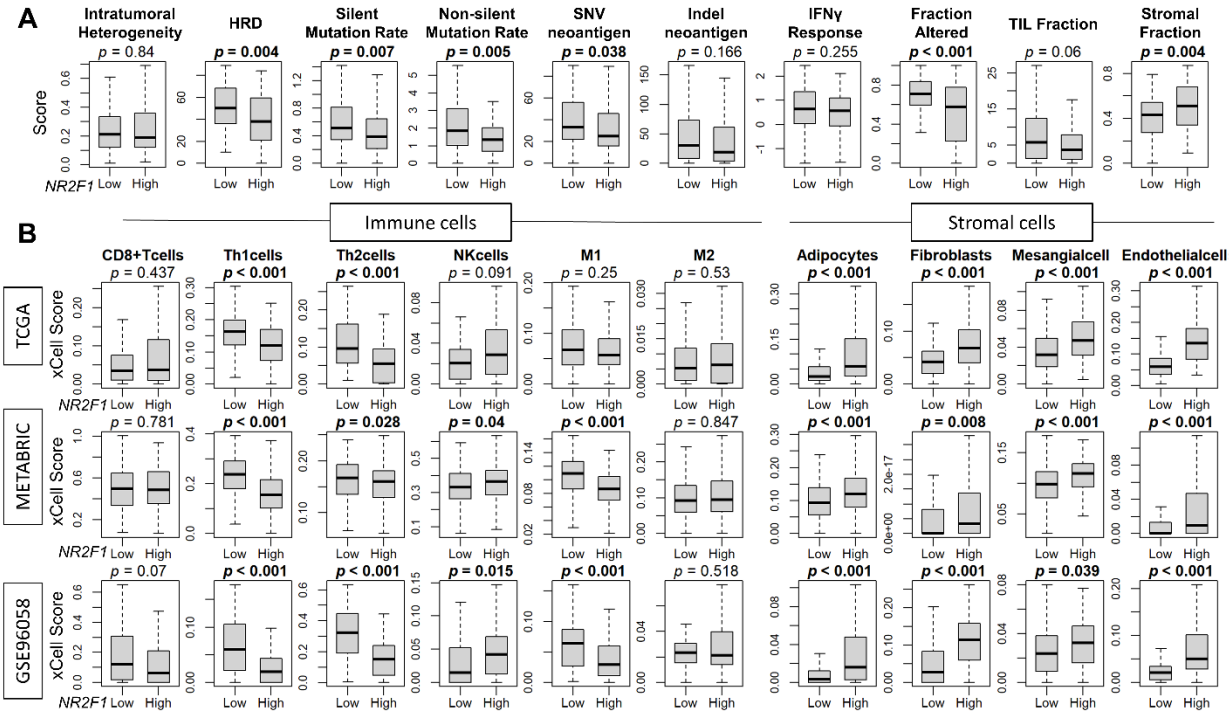


Supplementary Figure 2. Survival analysis between high and low *NR2F1* group in ER-positive/HER2-negative breast cancer. Kaplan-Meier curves of OS, DSS, and DFS based on the high and low *NR2F1* expression in ER-positive/HER2-negative breast cancer of three large cohorts. ER-positive/HER2-negative subtype was determined by immunohistochemistry. Log-rank test was used for the analysis, and significant p values are shown in bold. $p < 0.05$ was considered significant.

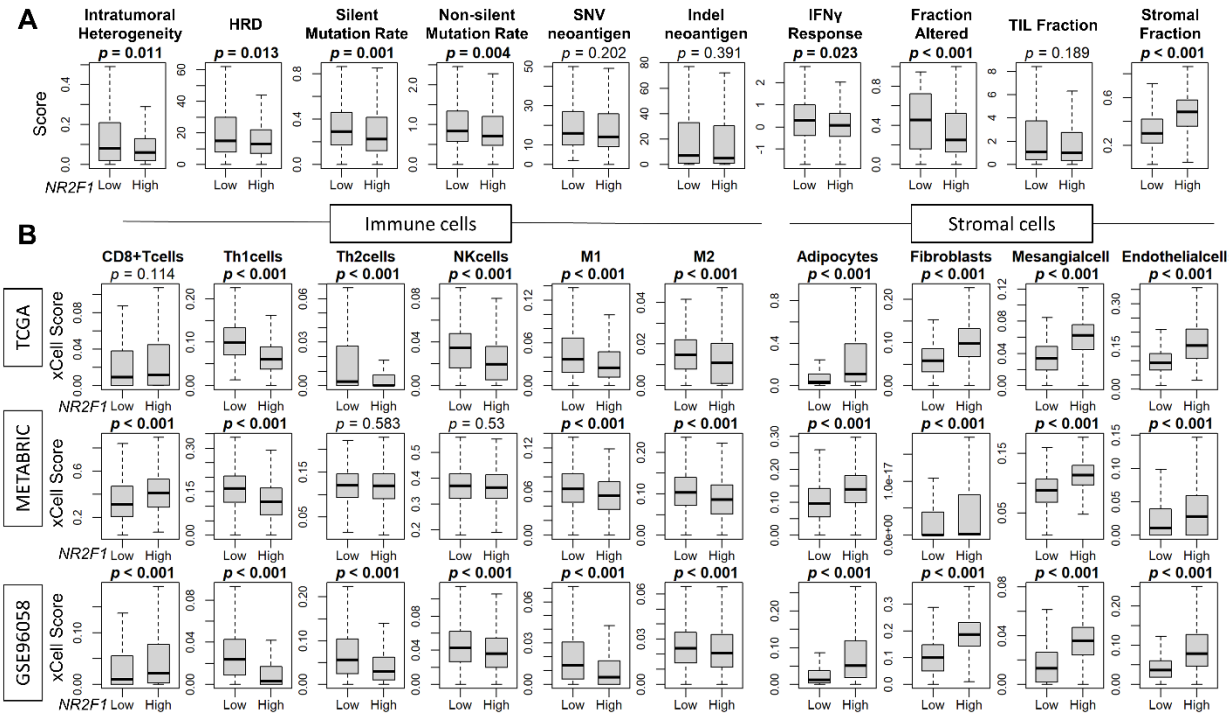


Supplementary Figure 3. Survival analysis between high and low *NR2F1* group in HER2 positive breast cancer. Kaplan-Meier curves of OS, DSS, and DFS based on the high and low *NR2F1* expression in HER2-positive breast cancer of three large cohorts. HER2-positive subtype was determined by immunohistochemistry. Log-rank test was used for the analysis, and significant p values are shown in bold. $p < 0.05$ was considered significant.

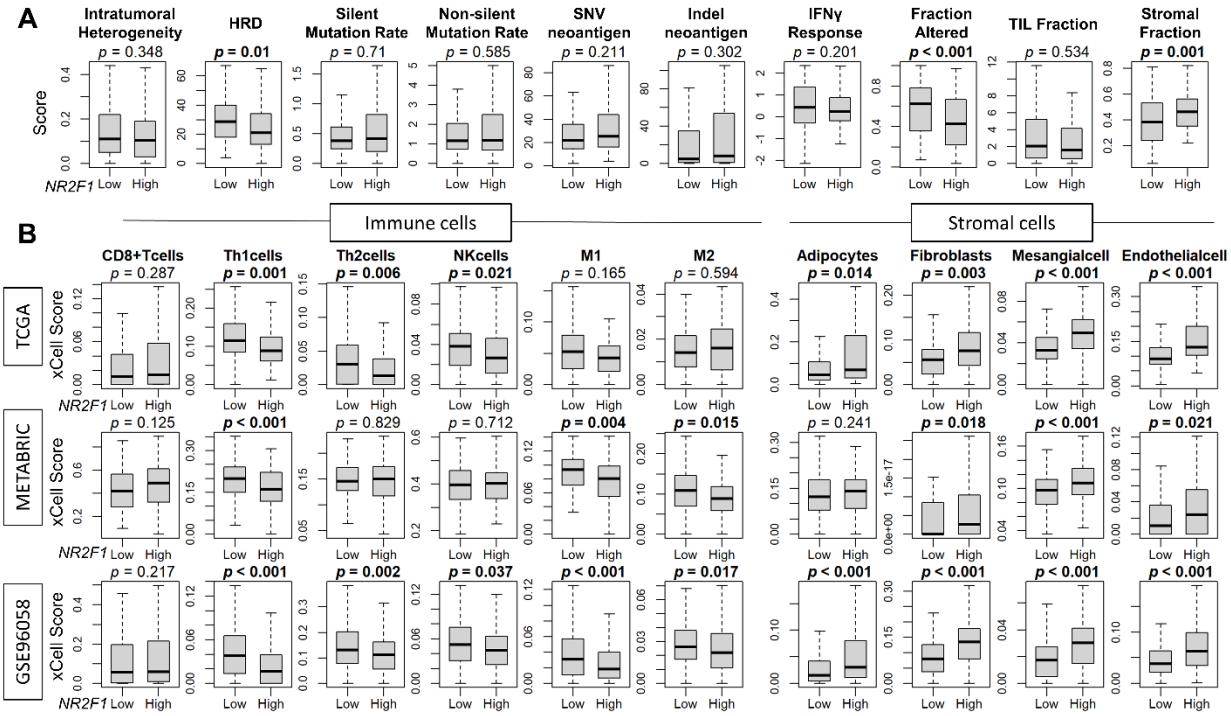
Results section (Page 9, line 280):
Further analysis by each immunohistological subtype showed the same trend that the fraction of stromal cells was significantly higher in the NR2F1 high group across multiple cohorts (Supplementary Figure 4-6).



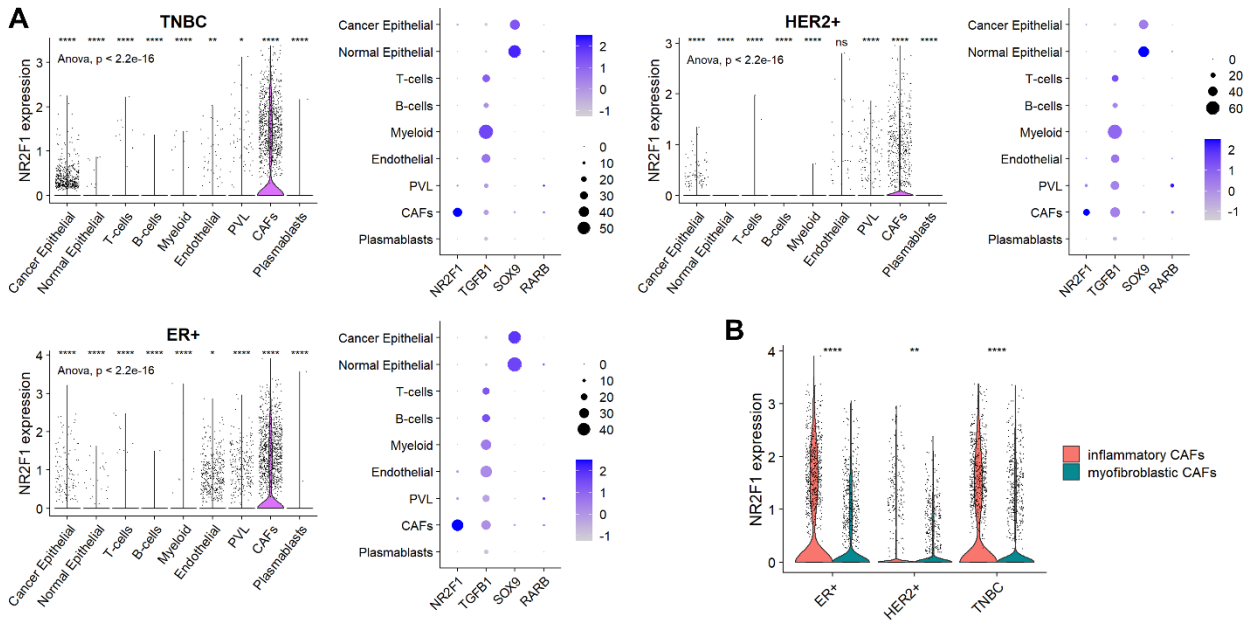
Supplementary Figure 4. Association of *NR2F1* with immunity in the tumor microenvironment of triple-negative breast cancer. (A) Boxplots showing various scores based on high and low *NR2F1* expression in triple-negative breast cancer of TCGA; Intratumoral heterogeneity, homologous recombination deficiency (HRD), silent/non-silent mutation rate, SNV/Indel neoantigen, Interferon gamma response, fraction altered, tumor-infiltrating lymphocytes (TIL) fraction, and stromal fraction. **(B)** Boxplots showing immune and stromal cell fractions between *NR2F1* high and low group in triple-negative breast cancer of three large cohorts. Man-Whitney U test was used to compare the two groups and p-values are shown in bold for significant results ($p < 0.05$).



Supplementary Figure 5. Association of *NR2F1* with immunity within the tumor microenvironment of ER-positive and HER2 negative breast cancer. (A) Boxplots showing various scores based on high and low *NR2F1* expression in ER-positive and HER2 negative breast cancer of TCGA; Intratumoral heterogeneity, homologous recombination deficiency (HRD), silent/non-silent mutation rate, SNV/Indel neoantigen, Interferon gamma response, fraction altered, tumor-infiltrating lymphocytes (TIL) fraction, and stromal fraction. **(B)** Boxplots showing immune and stromal cell fractions between *NR2F1* high and low group in ER-positive and HER2 negative breast cancer of three large cohorts. Man-Whitney U test was used to compare the two groups and p-values are shown in bold for significant results ($p < 0.05$).



Supplementary Figure 6. Association of *NR2F1* with immunity within the tumor microenvironment of HER2 positive breast cancer. (A) Boxplots showing various scores based on high and low *NR2F1* expression in HER2 positive breast cancer of TCGA; Intratumoral heterogeneity, homologous recombination deficiency (HRD), silent/non-silent mutation rate, SNV/Indel neoantigen, Interferon gamma response, fraction altered, tumor-infiltrating lymphocytes (TIL) fraction, and stromal fraction. **(B)** Boxplots showing immune and stromal cell fractions between *NR2F1* high and low group in HER2 positive breast cancer of three large cohorts. Man-Whitney U test was used to compare the two groups and p-values are shown in bold for significant results ($p < 0.05$).



Supplementary Figure 7. Expression of *NR2F1* and dormancy-related genes by immunohistological breast cancer subtypes in Cohort 2. (A) Violin plots showing *NR2F1* expression by cell type in triple-negative (TNBC), HER2 positive (HER2+), and ER-positive HER2 negative (ER+) tumors in single-cell Cohort 2. One dot represents one cell. After multi-group comparison, baseline *NR2F1* expression and expression in each cell type were compared in two groups by the one-way ANOVA test. Also, dot plots show the expression of *NR2F1*, *TGFB1*, *SOX9*, and *RARB* by cell type in each immunohistological subtype in single-cell Cohort 2. The size of each dot indicates the number of cells, and the purple intensity indicates the expression level. **(B)** The violin plot shows *NR2F1* expression in inflammatory CAFs (iCAFs) and myofibroblasts (myCAFs) by immunohistological subtypes in single-cell Cohort 2. Symbols in the figure mean as follows ns: $p > 0.05$, *: $p \leqq 0.05$, **: $p \leqq 0.01$, ***: $p \leqq 0.001$, ****: $p \leqq 0.0001$.

Comment 4:

The authors state "We demonstrated that the expression of NR2F1, RARB, and TGFB1 genes are higher in previously established dormant cells (D2OR murine breast cancer cells [44] compared to the proliferative cells (D2A1 cells) in both 2D and 3D cultures (Figure 1A, all p < 0.02)." This is not true for NR2F1 in 2D. Please correct.

Response 4:

We thank the reviewer for reading our manuscript closely and pointing out our oversight. As you indicated, we did not find any significance difference in 2D in Figure 1A and have corrected the results section as follows.

Results section (Page 4, line 157):

We demonstrated that the expression of the NR2F1 gene is higher in previously established dormant cells (D2OR murine breast cancer cells) [44] compared to the proliferative cells (D2A1 cells) in 3D cultures, and the expression of RARB and

TGFB1 genes are higher in dormant cells compared to the proliferative cells in both 2D and 3D cultures (Figure 1A, all $p < 0.02$)

Comment 5:
The manuscript needs mild English proofing, mainly the abstract.

Response 5:
We agree with the reviewers' suggestion that the manuscript needs mild English proofing. We have thoroughly reviewed our manuscript for grammar and spelling errors and mistakes were corrected. We have also corrected the errors in the abstract. The revised abstract is shown below.

Abstracts (Page 1, line 32):
Background: Tumor dormancy is a crucial mechanism responsible for the late recurrence of breast cancer. Thus, we investigated the clinical relevance of the expression of NR2F1, a known dormancy biomarker. Methods: A total of 6758 transcriptomes of bulk tumors from multiple breast cancer patient cohorts and two single-cell sequence cohorts were analyzed. Results: Breast cancer (BC) with high NR2F1 expression, enriched TGFβ signaling, multiple metastases, and stem cell-related pathways. Cell proliferation-related gene sets were suppressed and MKi67 expression was lower in high NR2F1 BC. In tumors with high Nottingham grade, NR2F1 expression was found to be lower. There was no consistent relationship between NR2F1 expression and metastasis or survival. Cancer mutation rates, immune responses, and immune cell infiltrations were lower in high NR2F1 tumors whereas the infiltration of stromal cells including cancer-associated fibroblasts (CAFs) was higher. Surprisingly, NR2F1 was predominantly expressed in CAFs, particularly inflammatory CAFs, ra-ther than in cancer cells, consistently in the two single-cell sequence cohorts. Conclusions: NR2F1 expression in breast cancer is associated with tumor dormancy traits and it is predominantly ex-pressed in CAFs in the tumor microenvironment.

We would like to thank the reviewer for his/her thoughtful and thorough review of our manuscript, which significantly strengthened our manuscript.