Figure S1

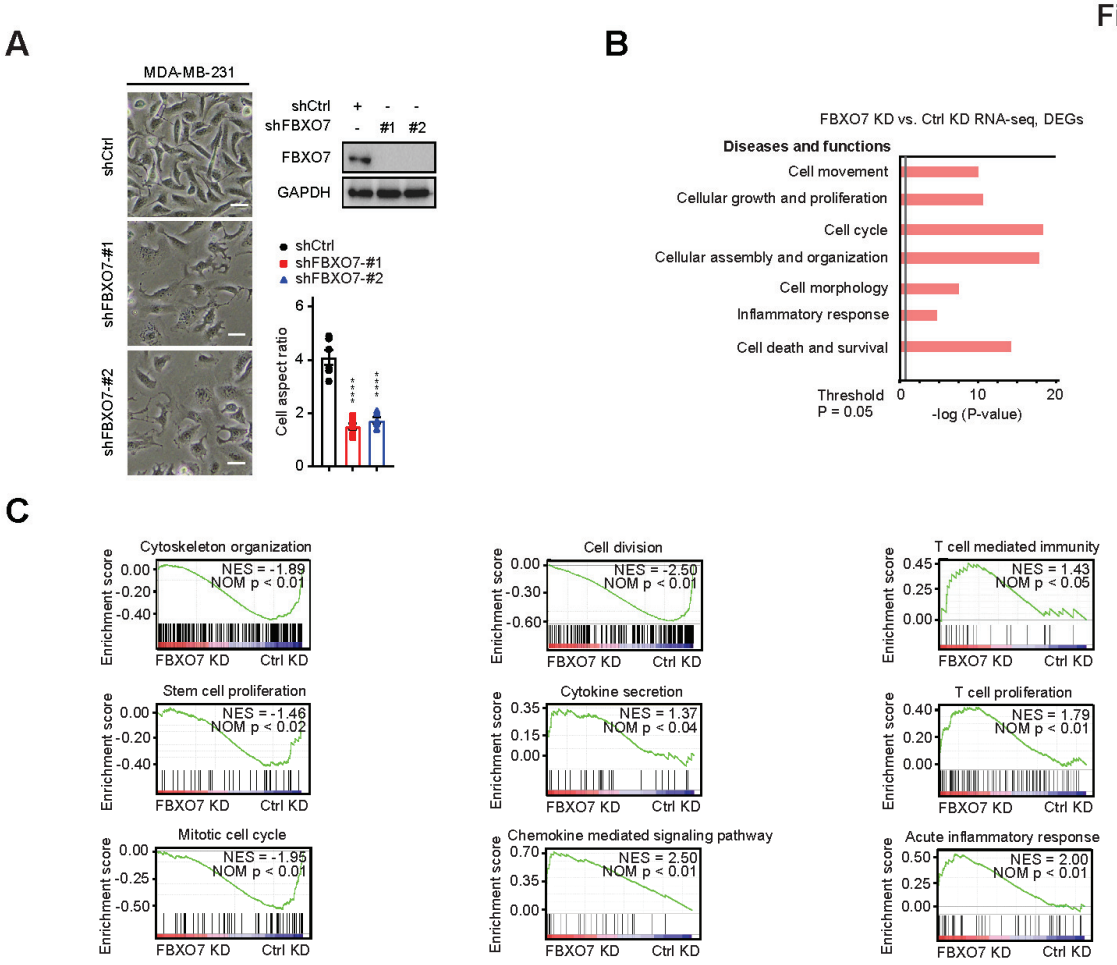
A
MDA-MB-231
shCtrl
shFBXO7-#1
shFBXO7-#2
shCtrl + - -
shFBXO7 - #1 #2
FBXO7
GAPDH
shCtrl
shFBXO7-#1
shFBXO7-#2
Cell aspect ratio
****
****
B
FBXO7 KD vs. Ctrl KD RNA-seq, DEGs
Diseases and functions
Cell movement
Cellular growth and proliferation
Cell cycle
Cellular assembly and organization
Cell morphology
Inflammatory response
Cell death and survival
Threshold
P = 0.05
-log (P-value)
C
Cytoskeleton organization
NES = -1.89
NOM p < 0.01
Cell division
NES = -2.50
NOM p < 0.01
T cell mediated immunity
NES = 1.43
NOM p < 0.05
Stem cell proliferation
NES = -1.46
NOM p < 0.02
Cytokine secretion
NES = 1.37
NOM p < 0.04
T cell proliferation
NES = 1.79
NOM p < 0.01
Mitotic cell cycle
NES = -1.95
NOM p < 0.01
Chemokine mediated signaling pathway
NES = 2.50
NOM p < 0.01
Acute inflammatory response
NES = 2.00
NOM p < 0.01
Enrichment score
FBXO7 KD
Ctrl KD

Figure S1. FBXO7 maintains mesenchymal and immune evasion phenotypes of cancer cells, related to Figure 1.
(A) Representative images of control and FBXO7 KD MDA-MB-231 cells (left panels). Scale bar, 10 μm. Immunoblots of indicated proteins and quantification of cell aspect ratio (right panels). GAPDH, loading control. Six cells from 3 different fields (2 for each) were randomly chosen for each sample (n = 6).
(B) Functional analysis of differentially expressed genes (DEGs) from FBXO7 KD vs. control KD RNA-seq in MDA-MB-231 cells.
(C) GSEA enrichment plots for select gene sets by RNA-seq (FBXO7 KD vs. control KD). NES and p values are shown.
Data represent mean ± SEM. ****$p < 0.0001$; one-way ANOVA followed by Tukey's multiple comparisons test (A).

Figure S2

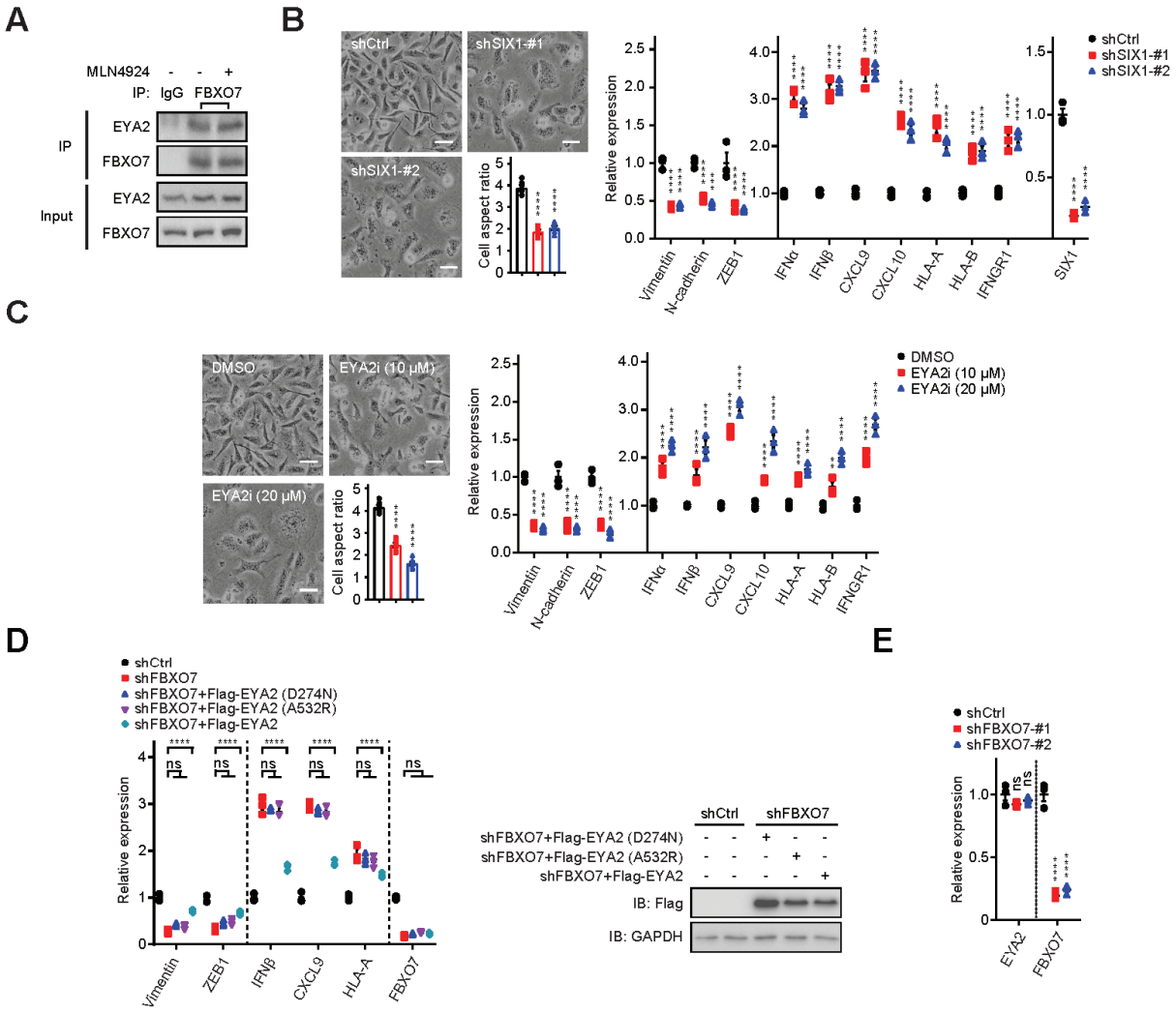
A
MLN4924 - - +
IP: IgG FBXO7
IP
EYA2
FBXO7
Input
EYA2
FBXO7
B
shCtrl
shSIX1-#1
shSIX1-#2
Cell aspect ratio
Relative expression
shCtrl
shSIX1-#1
shSIX1-#2
Vimentin
N-cadherin
ZEB1
IFNα
IFNβ
CXCL9
CXCL10
HLA-A
HLA-B
IFNGR1
SIX1
C
DMSO
EYA2i (10 μM)
EYA2i (20 μM)
Cell aspect ratio
Relative expression
DMSO
EYA2i (10 μM)
EYA2i (20 μM)
Vimentin
N-cadherin
ZEB1
IFNα
IFNβ
CXCL9
CXCL10
HLA-A
HLA-B
IFNGR1
D
shCtrl
shFBXO7
shFBXO7+Flag-EYA2 (D274N)
shFBXO7+Flag-EYA2 (A532R)
shFBXO7+Flag-EYA2
Relative expression
ns
Vimentin
ZEB1
IFNβ
CXCL9
HLA-A
FBXO7
shCtrl
shFBXO7
shFBXO7+Flag-EYA2 (D274N)
shFBXO7+Flag-EYA2 (A532R)
shFBXO7+Flag-EYA2
IB: Flag
IB: GAPDH
E
shCtrl
shFBXO7-#1
shFBXO7-#2
Relative expression
ns
EYA2
FBXO7

Figure S2. FBXO7 binds and stabilizes SIX1 co-transcriptional regulator EYA2, related to Figure 2.
(A) Co-IP of endogenous FBXO7 with EYA2 in HEK293T cells treated with vehicle or NEDDylation inhibitor MLN4924. IgG, IP control.
(B) Representative images (left panels) and RT-qPCR analysis of the indicated genes (right panel) in control and SIX1 KD MDA-MB-231 cells (n = 3). Scale bar, 100 μm. Cell aspect ratio (left panel). Six cells from 3 different fields (2 for each) were randomly chosen (n = 6).
(C) Representative images (left panels) and RT-qPCR analysis of the indicated genes (right panel) in vehicle or EYA2i-treated MDA-MB-231 cells (n = 3). Scale bar, 100 μm. Cell aspect ratio (left panel). Six cells from 3 different fields (2 for each) were randomly chosen (n = 6).
(D) RT-qPCR analysis of the indicated genes in control or FBXO7 KD MDA-MB-231 cells expressing EYA2, $EYA2^{D274N}$, or $EYA2^{A532R}$ (left panel) (n = 3). Immunoblots of indicated proteins (right panel). GAPDH, loading control.
(E) RT-qPCR analysis of the indicated genes in control or FBXO7 KD MDA-MB-231 cells (n = 3).
Data represent mean ± SEM. ns, not significant; **p < 0.01; ***p < 0.001; ****p < 0.0001 by one-way ANOVA followed by Tukey's multiple comparisons test (B and C, right panels), and two-way ANOVA followed by Tukey's multiple comparisons test (B and C, left panels; D, and E).

Figure S3

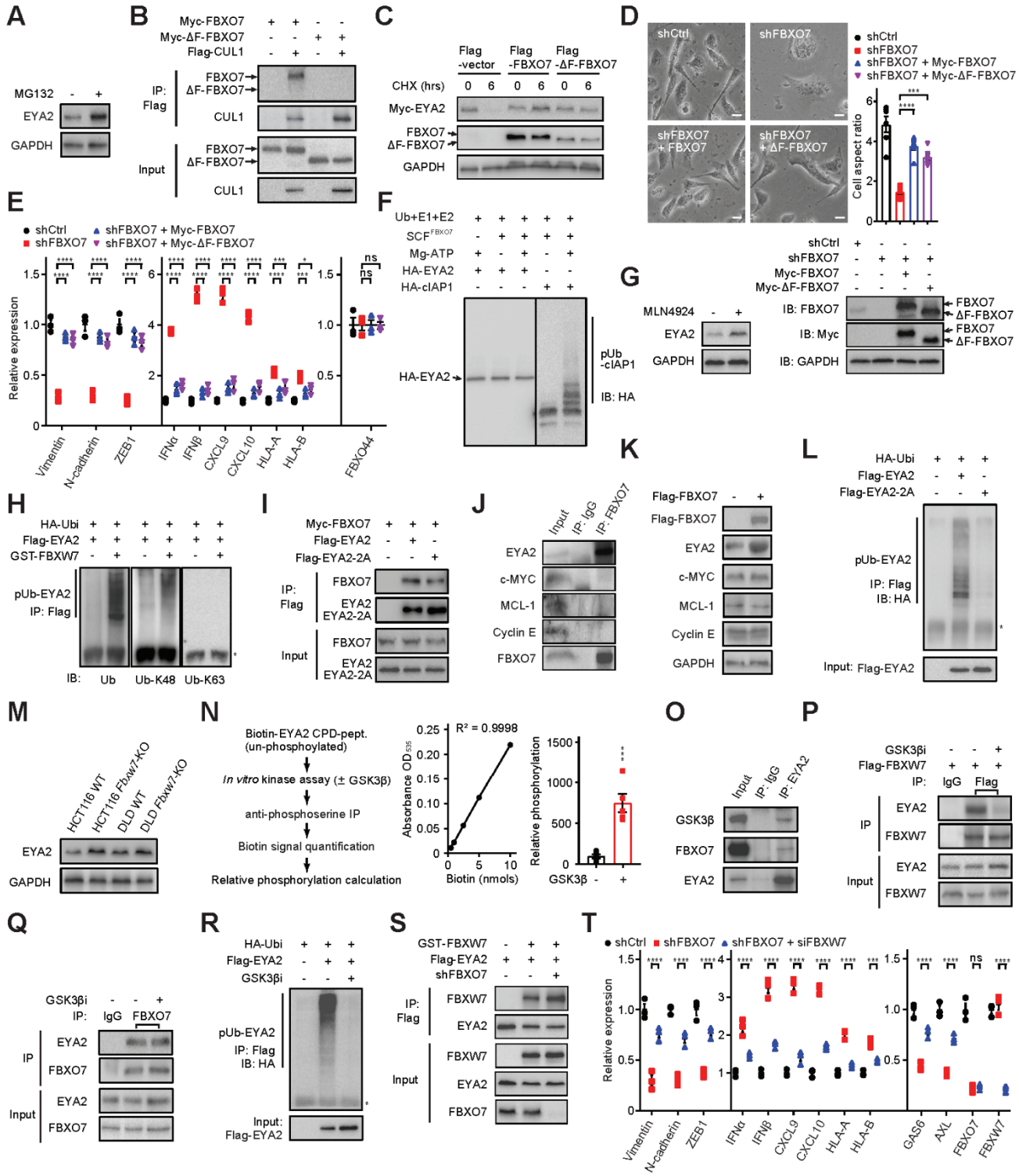
A
MG132 - +
EYA2
GAPDH
B
Myc-FBXO7 + + - -
Myc-ΔF-FBXO7 - - + +
Flag-CUL1 - + - +
IP: Flag
FBXO7
ΔF-FBXO7
CUL1
Input
FBXO7
ΔF-FBXO7
CUL1
C
Flag -vector
Flag -FBXO7
Flag -ΔF-FBXO7
CHX (hrs) 0 6 0 6 0 6
Myc-EYA2
FBXO7
ΔF-FBXO7
GAPDH
D
shCtrl
shFBXO7
shFBXO7 + FBXO7
shFBXO7 + ΔF-FBXO7
shCtrl
shFBXO7
shFBXO7 + Myc-FBXO7
shFBXO7 + Myc-ΔF-FBXO7
Cell aspect ratio
E
shCtrl
shFBXO7
shFBXO7 + Myc-FBXO7
shFBXO7 + Myc-ΔF-FBXO7
Relative expression
Vimentin
N-cadherin
ZEB1
IFNα
IFNβ
CXCL9
CXCL10
HLA-A
HLA-B
FBXO44
F
Ub+E1+E2 + + + + +
SCF FBXO7 - + + + +
Mg-ATP + - + - +
HA-EYA2 + + + - -
HA-cIAP1 - - - + +
HA-EYA2
pUb -cIAP1
IB: HA
G
shCtrl +
shFBXO7 - + + +
Myc-FBXO7 - - + -
Myc-ΔF-FBXO7 - - - +
MLN4924 - +
EYA2
GAPDH
IB: FBXO7
IB: Myc
IB: GAPDH
FBXO7
ΔF-FBXO7
FBXO7
ΔF-FBXO7
H
HA-Ubi + + + + + +
Flag-EYA2 + + + + + +
GST-FBXW7 - + - + - +
pUb-EYA2
IP: Flag
IB: Ub Ub-K48 Ub-K63
I
Myc-FBXO7 + + +
Flag-EYA2 - + -
Flag-EYA2-2A - - +
IP: Flag
FBXO7
EYA2 EYA2-2A
Input
FBXO7
EYA2 EYA2-2A
J
Input
IP: IgG
IP: FBXO7
EYA2
c-MYC
MCL-1
Cyclin E
FBXO7
K
Flag-FBXO7 - +
Flag-FBXO7
EYA2
c-MYC
MCL-1
Cyclin E
GAPDH
L
HA-Ubi + + +
Flag-EYA2 - + -
Flag-EYA2-2A - - +
pUb-EYA2
IP: Flag
IB: HA
Input: Flag-EYA2
M
HCT116 WT
HCT116 Fbxw7-KO
DLD WT
DLD Fbxw7-KO
EYA2
GAPDH
N
Biotin-EYA2 CPD-pept. (un-phosphoylated)
In vitro kinase assay (± GSK3β)
anti-phosphoserine IP
Biotin signal quantification
Relative phosphorylation calculation
R² = 0.9998
Absorbance OD595
Biotin (nmols)
Relative phosphorylation
GSK3β - +
O
Input
IP: IgG
IP: EYA2
GSK3β
FBXO7
EYA2
P
GSK3βi - - +
Flag-FBXW7 + + +
IP: IgG Flag
IP
EYA2
FBXW7
Input
EYA2
FBXW7
Q
GSK3βi - - +
IP: IgG FBXO7
IP
EYA2
FBXO7
Input
EYA2
FBXO7
R
HA-Ubi + + +
Flag-EYA2 - + +
GSK3βi - - +
pUb-EYA2
IP: Flag
IB: HA
Input: Flag-EYA2
S
GST-FBXW7 - + +
Flag-EYA2 + + +
shFBXO7 - - +
IP: Flag
FBXW7
EYA2
Input
FBXW7
EYA2
FBXO7
T
shCtrl
shFBXO7
shFBXO7 + siFBXW7
Relative expression
Vimentin
N-cadherin
ZEB1
IFNα
IFNβ
CXCL9
CXCL10
HLA-A
HLA-B
GAS6
AXL
FBXO7
FBXW7

Figure S3. FBXO7 protects EYA2 from SCF^{FBXW7}-dependent degradation, related to Figure 3.

(A) Immunoblot of EYA2 in HEK293T cells treated with vehicle or 26S proteasome inhibitor MG132. GAPDH, loading control.

(B) Co-IP of Flag-CUL1 with Myc-FBXO7 or Myc-ΔF-FBXO7 in MDA-MB-231 cells.

(C) Myc-EYA2 stability in MDA-MB-231 cells expressing control, WT FBXO7, or ΔF-FBXO7. GAPDH, loading control.

(D) Representative images of control or FBXO7 KD MDA-MB-231 cells expressing Myc-FBXO7 or Myc-ΔF-FBXO7 (left panels). Cell aspect ratio (top right panel). Six cells from 3 different fields (2 for each) were randomly chosen (n = 6). Immunoblots of indicated proteins (bottom right panel). GAPDH, loading control.

(E) RT-qPCR analysis of the indicated genes in control or FBXO7 KD MDA-MB-231 cells expressing Myc-FBXO7 or Myc-ΔF-FBXO7 (n = 3). FBXO44, control.

(F) *In vitro* ubiquitylation reactions of SCF^{FBXO7} with HA-EYA2. HA-cIAP, positive control.

(G) Immunoblot of EYA2 in HEK293T cells treated with vehicle or MLN4924. GAPDH, loading control.

(H) *In vivo* ubiquitylation reactions of HEK293T cells transfected with Flag-EYA2 and GST-FBXW7 vectors and probed with the indicated anti-ubiquitin antibodies. *, IgG heavy chain.

(I) Co-IP of Flag-EYA2 or Flag-EYA2-2A with Myc-FBXO7 in MDA-MB-231 cells.

(J) Co-IP of endogenous FBXO7 with EYA2 and known SCF^{FBXW7} substrates in HEK293T cells. IgG, IP control.

(K) Immunoblots of EYA2 and SCF^{FBXW7} substrates in HEK293T cells expressing control or Flag-FBXO7. GAPDH, loading control.

(L) *In vivo* ubiquitylation reactions of Flag-EYA2 and Flag-EYA2-2A in HEK293T cells. *, IgG heavy chain.

(M) Immunoblot of endogenous EYA2 in the indicated WT and *Fbxw7* KO colorectal cancer cells. GAPDH, loading control.

(N) Schematic (left panel), standard curve (middle panel), and quantification (right panel) of *in vitro* kinase assays for EYA2 CPD peptide incubated with/without recombinant GSK3β (n = 5).

(O) Co-IP of endogenous EYA2 with GSK3β in HEK293T cells. FBXO7, positive control. IgG, IP control.

(P and Q) Co-IP of Flag-FBXW7 (P) or endogenous FBXO7 (Q) with EYA2 in HEK293T cells treated with vehicle or GSK3βi. IgG, IP control.

(R) Immunoblot of pUb Flag-EYA2 in HEK293T cells treated with vehicle or GSK3βi. *, IgG heavy chain.

(S) Co-IP of Flag-EYA2 with GST-FBXW7 in control and FBXO7 KD MDA-MB-231 cells.

(T) RT-qPCR analysis of the indicated genes in control, FBXO7 KD, or FBXO7 + FBXW7 KD MDA-MB-231 cells (n = 3).

Data represent mean ± SEM. ns, not significant; *p < 0.05; ***p < 0.001; ****p < 0.0001 by one-way ANOVA followed by Tukey's multiple comparisons test (D), two-way ANOVA followed by Tukey's multiple comparisons test (E and T), and unpaired Student's *t*-test (N).

Figure S4

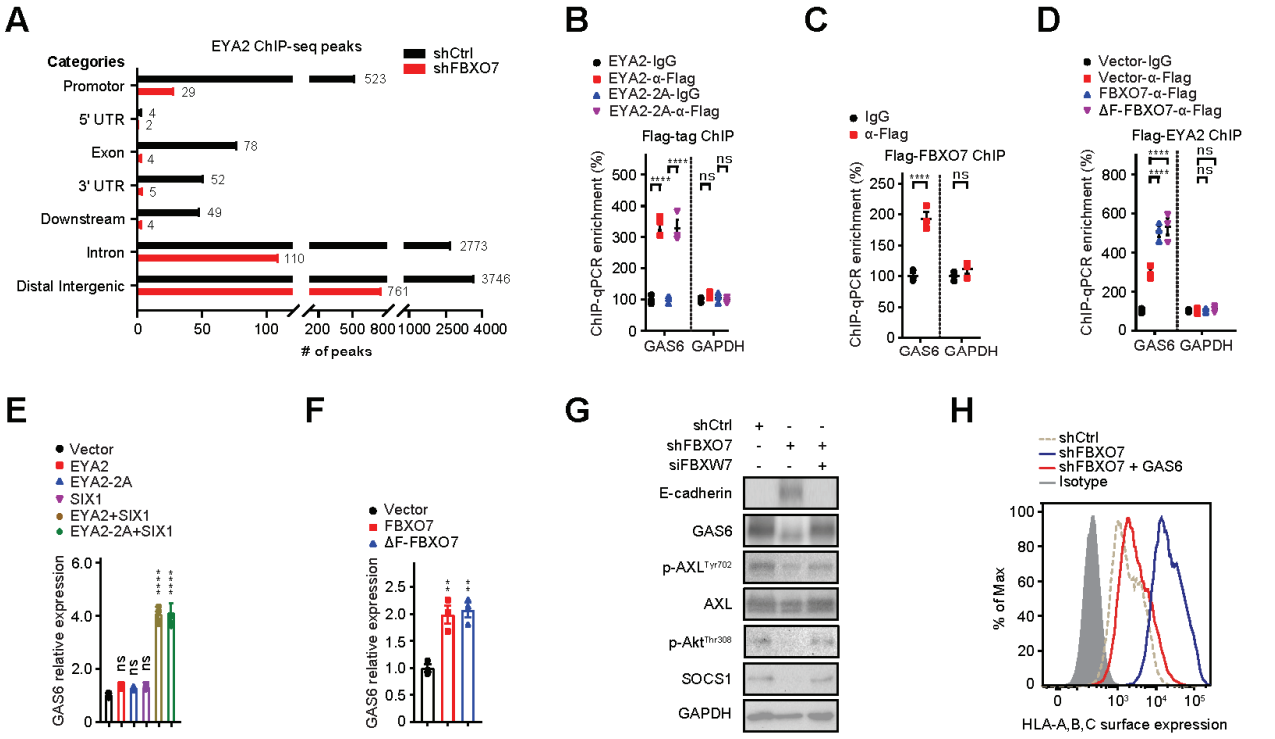
A
EYA2 ChIP-seq peaks
Categories
shCtrl
shFBXO7
Promotor
5' UTR
Exon
3' UTR
Downstream
Intron
Distal Intergenic
523
29
4
2
78
4
52
5
49
4
2773
110
3746
761
0
50
100
200
500
800
1000
2500
4000
# of peaks
B
EYA2-IgG
EYA2-α-Flag
EYA2-2A-IgG
EYA2-2A-α-Flag
Flag-tag ChIP
ChIP-qPCR enrichment (%)
GAS6
GAPDH
C
IgG
α-Flag
Flag-FBXO7 ChIP
ChIP-qPCR enrichment (%)
GAS6
GAPDH
D
Vector-IgG
Vector-α-Flag
FBXO7-α-Flag
ΔF-FBXO7-α-Flag
Flag-EYA2 ChIP
ChIP-qPCR enrichment (%)
GAS6
GAPDH
E
Vector
EYA2
EYA2-2A
SIX1
EYA2+SIX1
EYA2-2A+SIX1
GAS6 relative expression
F
Vector
FBXO7
ΔF-FBXO7
GAS6 relative expression
G
shCtrl
shFBXO7
siFBXW7
E-cadherin
GAS6
p-AXL
AXL
p-Akt
SOCS1
GAPDH
H
shCtrl
shFBXO7
shFBXO7 + GAS6
Isotype
% of Max
HLA-A,B,C surface expression

Figure S4. FBXO7 promotes EYA2/SIX1-mediated induction of AXL ligand GAS6, related to Figure 4.
(A) EYA2 ChIP-seq peaks in MDA-MB-231 cells by genomic features.
(B) ChIP analysis of Flag-EYA2 or Flag-EYA2-2A binding the *Gas6* promoter in MDA-MB-231 cells (n = 3). GAPDH, control. ChIP performed with anti-Flag antibody. IgG, IP control.
(C) ChIP analysis of Flag-FBXO7 binding to *Gas6* promoter in MDA-MB-231 cells (n = 3). GAPDH, control. ChIP performed with anti-Flag antibody. IgG, IP control.
(D) ChIP analysis of Flag-EYA2 binding the *Gas6* promoter in MDA-MB-231 cells expressing FBXO7 or ΔF-FBXO7 (n = 3). GAPDH, control. ChIP performed with anti-Flag antibody. IgG, IP control.
(E and F) RT-qPCR analysis of GAS6 in MCF7 cells transfected with the indicated vectors (n = 3).
(G) Immunoblots of AXL signaling proteins in control or FBXO7 KD MDA-MB-231 cells transfected with control or FBXW7 siRNAs. GAPDH, loading control.
(H) Flow cytometry analysis of HLA-A/B/C surface expression on control, FBXO7 KD, and FBXO7 KD + GAS6 MDA-MB-231 cells.
Data represent mean ± SEM. ns, not significant; **p < 0.01; ****p < 0.0001 by one-way ANOVA followed by Dunnett's multiple comparisons test (E and F), two-way ANOVA followed by Tukey's multiple comparisons test (B and D), and Sidak's multiple comparisons test (C).

Figure S5

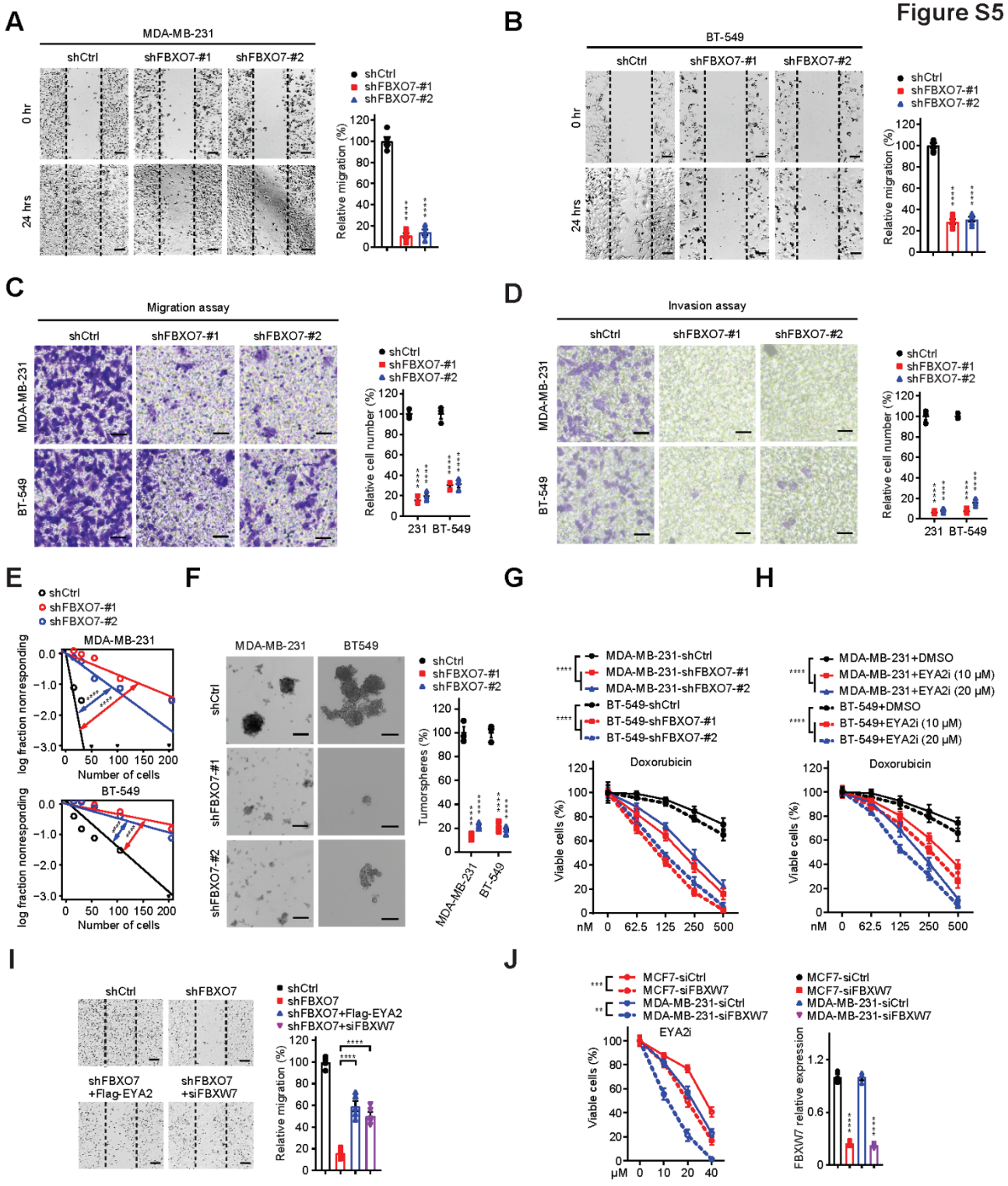
A
MDA-MB-231
shCtrl
shFBXO7-#1
shFBXO7-#2
0 hr
24 hrs
Relative migration (%)
B
BT-549
C
Migration assay
MDA-MB-231
BT-549
Relative cell number (%)
231 BT-549
D
Invasion assay
E
log fraction nonresponding
Number of cells
F
Tumorspheres (%)
G
MDA-MB-231-shCtrl
MDA-MB-231-shFBXO7-#1
MDA-MB-231-shFBXO7-#2
BT-549-shCtrl
BT-549-shFBXO7-#1
BT-549-shFBXO7-#2
Doxorubicin
Viable cells (%)
nM 0 62.5 125 250 500
H
MDA-MB-231+DMSO
MDA-MB-231+EYA2i (10 μM)
MDA-MB-231+EYA2i (20 μM)
BT-549+DMSO
BT-549+EYA2i (10 μM)
BT-549+EYA2i (20 μM)
I
shFBXO7
shFBXO7+Flag-EYA2
shFBXO7+siFBXW7
J
MCF7-siCtrl
MCF7-siFBXW7
MDA-MB-231-siCtrl
MDA-MB-231-siFBXW7
EYA2i
μM 0 10 20 40
FBXW7 relative expression

Figure S5. Targeting FBXO7/EYA2 decreases mesenchymal-associated phenotypes of cancer cells *in vitro*, related to Figure 5.

(A and B) Gap closure assays of control and FBXO7 KD MDA-MB-231 (A) and BT-549 (B) cells (left panels). Quantification of migration (right panels). Five fields were randomly chosen and quantified (n = 5).

(C and D) Boyden chamber assays of migration (C) and invasion (D) for the indicated control and FBXO7 KD cells (left panels). Quantification of data (right panels). Three fields were randomly chosen and quantified (n = 3).

(E) ELDA for indicated control and FBXO7 KD cells (n = 3).

(F) Representative images (left panels) and quantification (right panel) of tumorspheres of the indicated control and FBXO7 KD cells (n = 3). Scale bar, 50 μm.

(G and H) Viabilities of the indicated cells expressing control or FBXO7 shRNA (G) or treated with vehicle or EYA2i (H) upon doxorubicin treatment for 72 hrs (n = 3).

(I) Gap closure assays of control or FBXO7 KD MDA-MB-231 cells expressing Flag-EYA2 or transfected with FBXW7 siRNA (left panels). Quantification of migration (right panel). Five fields were randomly chosen and quantified (n = 5).

(J) Viabilities of the indicated control and FBXW7 KD cells treated with EYA2i (left panel). RT-qPCR analysis of FBXW7 (right panel) (n = 3).

Data represent mean ± SEM. **p < 0.01; ***p < 0.001; ****p < 0.0001 by one-way ANOVA followed by Dunnett's multiple comparisons test (A and B), Tukey's multiple comparisons test (I; J, right panel), two-way ANOVA followed by Dunnett's multiple comparisons test (C and D), Tukey's multiple comparisons test (F, G, H; J, left panel), and χ^2 test for pair-wise differences (E).

Figure S6

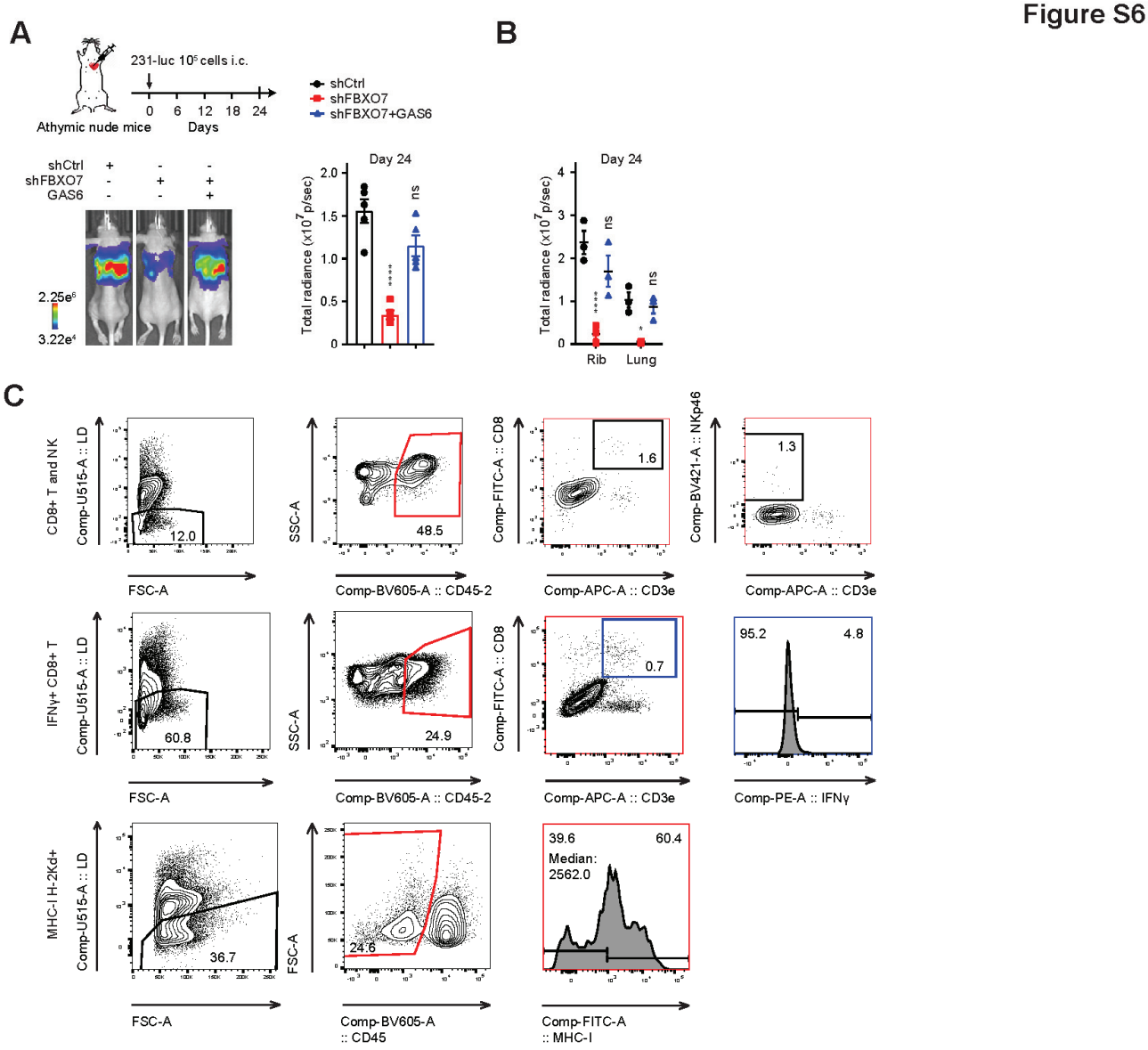
A
231-luc 10^5 cells i.c.
0 6 12 18 24
Athymic nude mice
Days
shCtrl
shFBXO7
shFBXO7+GAS6
shCtrl + - -
shFBXO7 - + +
GAS6 - - +
2.25e6
3.22e4
Day 24
Total radiance (x10^7 p/sec)
ns
****
B
Day 24
Total radiance (x10^7 p/sec)
ns
****
*
Rib
Lung
C
CD8+ T and NK
Comp-U515-A :: LD
12.0
FSC-A
SSC-A
48.5
Comp-BV605-A :: CD45-2
Comp-FITC-A :: CD8
1.6
Comp-APC-A :: CD3e
Comp-BV421-A :: NKp46
1.3
Comp-APC-A :: CD3e
IFNγ+ CD8+ T
Comp-U515-A :: LD
60.8
FSC-A
SSC-A
24.9
Comp-BV605-A :: CD45-2
Comp-FITC-A :: CD8
0.7
Comp-APC-A :: CD3e
95.2
4.8
Comp-PE-A :: IFNγ
MHC-I H-2Kd+
Comp-U515-A :: LD
36.7
FSC-A
FSC-A
24.6
Comp-BV605-A :: CD45
39.6
60.4
Median: 2562.0
Comp-FITC-A :: MHC-I

Figure S6. FBXO7/EYA2 inhibition attenuates tumor growth, boosts antitumor immunity, and enhances ICB therapy response, related to Figure 6.

(A and B) Representative bioluminescence images of immunocompromised mice 24 days post-intracardiac injection with control, FBXO7 KD, and FBXO7 KD + GAS6 MDA-MB-231-*luc* cells (A, bottom left panel). Schematic of injection (A, top left panel). Plot of total body radiance (A, right panel) (n = 5). Plot of organ radiance (B) (n = 3).

(C) Flow cytometry gating strategies for detection of tumor-infiltrating $CD8^+$ T and NK cells (top panels), $IFN\gamma^+CD8^+$ T cells (middle panels), and MHC-I $H\text{-}2Kd^+$ 4T1-*luc* tumor cells (bottom panels).

Data represent mean ± SEM. ns, not significant; *p < 0.05; ****p < 0.0001 by one-way ANOVA followed by Tukey's multiple comparisons test (A), and Tukey's multiple comparisons test (B).

Figure S7

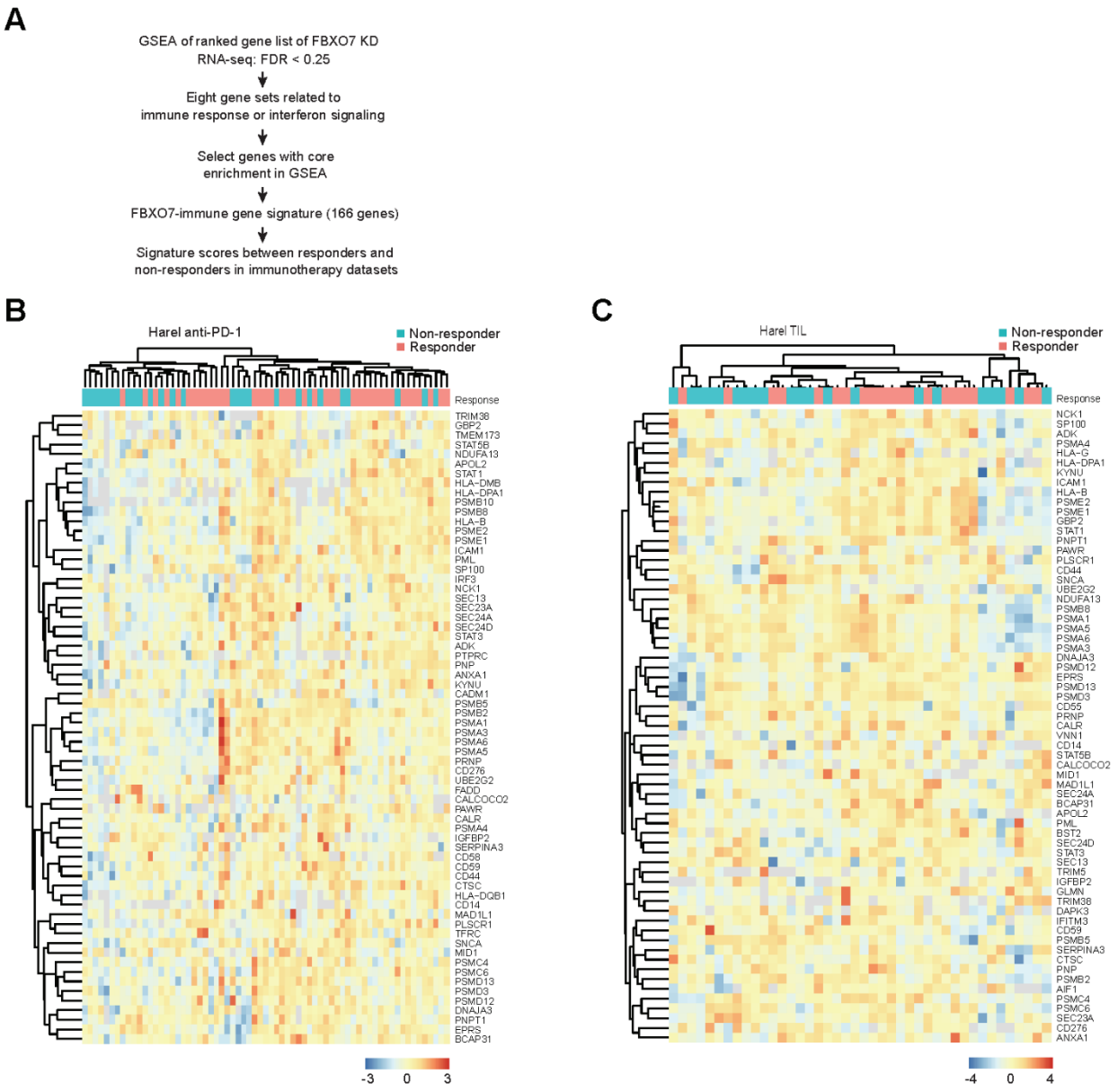
A
GSEA of ranked gene list of FBXO7 KD
RNA-seq: FDR < 0.25
Eight gene sets related to
immune response or interferon signaling
Select genes with core
enrichment in GSEA
FBXO7-immune gene signature (166 genes)
Signature scores between responders and
non-responders in immunotherapy datasets
B
Harel anti-PD-1
Non-responder
Responder
Response
TRIM38
GBP2
TMEM173
STAT5B
NDUFA13
APOL2
STAT1
HLA-DMB
HLA-DPA1
PSMB10
PSMB8
HLA-B
PSME2
PSME1
ICAM1
PML
SP100
IRF3
NCK1
SEC13
SEC23A
SEC24A
SEC24D
STAT3
ADK
PTPRC
PNP
ANXA1
KYNU
CADM1
PSMB5
PSMB2
PSMA1
PSMA3
PSMA6
PSMA5
PRNP
CD276
UBE2G2
FADD
CALCOCO2
PAWR
CALR
PSMA4
IGFBP2
SERPINA3
CD58
CD59
CD44
CTSC
HLA-DQB1
CD14
MAD1L1
PLSCR1
TFRC
SNCA
MID1
PSMC4
PSMC6
PSMD13
PSMD3
PSMD12
DNAJA3
PNPT1
EPRS
BCAP31
-3
0
3
C
Harel TIL
Non-responder
Responder
Response
NCK1
SP100
ADK
PSMA4
HLA-G
HLA-DPA1
KYNU
ICAM1
HLA-B
PSME2
PSME1
GBP2
STAT1
PNPT1
PAWR
PLSCR1
CD44
SNCA
UBE2G2
NDUFA13
PSMB8
PSMA1
PSMA5
PSMA6
PSMA3
DNAJA3
PSMD12
EPRS
PSMD13
PSMD3
CD55
PRNP
CALR
VNN1
CD14
STAT5B
CALCOCO2
MID1
MAD1L1
SEC24A
BCAP31
APOL2
PML
BST2
SEC24D
STAT3
SEC13
TRIM5
IGFBP2
GLMN
TRIM38
DAPK3
IFITM3
CD59
PSMB5
SERPINA3
CTSC
PNP
PSMB2
AIF1
PSMC4
PSMC6
SEC23A
CD276
ANXA1
-4
0
4

Figure S7. FBXO7 associates with mesenchymal and immune-suppressive phenotypes in cancer patient datasets, related to Figure 7.
(A) Generation of FBXO7-immune gene signature (166 immune-related genes) based on GSEA analysis of FBXO7 KD RNA-seq data.
(B and C) Heatmaps of FBXO7-immune gene signature differentially enriched in “responder” versus “non-responder” patients in Harel anti-PD-1 (B) and Harel TIL therapies (C) datasets.