Supplemental data for

TYRO3 induces anti-PD-1/PD-L1 therapy resistance by limiting innate immunity and tumoral ferroptosis

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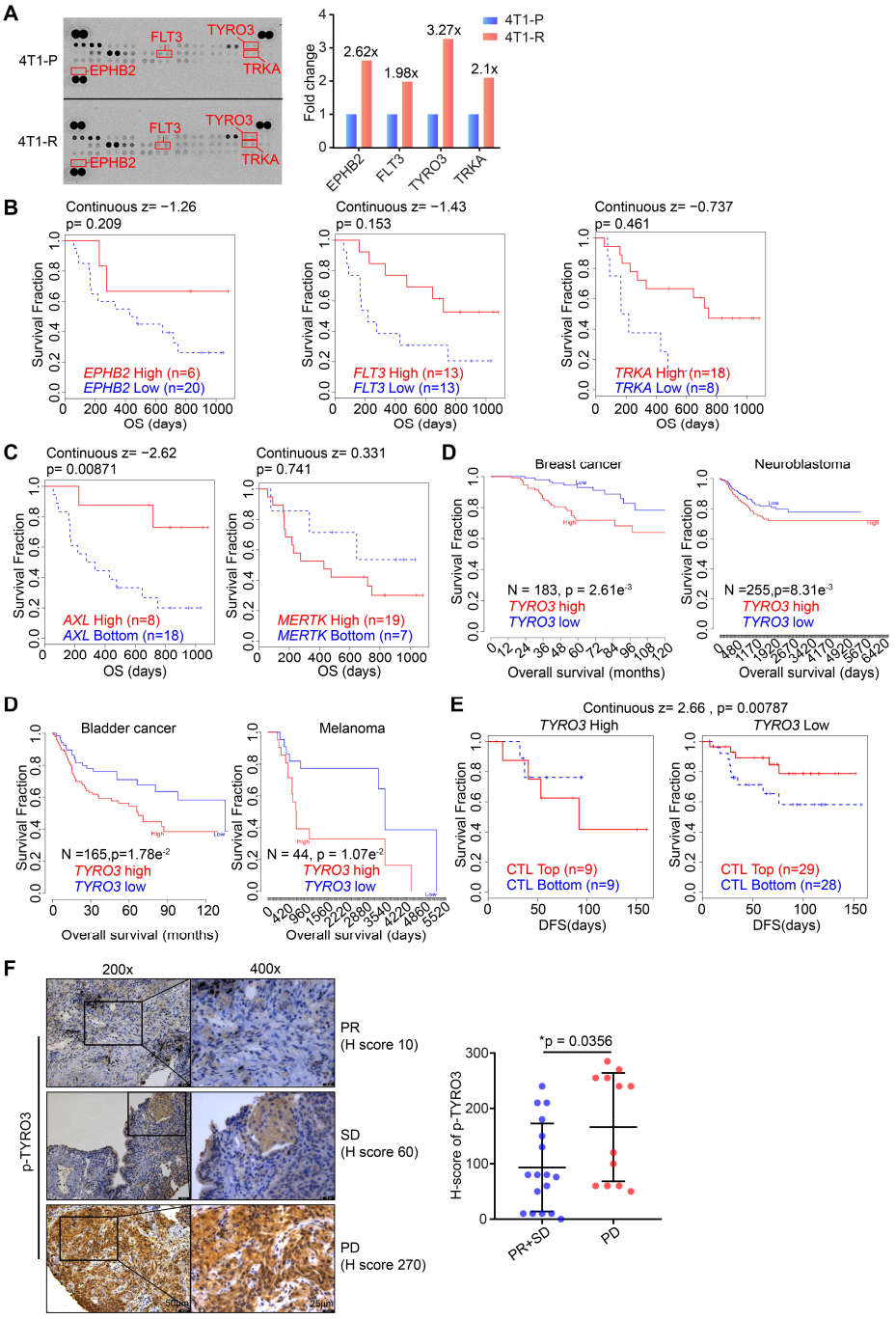
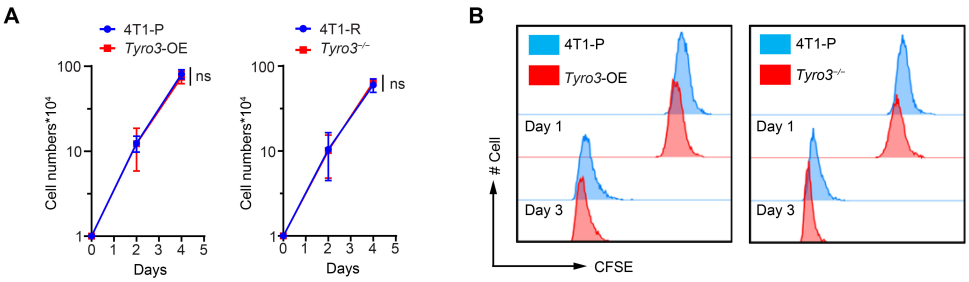
This PDF file includes:

Extended Data: Supplemental Figures 1-4

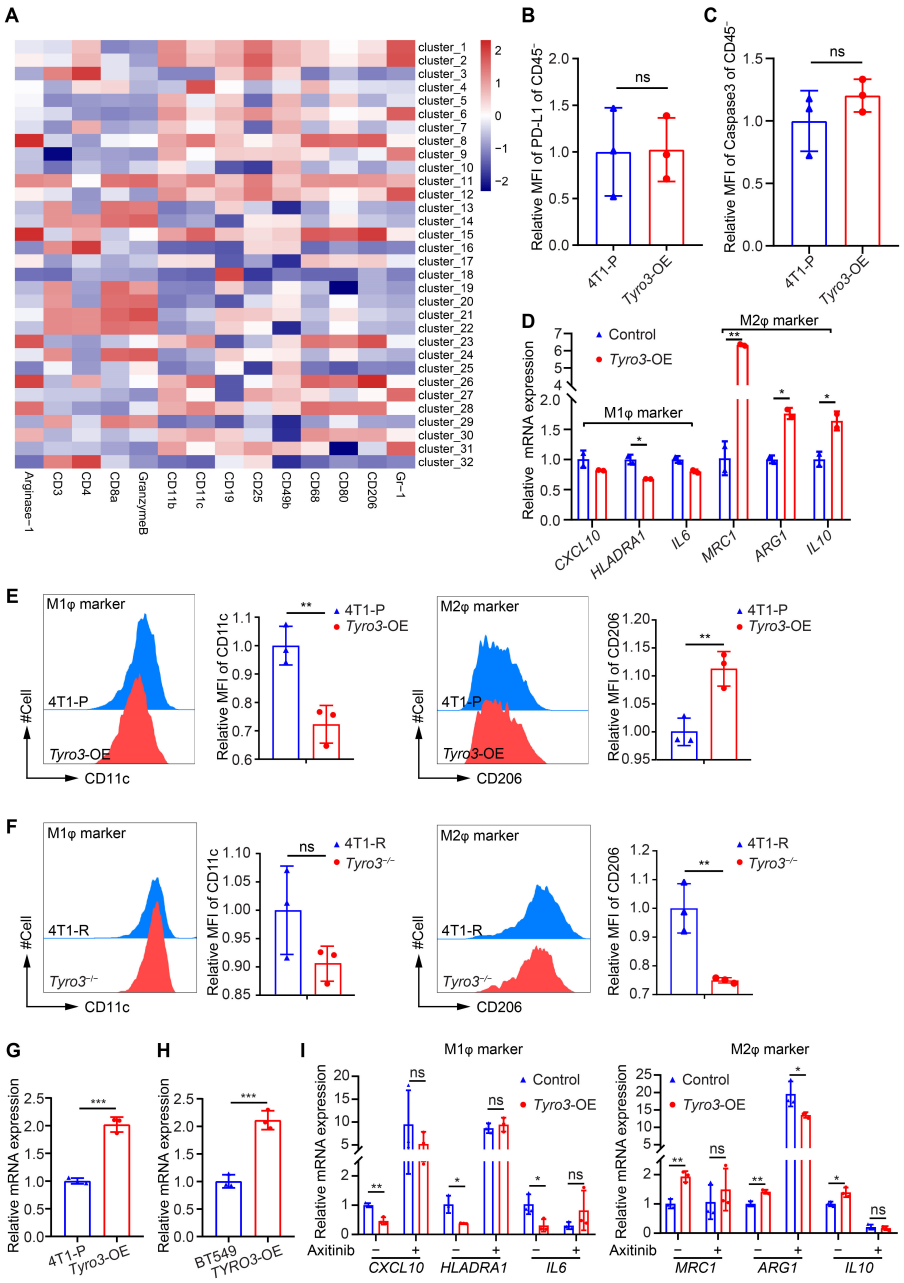

A
4T1-P
4T1-R
FLT3
TYRO3
EPHB2
TRKA
Fold change
2.62x
1.98x
3.27x
2.1x
EPHB2
FLT3
TYRO3
TRKA
B
Continuous z= −1.26
p= 0.209
EPHB2 High (n=6)
EPHB2 Low (n=20)
Continuous z= −1.43
p= 0.153
FLT3 High (n=13)
FLT3 Low (n=13)
Continuous z= −0.737
p= 0.461
TRKA High (n=18)
TRKA Low (n=8)
Survival Fraction
OS (days)
C
Continuous z= −2.62
p= 0.00871
AXL High (n=8)
AXL Bottom (n=18)
Continuous z= 0.331
p= 0.741
MERTK High (n=19)
MERTK Bottom (n=7)
D
Breast cancer
N = 183, p = 2.61e-3
TYRO3 high
TYRO3 low
Overall survival (months)
Neuroblastoma
N =255,p=8.31e-3
TYRO3 high
TYRO3 low
Overall survival (days)
D
Bladder cancer
N =165,p=1.78e-2
TYRO3 high
TYRO3 low
Overall survival (months)
Melanoma
N = 44, p = 1.07e-2
TYRO3 high
TYRO3 low
Overall survival (days)
E
Continuous z= 2.66 , p= 0.00787
TYRO3 High
CTL Top (n=9)
CTL Bottom (n=9)
TYRO3 Low
CTL Top (n=29)
CTL Bottom (n=28)
DFS(days)
F
200x
400x
p-TYRO3
PR
(H score 10)
SD
(H score 60)
PD
(H score 270)
*p = 0.0356
H-score of p-TYRO3
PR+SD
PD

Supplemental Figure 1. TYRO3 expression is correlated with anti-PD-1/PD-L1 resistance.

(**A**) Relative expression of the indicated kinases in 4T1-P and 4T1-R tumor cells by RTK kinase array. (**B**) Overall survival in melanoma patients who received PD-1 antibody therapy with high and low *EPHB2*, *FLT3*, and *TRKA* mRNA expression. (**C**) Overall survival in melanoma patients who received PD-1 antibody therapy with high and low *AXL*/*MERTK* mRNA expression. (**D**) Overall survival with high and low mRNA *TYRO3* expression in breast cancer, neuroblastoma, bladder cancer, and melanoma patients. (**E**) Relationship between overall survival and cytotoxic T lymphocyte levels in breast cancer patients with high and low mRNA *TYRO3* expression. (**F**) IHC staining of p-TYRO3 in lung cancer patients who received anti-PD-1/PD-L1 therapy. Resistant cases n = 12. Non-resistant cases n = 17. *p = 0.0356, two-way unpaired t-test. Scale bars: left 50μm, right 25μm.

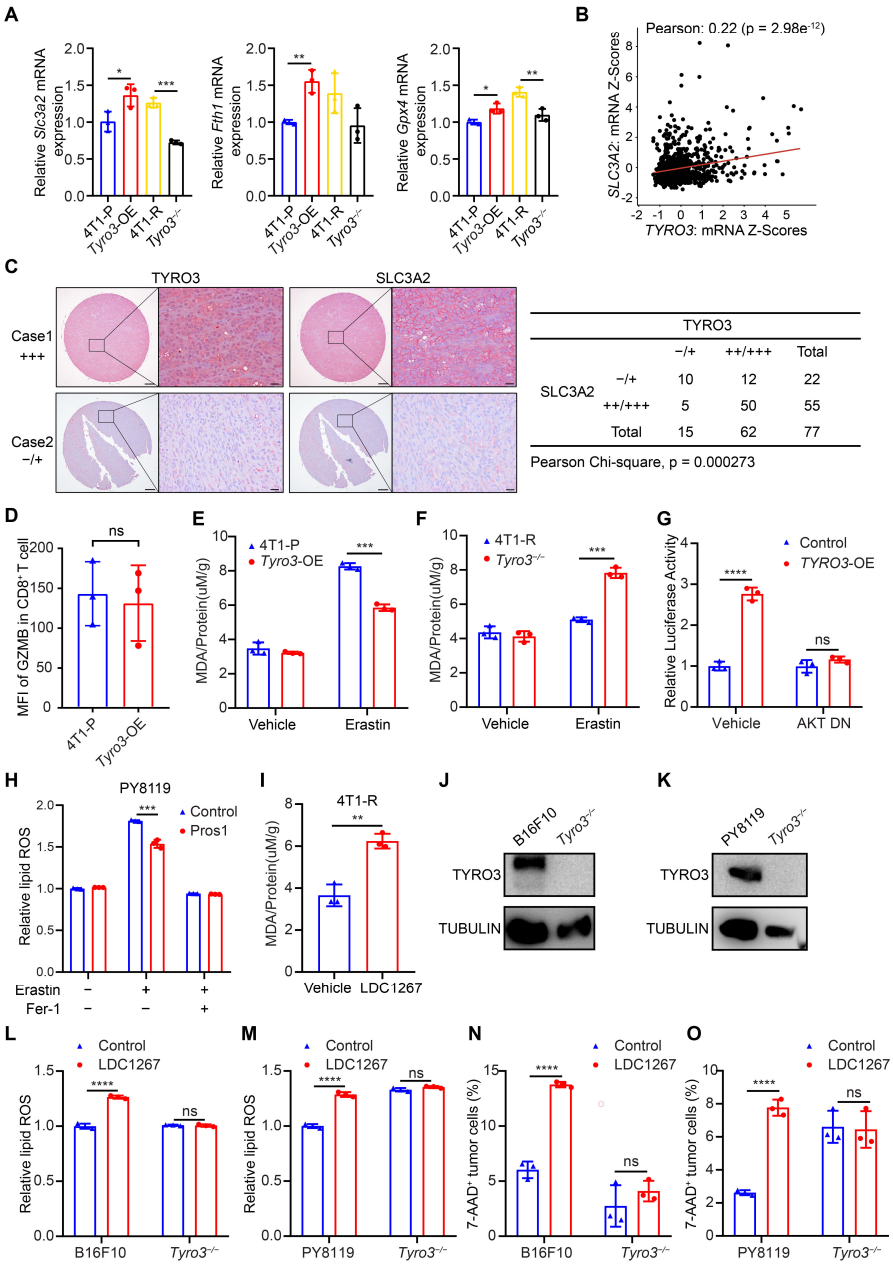


Supplemental Figure 2. Cell proliferation assay and pathways downstream of TYRO3. (**A**) Cell proliferation of *Tyro3*-OE and *Tyro3*$^{-/-}$ cells compared with their respective control cells, n = 3. NS p = 0.63, ns p = 0.72, two-way ANOVA. (**B**) Carboxyfluorescein succinimidyl ester (CFSE) staining of 4T-1P, *Tyro3*-OE, 4T1-R, and *Tyro3*$^{-/-}$ cells. Samples were stained on day 1 and collected on day 3 followed by flow cytometric analysis.



Supplemental Figure 3. TYRO3 favors anti-inflammatory tumor microenvironment. (**A**) Heat map showing normalized marker expression of each immune cell cluster from mass cytometric analysis. (**B**) Relative MFI of PD-L1 in tumor cells from anti-PD-1 treated 4T1-P and *Tyro3*-OE tumors, ns p = 0.96, two-way unpaired t-test. (**C**) Relative MFI of Caspase-3 in tumor cells from anti-PD-1 treated 4T1-P and *Tyro3*-OE tumors. Ns p = 0.356, two-way unpaired t-test.

(**D**) Relative mRNA expression of M1/M2 macrophage marker, *HLADRA1* *p = 0.02, *MRC1* **p = 0.001, *ARG1* *p = 0.011, *IL10* *p = 0.046, n = 2, two-tailed unpaired t-test. (**E**) Relative MFI of CD11c and CD206 in bone marrow–derived macrophages (BMDMs) cultured in the conditioned medium (CM) from 4T-1P or *Tyro3*-OE tumor cells, n = 3. CD11c **p = 0.0072, CD206 **p = 0.0078, and (**F**) in BMDMs cultured in the CM from 4T1-R/*Tyro3*$^{-/-}$ cells, n = 3. CD11c, ns p = 0.123, CD206 **p = 0.0073. Two-tailed unpaired t-test. (**G**) Relative *VEGF* mRNA expression in 4T1-P and *Tyro3*-OE cells, n = 3. ***p = 0.0003, and in (**H**) BT549 and *TYRO3*-OE cells, n = 3. ***p = 0.0008. Two-tailed unpaired t-test. (**I**) Relative mRNA expression of M1/M2 macrophage marker, *CXCL10* **p = 0.002, *CXCL10* plus axitinib ns p = 0.39, *HLADRA1* *p = 0.019, *HLADRA1* plus axitinib ns p = 0.5, *IL6* *p = 0.03, *IL6* plus axitinib ns p = 0.26; *MRC1* **p = 0.002, *MRC1* plus axitinib ns p = 0.47, *ARG1* **p = 0.003, *ARG1* plus axitinib *p = 0.047, *IL10* *p = 0.02, *IL10* plus axitinib ns p = 0.6; n = 3, two-tailed unpaired t-test.



Supplemental Figure 4. TYRO3 inhibits tumor cell ferroptosis. (**A**) Relative mRNA expression of *Slc3a2*, *Fth1* and *Gpx4* in *Tyro3*-OE and *Tyro3*$^{-/-}$ cells, n = 3. *Slc3a2*: 4T1-P vs. *Tyro3*-OE *p = 0.03, 4T1-R vs *Tyro3*$^{-/-}$, ***p = 0.0002; *Fth1*: 4T1-P vs. *Tyro3*-OE, **p = 0.003; *Gpx4*: 4T1-P vs. *Tyro3*-OE *p = 0.014, 4T1-R vs *Tyro3*$^{-/-}$, **p = 0.006, two-tailed unpaired t-test. (**B**) Co-expression of *SLC3A2* and *TYRO3* in breast cancer patients, Pearson = 0.22, ****p = 2.98e-

[12]. (**C**) IHC staining of SLC3A2 and TYRO3 in melanoma tissue array, n = 77. Pearson Chi-square, ***p = 0.000273. Scale bars: left 200μm, right 20μm. (**D**) MFI of Granzyme B in $CD8^+$ T cells in anti-PD-1 treated 4T1-P and *Tyro3*-OE tumors, n = 3. Ns p = 0.75, two-tailed unpaired t-test. (**E**) MDA concentration in 4T1-P and *Tyro3*-OE cells, cells were treated by 10uM erastin for 24 hours. MDA content was normalized to protein concentration, n = 3. ***p = 0.0001, two-tailed unpaired t-test. (**F**) MDA concentration in 4T1-R and $Tyro3^{-/-}$ cells, n = 3. ***p = 0.00013, two-tailed unpaired t-test. (**G**) Dual-luciferase reporter assay of the ARE-reporter. Cells were transfected with *TYRO3* overexpression plasmid, with or without AKT dominant-negative (AKT-DN) plasmid, n = 3. ****p < 0.0001, ns p = 0.16, two-tailed unpaired t-test. (**H**) Relative lipid ROS in PY8119 cells that were primed with or without 200 nM Pros1 for 24h then treated with 2μM erastin and/or 5μM fer-1 for 8h, n = 3. ***p = 0.0006, two-tailed unpaired t-test. (**I**) Effect of LDC1267 on MDA concentration in 4T1-R cells. Cells were treated by 5μM LDC1267 for 24h, n = 3. **p = 0.002, two-tailed unpaired t-test. (**J**) Western blot analysis of TYRO3 expression in B16F10 and $Tyro3^{-/-}$ cells; and in (**K**) PY8119 and $Tyro3^{-/-}$ cells. TUBULIN was used as a loading control. (**L**) Relative lipid ROS in B16F10 or $Tyro3^{-/-}$ cells treated with 5μM LDC1267 for 12h, n = 3. ****p < 0.0001, ns p = 0.67; two-tailed unpaired t-test. (**M**) Relative lipid ROS in PY8119 or $Tyro3^{-/-}$ cells treated with 5μM LDC1267 for 12h, n = 3. ****p < 0.0001, ns p = 0.08; two-tailed unpaired t-test. (**N**) The percentage of $7\text{-}AAD^+$ cells in B16F10 cells or $Tyro3^{-/-}$ cells treated with 5μM LDC1267 for 24h, n = 3. ****p < 0.0001, ns p = 0.33; two-tailed unpaired t-test. (**O**) The percentage of $7\text{-}AAD^+$ cells in PY8119 cells or $Tyro3^{-/-}$ cells treated with 5μM LDC1267 for 24h, n = 3. ****p < 0.0001, ns p = 0.85; two-tailed unpaired t-test.