SUPPLEMENTARY FIGURE LEGENDS

FIGURE S1. **Effect of DGKη siRNA on ERK1/2 phosphorylation.** *A* and *B*, HeLa cells were transfected with control siRNA or DGKη siRNA #1 (*A*), #2 (*A*) or #3 (*B*). After 72 h, the cells were serum-starved for 5 h and stimulated with 100 ng/ml of EGF for 5 min. Phospho-ERK1/2, ERK1/2, DGKη, and actin were detected using their specific antibodies by Western blot (WB). *C*, HEK293 cells were transfected with control siRNA or DGKη siRNA #1. After 72 h, the cells were serum-starved for 5 h and stimulated with 100 ng/ml of EGF for 2 min. Phospho-ERK1/2, ERK1/2, DGKη, and β-actin were detected using their specific antibodies by Western blot (WB).

FIGURE S2. **Silencing of DGKδ expression does not affect activation of MEK1/2 by RasV12.** HeLa cells were transfected with control siRNA or DGKδ siRNA. After 24 h, the cells were transfected with pcDNA3.1 or pCMV-H-RasV12. After 48 h of transfection with the expression plasmids, the cells were serum-starved for 5 h. Phospho-MEK1/2, MEK1/2, DGKδ, Ras and β-actin in the cell lysates were detected by Western blot (WB). The bands marked by asterisk represent non-specific bands reacting with anti-DGKδ antibody.

FIGURE S3. **Effect of DGKη siRNA on C-Raf binding to Ras.** HeLa cells were transfected with control siRNA or DGKη siRNA #1. After 24 h, the cells were transiently transfected with pCMV-H-RasV12 or pCMV-H-RasN17. After 24 h of transfection with the expression plasmids, the cells were serum-starved for 5 h. Endogenous C-Raf in the cell lysates was immunoprecipitated with anti-C-Raf antibody. RasV12 or RasN17 binding to C-Raf was detected using anti-Ras antibody by Western blot (WB). TCL, total cell lysates; IP, immunoprecipitate.

FIGURE S4. **DGKη exhibits no effect on S338 phosphorylation of C-Raf, and modulates MEK1/2 activation by a constitutively active mutant of C-Raf.** The HeLa cells were transfected with control siRNA or DGKη siRNA #1. *A*, after 72 h, the cells were serum-starved for 5 h and stimulated with 100 ng/ml of EGF for the indicated times. Ser338 phosphorylation of C-Raf was analyzed using Western blot (WB). Upper panels: representative results of Western blot analysis are shown. Bottom panel: the phospho-C-Raf levels are shown as means ± SD of three independent experiments. *B*, after 24 h of transfection with the siRNAs, cells were transfected with pcDNA3.1 (–) or pCMV-C-Raf-DDED, where Ser338/Tyr341/Thr491/Ser494 were replaced with Asp/Asp/Glu/Asp, respectively (C-Raf-DDED). After 24 h of transfection with the expression plasmids, the cells were serum-starved for 5 h. The cells transfected with pcDNA3.1 were stimulated with 100 ng/ml of EGF as controls (EGF) for 2 min. Phospho-MEK1/2, MEK1/2, and C-Raf were detected by Western blot analysis using their specific antibodies. Upper panels: representative results of Western blot analysis are shown. Bottom panel: phospho-MEK1/2 levels were quantified by densitometry. Phospho-MEK1/2 levels in the cells, which were transfected with control siRNA and pcDNA3.1 and stimulated with EGF for 2 min, were set to 100. The data are shown as means ± SD of three independent experiments.

FIGURE S5. **DGKη regulates B-Raf/C-Raf heterodimerization independently or downstream of 14-3-3 proteins.** The HeLa cells were transfected with control siRNA or DGKη siRNA #1. After 72 h, the cells were serum-starved for 5 h and stimulated with 100 ng/ml of EGF for 2 min. Endogenous C-Raf in the cell lysates was immunoprecipitated with anti-C-Raf antibody. 14-3-3 proteins associated with C-Raf were detected using Western blot (WB). Normal rabbit IgG was used as a negative control. TCL, total cell lysates; IP, immunoprecipitate.

FIGURE S6. **Effect of U0126 on C-Raf kinase activities in cells transfected with DGKη-siRNA.** The HeLa cells were transfected with control siRNA or DGKη siRNA #1. After 72 h, the cells were

serum-starved for 5 h and incubated with DMSO (–) or 10 μM of U0126 for 30 min. EGF stimulation was performed at a concentration of 100 ng/ml of EGF for 2 min. Endogenous C-Raf in the cell lysates was immunoprecipitated with anti-C-Raf antibody. C-Raf kinase activities of the immunoprecipitants were measured *in vitro* as described in Figure 5. TCL, total cell lysates; WB, Western blot.

FIGURE S7. **DGKη is required for heterodimerization of B-Raf with C-Raf induced by RasV12 expression.** The HeLa cells were transfected with control or DGKη siRNA. After 24 h, cells were transiently transfected with pcDNA3.1 or pCMV-H-RasV12. After 24 h of transfection with the expression plasmids, the cells were serum-starved for 5 h. Endogenous C-Raf in the cell lysates was immunoprecipitated with anti-C-Raf antibody. B-Raf associated with C-Raf was detected using Western blot (WB). TCL, total cell lysates; IP, immunoprecipitate.

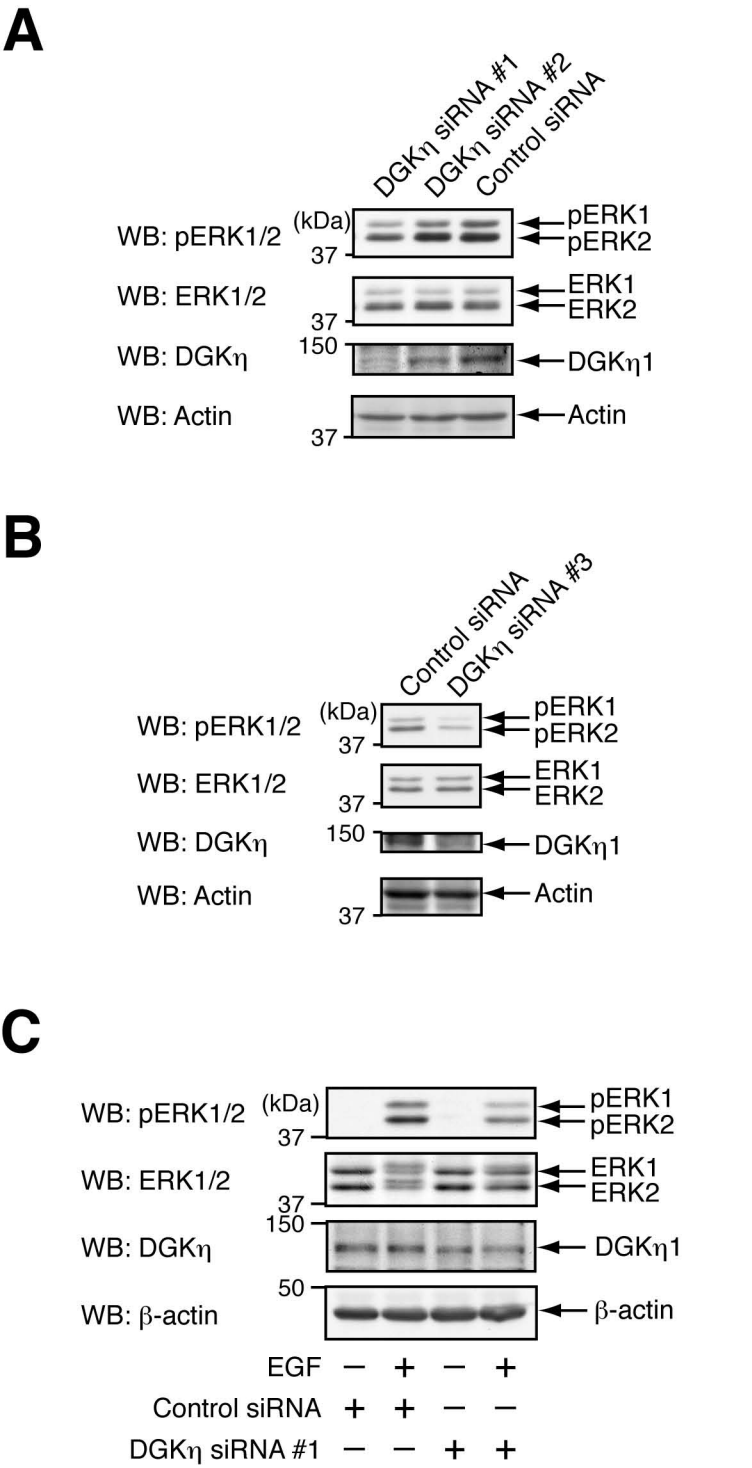

A
DGKη siRNA #1
DGKη siRNA #2
Control siRNA
WB: pERK1/2
(kDa)
37
pERK1
pERK2
WB: ERK1/2
37
ERK1
ERK2
WB: DGKη
150
DGKη1
WB: Actin
37
Actin
B
Control siRNA
DGKη siRNA #3
WB: pERK1/2
(kDa)
37
pERK1
pERK2
WB: ERK1/2
37
ERK1
ERK2
WB: DGKη
150
DGKη1
WB: Actin
37
Actin
C
WB: pERK1/2
(kDa)
37
pERK1
pERK2
WB: ERK1/2
37
ERK1
ERK2
WB: DGKη
150
DGKη1
WB: β-actin
50
β-actin
EGF − + − +
Control siRNA + + − −
DGKη siRNA #1 − − + +

Figure S1

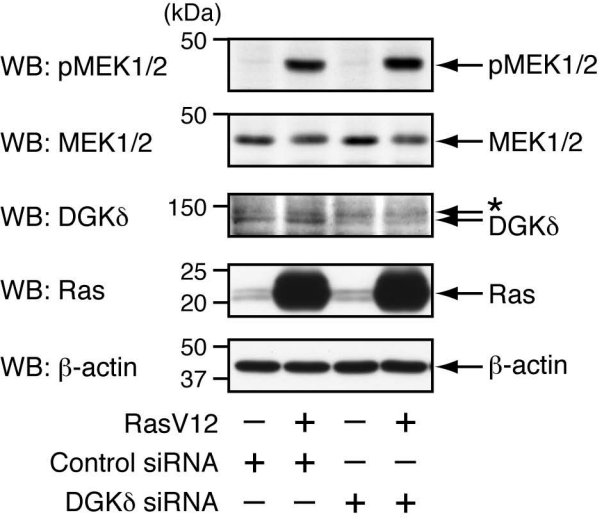

(kDa)
50
WB: pMEK1/2
pMEK1/2
50
WB: MEK1/2
MEK1/2
150
WB: DGKδ
*
DGKδ
25
20
WB: Ras
Ras
50
37
WB: β-actin
β-actin
RasV12 − + − +
Control siRNA + + − −
DGKδ siRNA − − + +

Figure S2

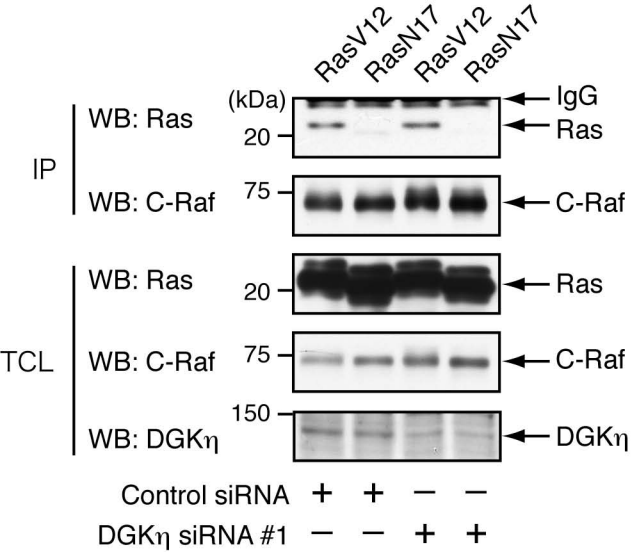
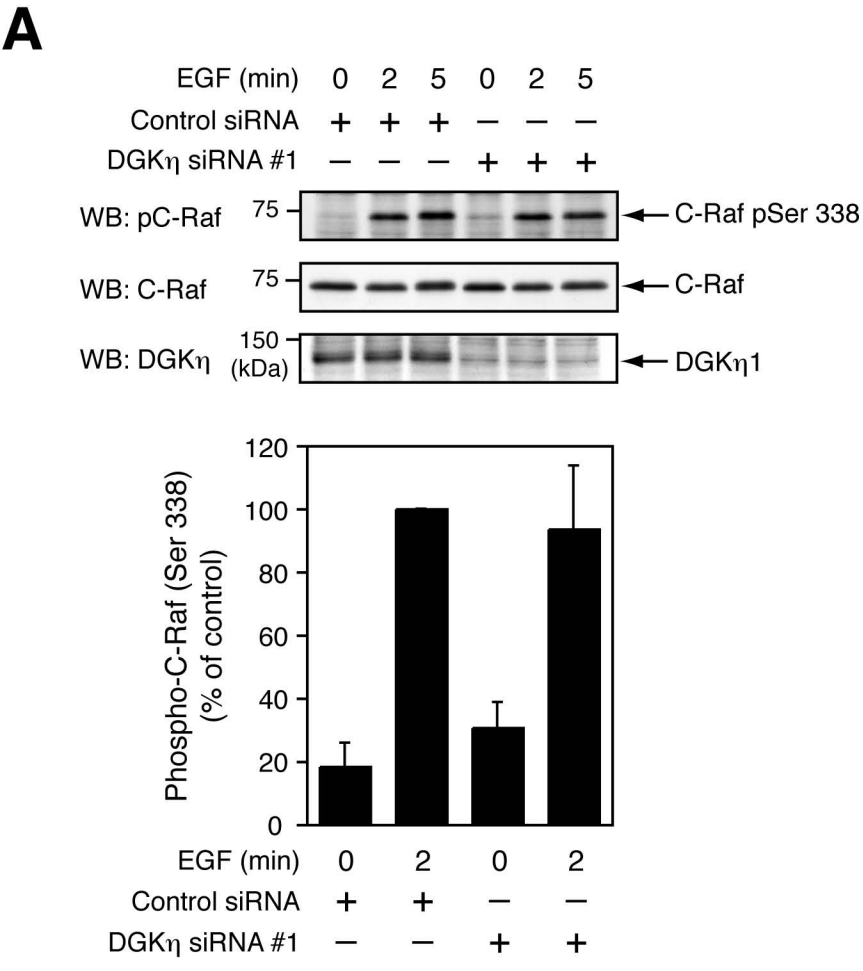

RasV12
RasN17
RasV12
RasN17
(kDa)
IP
WB: Ras
20
IgG
Ras
WB: C-Raf
75
C-Raf
TCL
WB: Ras
20
Ras
WB: C-Raf
75
C-Raf
WB: DGKη
150
DGKη
Control siRNA + + − −
DGKη siRNA #1 − − + +

Figure S3


A
EGF (min) 0 2 5 0 2 5
Control siRNA + + + – – –
DGKη siRNA #1 – – – + + +
WB: pC-Raf
75
C-Raf pSer 338
WB: C-Raf
75
C-Raf
WB: DGKη
150
(kDa)
DGKη1
Phospho-C-Raf (Ser 338)
(% of control)
120
100
80
60
40
20
0
EGF (min) 0 2 0 2
Control siRNA + + – –
DGKη siRNA #1 – – + +

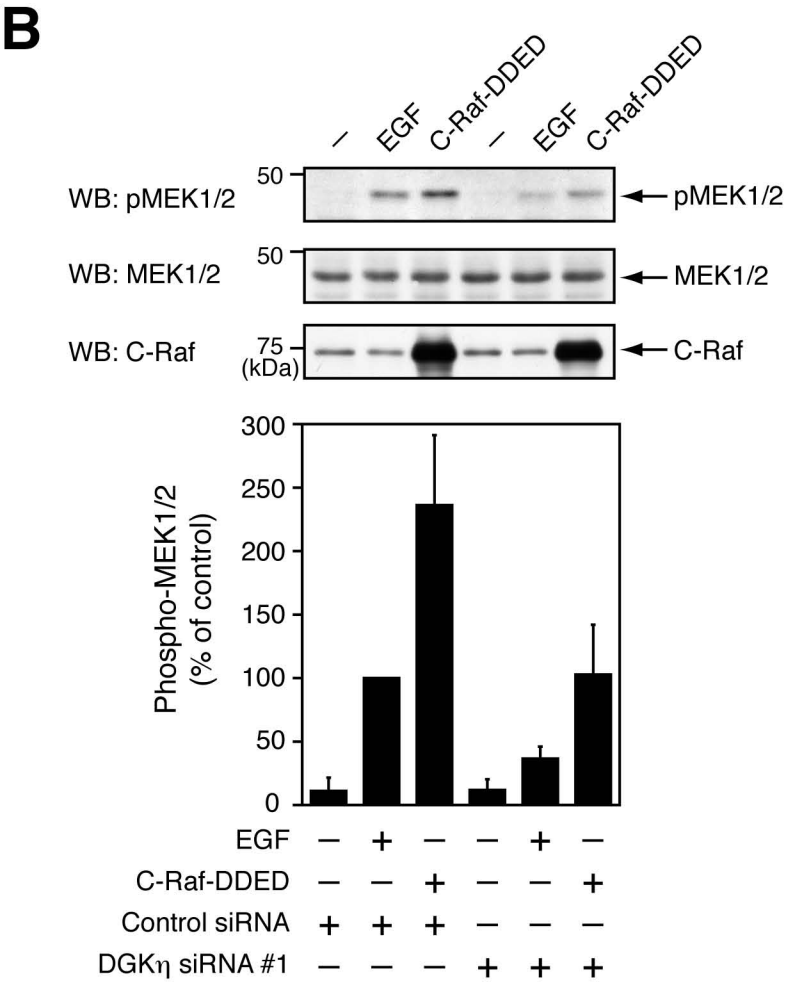

B
–
EGF
C-Raf-DDED
–
EGF
C-Raf-DDED
WB: pMEK1/2
50
pMEK1/2
WB: MEK1/2
50
MEK1/2
WB: C-Raf
75
(kDa)
C-Raf
Phospho-MEK1/2
(% of control)
300
250
200
150
100
50
0
EGF – + – – + –
C-Raf-DDED – – + – – +
Control siRNA + + + – – –
DGKη siRNA #1 – – – + + +

Figure S4

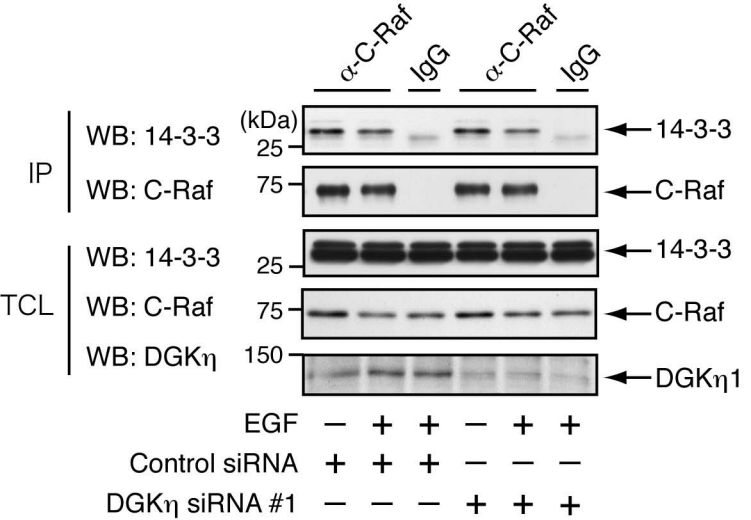

α-C-Raf
IgG
α-C-Raf
IgG
IP
WB: 14-3-3
WB: C-Raf
TCL
WB: 14-3-3
WB: C-Raf
WB: DGKη
(kDa)
25
75
25
75
150
14-3-3
C-Raf
14-3-3
C-Raf
DGKη1
EGF − + + − + +
Control siRNA + + + − − −
DGKη siRNA #1 − − − + + +

Figure S5

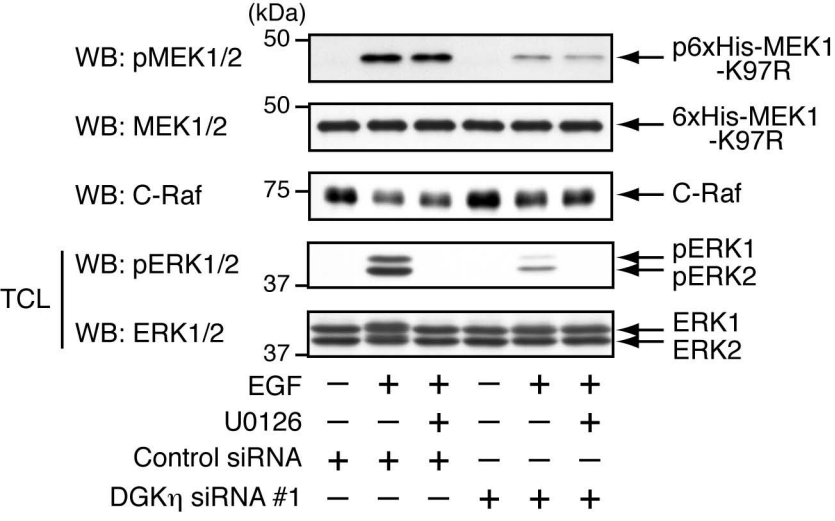

(kDa)
50
WB: pMEK1/2
p6xHis-MEK1
-K97R
50
WB: MEK1/2
6xHis-MEK1
-K97R
75
WB: C-Raf
C-Raf
TCL
WB: pERK1/2
37
pERK1
pERK2
WB: ERK1/2
37
ERK1
ERK2
EGF − + + − + +
U0126 − − + − − +
Control siRNA + + + − − −
DGKη siRNA #1 − − − + + +

Figure S6

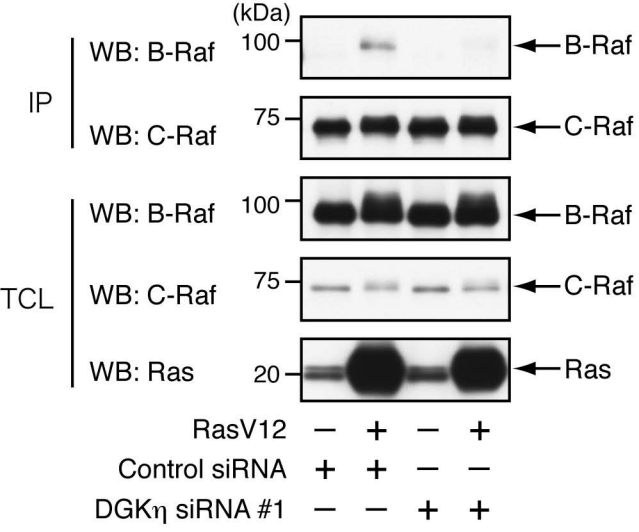

(kDa)
IP
WB: B-Raf
WB: C-Raf
TCL
WB: B-Raf
WB: C-Raf
WB: Ras
100
75
100
75
20
B-Raf
C-Raf
B-Raf
C-Raf
Ras
RasV12 − + − +
Control siRNA + + − −
DGKη siRNA #1 − − + +

Figure S7