iScience, Volume *24*

Supplemental information

CHK1 inhibitor sensitizes resistant colorectal cancer stem cells to nortopsentin

Simone Di Franco, Barbara Parrino, Miriam Gaggianesi, Vincenzo Davide Pantina, Paola Bianca, Annalisa Nicotra, Laura Rosa Mangiapane, Melania Lo Iacono, Gloria Ganduscio, Veronica Veschi, Ornella Roberta Brancato, Antonino Glaviano, Alice Turdo, Irene Pillitteri, Lorenzo Colarossi, Stella Cascioferro, Daniela Carbone, Camilla Pecoraro, Micol Eleonora Fiori, Ruggero De Maria, Matilde Todaro, Isabella Screpanti, Girolamo Cirrincione, Patrizia Diana, and Giorgio Stassi

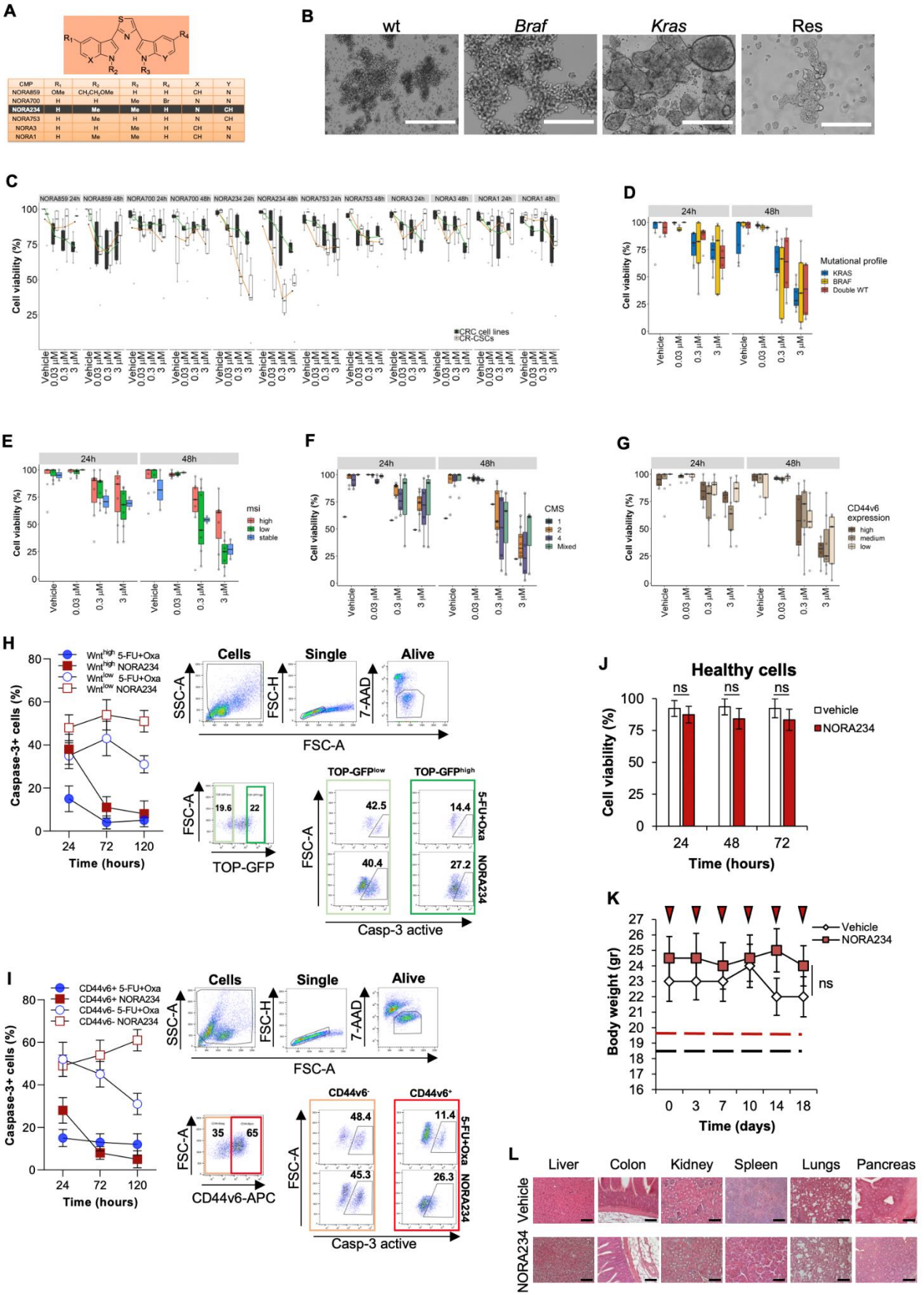
A
CMP | R1 | R2 | R3 | R4 | X | Y
NORA859 | OMe | CH2CH2OMe | H | H | CH | N
NORA700 | H | H | Me | Br | N | N
NORA234 | H | Me | Me | H | N | CH
NORA753 | H | Me | H | H | N | CH
NORA3 | H | H | Me | H | CH | N
NORA1 | H | Me | Me | H | CH | N
B
wt
Braf
Kras
Res
C
Cell viability (%)
CRC cell lines
CR-CSCs
Vehicle
0.03 µM
0.3 µM
3 µM
D
24h
48h
Cell viability (%)
Mutational profile
KRAS
BRAF
Double WT
E
msi
high
low
stable
F
CMS
1
2
4
Mixed
G
CD44v6 expression
high
medium
low
H
Wnthigh 5-FU+Oxa
Wnthigh NORA234
Wntlow 5-FU+Oxa
Wntlow NORA234
Caspase-3+ cells (%)
Time (hours)
Cells
Single
Alive
SSC-A
FSC-H
7-AAD
FSC-A
TOP-GFP
TOP-GFPlow
TOP-GFPhigh
19.6
22
42.5
40.4
14.4
27.2
5-FU+Oxa
NORA234
Casp-3 active
I
CD44v6+ 5-FU+Oxa
CD44v6+ NORA234
CD44v6- 5-FU+Oxa
CD44v6- NORA234
CD44v6-APC
CD44v6-
CD44v6+
35
65
48.4
45.3
11.4
26.3
J
Healthy cells
ns
vehicle
NORA234
Cell viability (%)
Time (hours)
K
Body weight (gr)
Time (days)
Vehicle
NORA234
L
Liver
Colon
Kidney
Spleen
Lungs
Pancreas
Vehicle
NORA234

Figure S1: NORA234 treatment impaired CRC cell proliferation, sparing healthy cells, Related to Figure 1 and Figure 2.

(A) Chemical structure of synthetic nortopsentin analogs. *(lower panel)* Table of the chemical group substitutions present in the six alkaloid derivatives.

(B) Representative contrast phase images of CR-CSphCs isolated from 4 different CRC patients (CR-CSphCs #3, #9, #21, #R4). Scale bars, 400 µm.

(C) Cell viability percentage of CRC cell lines and CR-CSphCs treated with vehicle, or the indicated concentration of NORA859, NORA700, NORA234, NORA753, NORA3 and NORA1, up to 48 hours.

(D-G) Cell viability analysis of in 29 CR-CSphC lines clustered according to the indicated mutational backgroud (D), MSI status (E), CMS profile (F), or CD44v6 expression (low ≤30%, medium 31-69% and high ≥70%) (G), treated with vehicle or the indicated concentration of NORA234 for 48 hours.

(H-I) Representative flow cytometry analysis of active Casp-3 positivity in TOP-GFP$^{high/low}$ (H), or CD44v6$^{+/-}$ (I), CR-CSphCs treated with vehicle, 5-FU in combination with oxaliplatin, or NORA234, up to 120 hours. (*right panels*) Representative gating strategies for the analysis of active Casp-3 positive cells in TOP-GFP$^{high/low}$ (H), or CD44v6$^{+/-}$ (I) CR-CSphCs, treated as previously described, for 120 hours.

(J) Cell viability percentage of healthy cells (IMEC and AD-MSCs) treated as in (D-G), up to 72 hours.Statistical significance between 2groups (n=6) was determined by unpaired Student's t-test (2-tailed).ns, non-significant.

(K) Weight of mice treated with vehicle or NORA234 (8 mg/kg) at the indicated time points (red arrow heads) up to 18 days. Data are mean ± S.D (n=3 mice for group). Statistical significance between 2 groups was determined by unpaired Student's t-test (2-tailed).ns, non-significant.

(L) Representative H&E staining of liver, colon, kidney, spleen, lungs, and pancreas of mice treated as in (K). Scale bars, 200 µm.

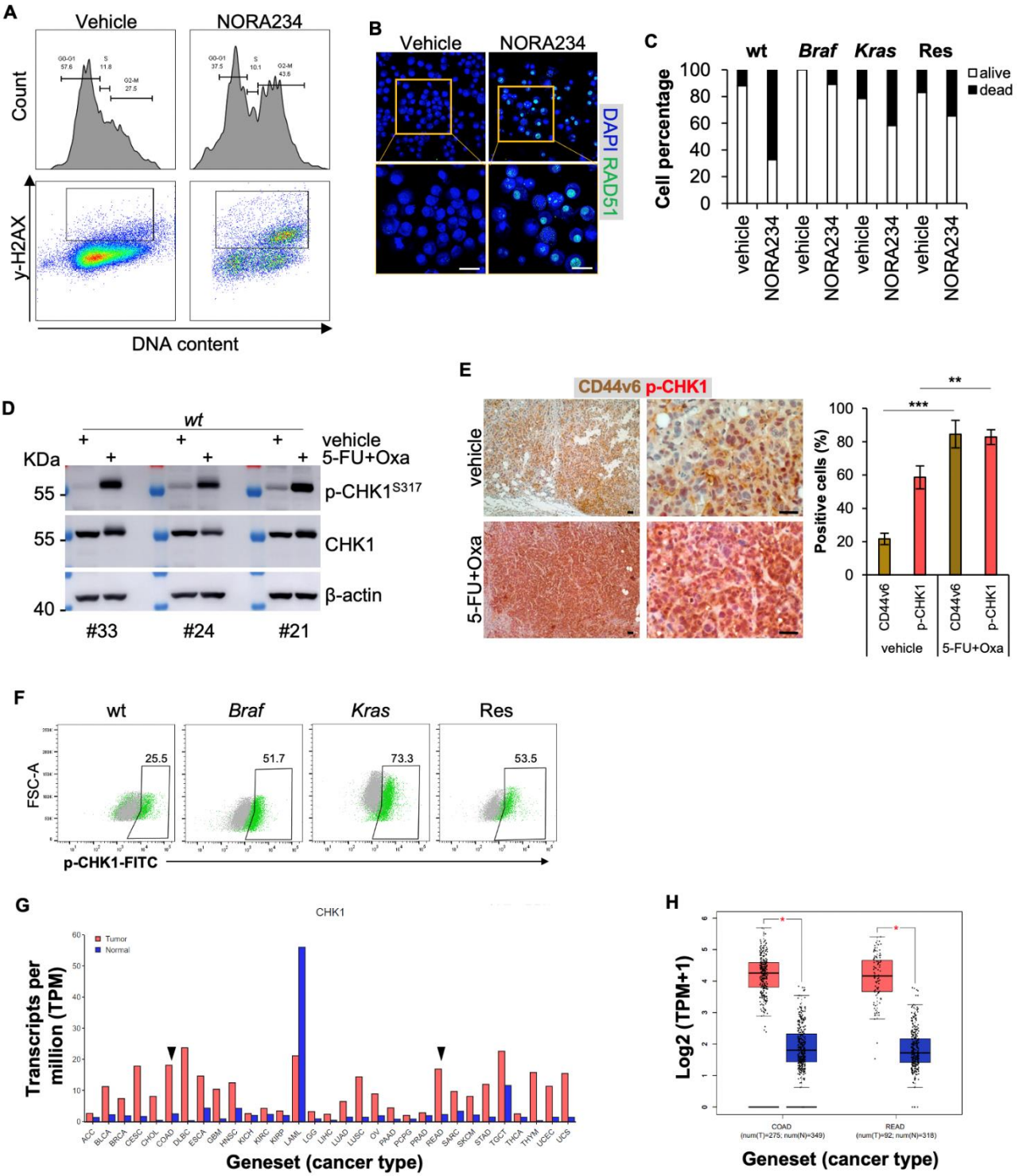
A
Vehicle
NORA234
Count
y-H2AX
DNA content
B
Vehicle
NORA234
DAPI RAD51
C
wt
Braf
Kras
Res
alive
dead
Cell percentage
vehicle
NORA234
D
wt
vehicle
5-FU+Oxa
KDa
p-CHK1S317
CHK1
β-actin
#33
#24
#21
E
CD44v6 p-CHK1
vehicle
5-FU+Oxa
Positive cells (%)
CD44v6
p-CHK1
F
wt
Braf
Kras
Res
FSC-A
p-CHK1-FITC
25.5
51.7
73.3
53.5
G
CHK1
Tumor
Normal
Transcripts per million (TPM)
Geneset (cancer type)
H
Log2 (TPM+1)
COAD
READ
Geneset (cancer type)
I
Pt#1
Pt#2
Pt#3
Pt#4
Pt#5
KDa
N T
CHK1
CHK2
p53
β-actin

Figure S2: CHK1 is highly expressed in CRC, Related to Figure 3.

(A) Representative cell cycle analysis in CR-CSphCs (#21) treated with vehicle or NORA234 for 24 hours. The percentage of cells in G0-G1, S and G2-M cell cycle phase is indicated.*(lower panel)* Representative flow cytometry analysis of γ-H2AX positivity in cells (CR-CSphC#21) treated for 24 hours as previously described and stained with PI.

(B) Representative immunofluorescence analysis of RAD51 in CR-CSphCs (#21) treated with vehicle or NORA234 for 48 hours. Nuclei were counterstained with DAPI. Scale bars, 20 μm.

(C) Cell percentage of alive and dead CR-CSphCs isolated from wt (#21), *Braf* (#3), *Kras* (#9) or chemoresistant (#R4) CRC patients, treated with vehicle or NORA234 for 48 hours.

(D) Immunoblot analysis of p-CHK1 and CHK1 in wt CR-CSphCs (CSphC #21, #24, and #33) treated for 24 hours, as indicated. β-actin was used as loading control.

(E) Immunohistochemical analysis of CD44v6 (brown) and p-CHK1 (red) on paraffin-embedded sections of tumor xenografts generated by subcutaneous injection of wt CR-CSphCs (#21), treated for 4 weeks (from 6^{th} to 9^{th} week) with vehicle or 5-FU in combination with oxaliplatin. (*right panel*) Percentage of CD44v6 and p-CHK1 positive cells. Data are mean ± S.D (n=6 mice for group). Statistical significance between 2 groups was determined by unpaired Student's t-test (2-tailed). ** $p \leq 0.01$; *** $p \leq 0.001$.

(F) Representative flow cytometry analysis of p-CHK1 on wt (#58), *Braf* (#3), *Kras* (#11) and chemoresistant (#R2) CR-CSphCs. Grey color indicates cells stained with IMC.

(G) Transcriptomic analysis, expressed as transcripts per million (TPM), of CHK1 in different datasets of normal and tumor tissue from GEPIA database.

(H) Boxplot analysis of CHK1 expression in colon (COAD) and rectal (READ) cancer datasets of normal ad tumor tissue from GEPIA database.

(I) Immunoblot analysis of CHK1, CHK2 and p53 in 5 representative colorectal tumor specimens (T) and adjacent-normal counterparts (N). β-actin was used as loading control.

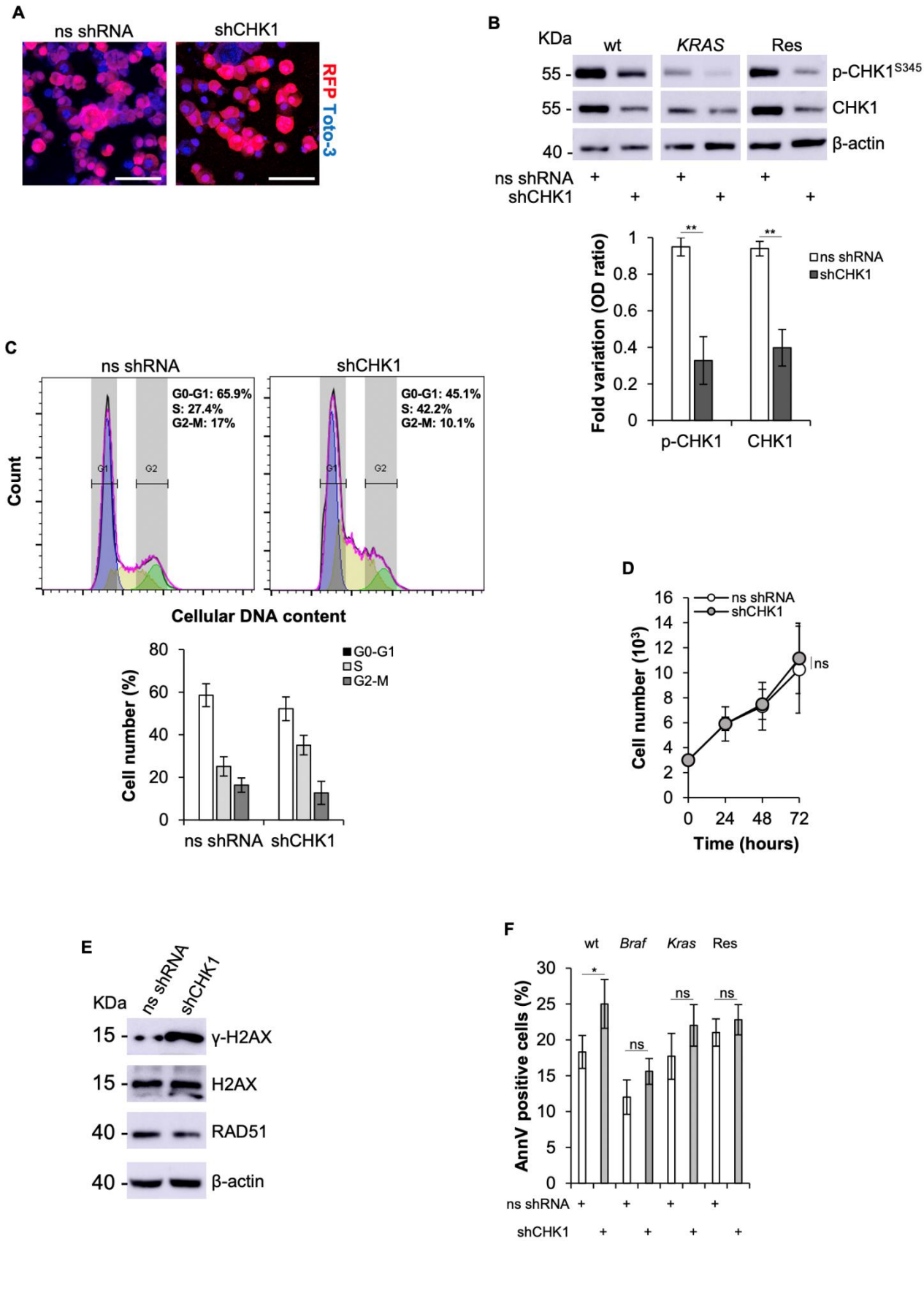
A
ns shRNA
shCHK1
RFP Toto-3
B
KDa
wt
KRAS
Res
55 -
55 -
40 -
p-CHK1S345
CHK1
β-actin
ns shRNA + + +
shCHK1 + + +
Fold variation (OD ratio)
1
0.8
0.6
0.4
0.2
0
**
**
ns shRNA
shCHK1
p-CHK1
CHK1
C
ns shRNA
shCHK1
G0-G1: 65.9%
S: 27.4%
G2-M: 17%
G0-G1: 45.1%
S: 42.2%
G2-M: 10.1%
G1
G2
Count
Cellular DNA content
Cell number (%)
80
60
40
20
0
G0-G1
S
G2-M
ns shRNA
shCHK1
D
Cell number (10³)
16
14
12
10
8
6
4
2
0
ns shRNA
shCHK1
ns
0
24
48
72
Time (hours)
E
KDa
ns shRNA
shCHK1
15 -
15 -
40 -
40 -
γ-H2AX
H2AX
RAD51
β-actin
F
wt
Braf
Kras
Res
AnnV positive cells (%)
30
25
20
15
10
5
0
*
ns
ns
ns
ns shRNA + + + +
shCHK1 + + + +

Figure S3: Silencing of CHK1 promotes the acquisition of DNA damage in CR-CSphCs, Related to Figure 4.

(A) Representative fluorescence analysis of CR-CSphCs (#21) transduced with ns shRNA control or shCHK1. Nuclei were counterstained with Toto-3.Scale bars, 100 μm.

(B) Representative immunoblot analysis and quantification of p-CHK1^{S345} and CHK1 expression in CR-CSphCs transduced as in (A). β-actin was used as loading control. Data are expressed as mean ± SD of three independent experiments using cells isolated from 3 different CRC patients (CR-CSphCs #9, #21, #R4). Statistical significance between 2 groups was determined by unpaired Student's t-test (2-tailed). ** $p \leq 0.01$.

(C) Representative cell cycle analysis in CR-CSphCs (#3) transduced as in (A). Data show percentage of cell number in G0-G1 (blue color), S (yellow color), and G2-M (green color) cell cycle phase. Data are expressed as mean ± SD of three independent experiments using cells isolated from 4 different CRC patients (CR-CSphCs #3, #9, #21, #R4).

(D) Cell growth kinetics of CR-CSphCs transduced as in (A), up to 3 days. Data represent the mean ± SD of three independent experiments using 4 different CR-CSphCs (#3, #9, #21, #R4).Statistical significance between 2 groups was determined by unpaired Student's t-test (2-tailed). ns, non-significant.

(E) Representative immunoblot analysis and quantification of y-H2AX, H2AX and RAD51 expression in CR-CSphCs (#21) transduced as in (A). β-actin was used as loading control.

(F) Percentage of AnnexinV positivity in CR-CSphCs transduced as in (A). Data represent mean ± S.D. of 3 independent experiments performed with cells isolated from wt (#21), *Braf* (#3), *Kras* (#9) or chemoresistant (#R4) CRC patients. Statistical significance between 2 groups was determined by unpaired Student's t-test (2-tailed). ns, non-significant; * $p \leq 0.05$.

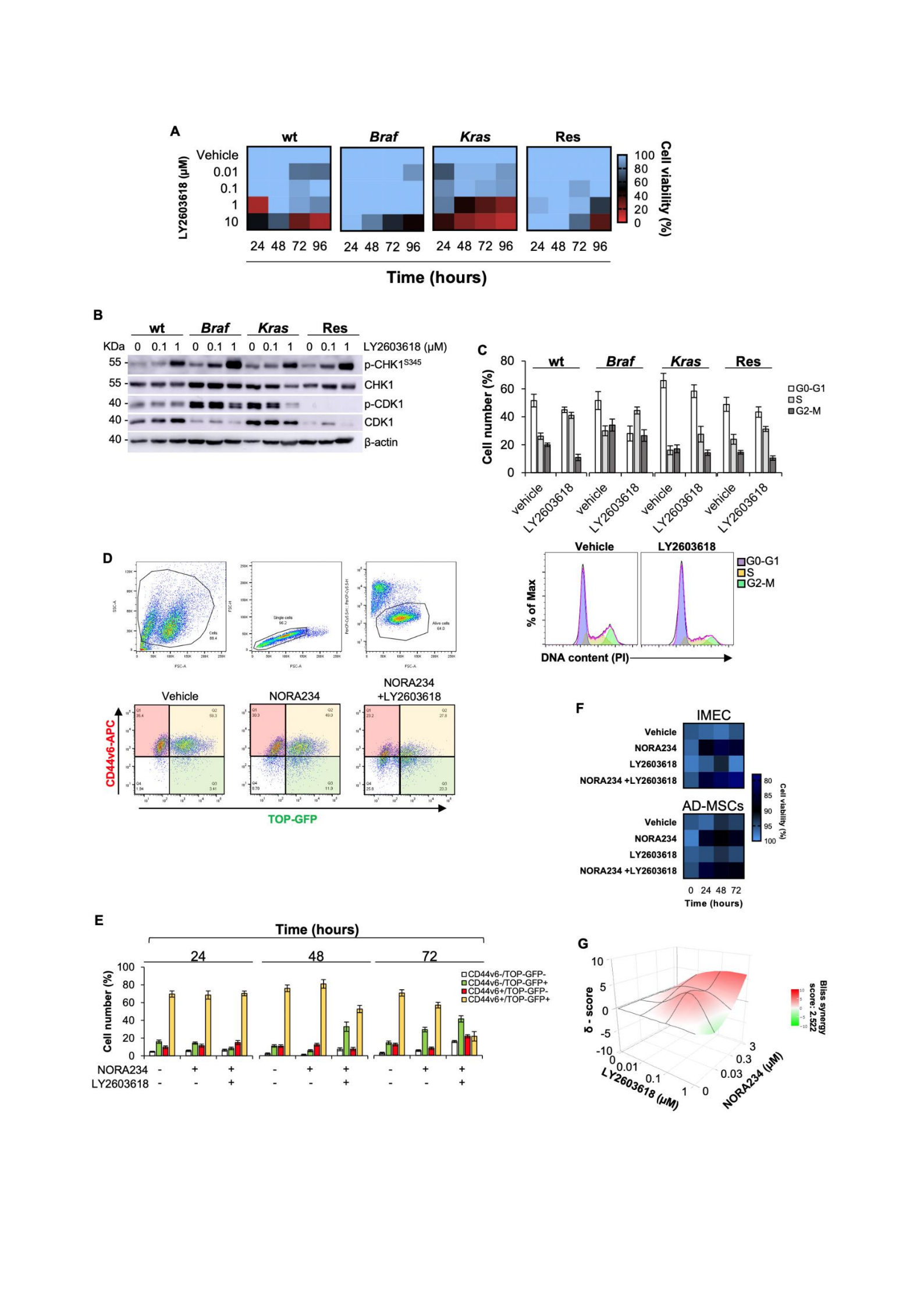
A
wt
Braf
Kras
Res
LY2603618 (μM)
Vehicle
0.01
0.1
1
10
24 48 72 96
Time (hours)
Cell viability (%)
100
80
60
40
20
0
B
wt
Braf
Kras
Res
KDa
0 0.1 1 0 0.1 1 0 0.1 1 0 0.1 1
LY2603618 (μM)
55
p-CHK1S345
55
CHK1
40
p-CDK1
40
CDK1
40
β-actin
C
wt
Braf
Kras
Res
Cell number (%)
G0-G1
S
G2-M
vehicle
LY2603618
Vehicle
LY2603618
% of Max
DNA content (PI)
D
Vehicle
NORA234
NORA234 +LY2603618
CD44v6-APC
TOP-GFP
E
Time (hours)
24
48
72
Cell number (%)
CD44v6-/TOP-GFP-
CD44v6-/TOP-GFP+
CD44v6+/TOP-GFP-
CD44v6+/TOP-GFP+
NORA234
LY2603618
F
IMEC
Vehicle
NORA234
LY2603618
NORA234 +LY2603618
AD-MSCs
Vehicle
NORA234
LY2603618
NORA234 +LY2603618
Cell viability (%)
0 24 48 72
Time (hours)
G
δ - score
LY2603618 (μM)
NORA234 (μM)
Bliss synergy score: 2.522

Figure S4: CHK1 inhibition in combination with NORA234 targets CD44v6 positive CRC cells, Related to Figure 4.

(A) Viability heatmap of cells treated withvehicle or Rabusertib (LY2603618), up to 96 hours. Data are expressed as mean of three independent experiments using 4 different CRC patients (CR-CSphCs#3, #9, #21, #R4).

(B) Representative immunoblot analysis of $pCHK1^{S345}$, CHK1, pCDK1 and CDK1 on 4 different CR-CSphCs isolated from wt (#21), *Braf* (#3), *Kras* (#9) or chemoresistant (#R4) CRC patients treated with the indicated concentration of LY2603618 for 48 hours. β-actin was used as loading control.

(C) Cell cycle analysis in CR-CSphCs treated with vehicle or LY2603618, for 24 hours. The graph represents the percentage of cell number in G0-G1, S, and G2-M phases. Data are expressed as mean ± SD of three independent experiments performed using 4 different CRC patients (CR-CSphCs#3, #9, #21, #R4). *(lower panels)* Representative cell cycle analysis of CR-CSphCs treated with vehicle or LY2603618, for 24 hours.

(D) Gate strategies for flow cytometry analysis of CR-CSphCs treated with vehicle or NORA234, alone and in combination with LY2603618, for 48 hours. (*lower panels*) Representative flow cytometry analysis of CD44v6 positivity in CR-CSphCs (#8, #9) transduced with TOP-GFP and treated as previously described.

(E) Cell number percentage of CD44v6/TOP-GFP positivity of CR-CSphCs treated as in (D). Data are expressed as mean ± SD of three independent experiments performed using 2 different CR-CSphCs (#8, #9).

(F) Cell viability percentage of healthy cells treated alone or in combination with NORA234 and Rabusertib (LY2603618), up to 72 hours. Data are expressed as mean of six independent experiments using IMEC or AD-MSCs.

(G) 3D synergy map of cell viability of Bliss score in cells treated as in (F), at the indicated doses, for 48 hours. Data are expressed as mean of three independent experiments using IMEC and AD-MSCs.

Supplementary Table S1. CR-CSCs and their CMS, MSI profile, CD44v6 expression and mutational profiles, Related to Figure 1-4.