Circadian clock-controlled diurnal oscillation of Ras/ERK signaling in mouse liver

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Abstract: Accumulating evidence indicates that ERK MAP kinase signaling plays an important role in the regulation of the circadian clock, especially in the clock-resetting mechanism in the suprachiasmatic nucleus (SCN) in mammals. Previous studies have also shown that ERK phosphorylation exhibits diurnal variation in the SCN. However, little is known about circadian regulation of ERK signaling in peripheral tissues. Here we show that the activity of Ras/ERK signaling exhibits circadian rhythms in mouse liver. We demonstrate that Ras activation, MEK phosphorylation, and ERK phosphorylation oscillate in a circadian manner. As the oscillation of ERK phosphorylation is lost in *Cry1/Cry2* double-knockout mice, Ras/ERK signaling should be under the control of the circadian clock. Furthermore, expression of MAP kinase phosphatase-1 (*Mkp-1*) shows diurnal changes in liver. These results indicate that Ras/ERK signaling is strictly regulated by the circadian clock in liver, and suggest that the circadian oscillation of the activities of Ras, MEK, and ERK may regulate diurnal variation of liver function and/or homeostasis.

Keywords: circadian rhythms, ERK, Ras, phosphorylation, liver

Introduction

Circadian rhythms regulate a variety of physiological and metabolic processes in diverse organisms.[1)] In mammals, circadian rhythms are driven by interlocked transcriptional-translational feedback loops (TTFLs) that involve a set of clock proteins including PER1, PER2, PER3, CRY1, CRY2, CLOCK, BMAL1, RORα, REV-ERBα, and E4BP4.[2),3)] The TTFL-based core clock machinery resides not only in the mammalian central pacemaker, the suprachiasmatic nucleus (SCN) of hypothalamus, but also in almost all peripheral tissues in the body.[4)] The circadian clock in the SCN plays a critical role in light-induced resetting of circadian rhythms *via* the retinohypothalamic tract and outputs signals to control other peripheral oscillators. On the other hand, peripheral oscillators should regulate diurnal changes of tissue-specific physiological processes and functions as demonstrated in several tissues such as liver, heart, and pancreas.[5)–7)]

The ERK MAP kinase signaling pathway regulates diverse physiological processes including cellular proliferation and differentiation.[8)–11)] A body of evidence indicates that ERK signaling is also involved in the regulation of circadian rhythms.[12),13)] For example, it has been reported that, in the SCN, ERK is phosphorylated in response to the photic signal from the retina, and that ERK activation is involved in the light-induced resetting of circadian rhythms.[14)] Moreover, ERK signaling has been shown to have an ability to reset the circadian clock in cultured fibroblasts.[15)] Thus, the ERK pathway plays an important role in the clock-resetting mechanisms of mammarian circadian rhythms.

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Intriguingly, it has been demonstrated that phosphorylation levels of ERK exhibit circadian variations in several tissues including the mouse SCN, the rat pineal grand, the hamster SCN, the chick pineal grand and retina, and bullfrog retina.[14),16)–22)] Recent studies have also demonstrated that ERK phosphorylation is under the regulation of the circadian clock in hippocampus, in which ERK activity rhythms are likely to be important for memory persistence.[23)] However, circadian regulation of ERK signaling in other peripheral tissues is not well understood.

In this study, we demonstrate that Ras activation, MEK phosphorylation, and ERK phosphorylation exhibit circadian rhythms in mouse liver and ERK phosphorylation rhythms are controlled by circadian core clock components. We also found clear rhythms of MAP kinase phosphatase-1 (*Mkp-1*) expression in liver. These results suggest overall circadian regulation of Ras-ERK MAP kinase signaling in liver.

Results

To examine the circadian profile of activation of ERK MAP kinase in liver, mice were maintained under 12 h light/12 h dark (LD) cycles for 2 weeks and then livers were harvested every 4 h in LD conditions. Immunoblotting analysis of liver lysates demonstrated that phosphorylation of ERK1/2 shows diurnal variations, peaking at zeitgeber time (ZT) 6 (light-on at ZT0 and light-off at ZT12) (Fig. 1A). The protein amount of ERK1/2 was constant throughout the day. This result is consistent with data reported previously.[24)] Then, we checked whether the oscillation of ERK phosphorylation occurs under constant dark (DD) conditions. Mice were entrained to LD cycles for 2 weeks and moved to DD conditions. On the first day in DD conditions, livers were harvested every 4 h. The result clearly demonstrates that there was a robust circadian oscillation of ERK phosphorylation, which peaks at circadian time (CT) 6 (CT0 corresponds to the time 12 h after light-off) as observed in LD conditions (Fig. 1B). We also found that the observed rhythms of ERK phosphorylation lasted at least 2 days in DD conditions (Fig. 1C). These results indicate that ERK phosphorylation rhythms in liver are self-sustained and oscillate even without external time cues. Given the expression profiles of clock genes and clock-controlled genes such as *Per1*, *Per2*, *Dbp*, *Bmal1*, and *E4bp4* showing clear circadian rhythms in DD conditions (Fig. 1D), the peak time of ERK phosphorylation preceded that of *Dbp* and *Per1* mRNA expression in liver.

ERK phosphorylation is regulated by the upstream signaling factors such as Ras and MEK1/2. Thus, we examined the activation of these factors in mouse liver. As a result, the amount of Ras-GTP, a GTP-bound active form of Ras, oscillated in a circadian manner, peaking at CT6 in DD condition (Fig. 2A), while the total amount of Ras was constant throughout the day. Phosphorylation of MEK1/2 also exhibited a circadian oscillation, peaking at CT6 (Fig. 2B). These results clearly indicate that the activities of Ras, MEK, and ERK all show circadian rhythms in liver and strongly suggest that the circadian oscillation of ERK phosphorylation is caused by the upstream Ras activation rhythms.

To investigate whether ERK phosphorylation rhythms are controlled by the circadian core clock components, we examined a time profile of ERK phosphorylation in the arrhythmic *Cry1/Cry2* double-knockout mice in DD conditions. Phosphorylation of ERK in these animals exhibited no circadian variations (Fig. 3). Thus, circadian rhythms of ERK phosphorylation in liver are controlled by the circadian clock.

ERK phosphorylation is known to be reversed by dual-specificity MAP kinase phosphatases (Mkps).[25),26)] These Mkps show the distinct substrate speificity for ERK1/2, p38, and JNK. ERK1/2 have been shown to be dephosphorylated by several Mkps including *Mkp-1*, *-2*, *-3*, *-4*, *PAC-1*, *hVH3*, and *Pyst2*. We assessed the possibility that expression of Mkps is regulated in a circadian manner. Among the Mkp family, *Mkp-1* showed robust circadian rhythms in its mRNA expression levels in liver (Fig. 4A), as in the mouse SCN.[27)] The circadian oscillation of *Mkp-1* expression lasted at least two days in DD conditions (Fig. 4B). Other Mkps that dephosphorylate ERK1/2 did not show clear circadian rhythms (Fig. 4C). Expression of *Mkp-5* and *Mkp-7* that dephosphorylate p38 and JNK but not ERK1/2 showed no circadian variations (Fig. 4C). Expression of other low molecular weight dual-specificity phosphatases such as *Mkp-6*, *VHR*, and *JKAP* also did not show circadian rhythms (Fig. 4C). These results indicate that, among Mkp members, *Mkp-1* is strongly regulated by the circadian clock in liver.

Discussion

In this study, we demonstrate that ERK phosphorylation, Ras activation, and MEK phosphorylation all show circadian oscillations in mouse liver.

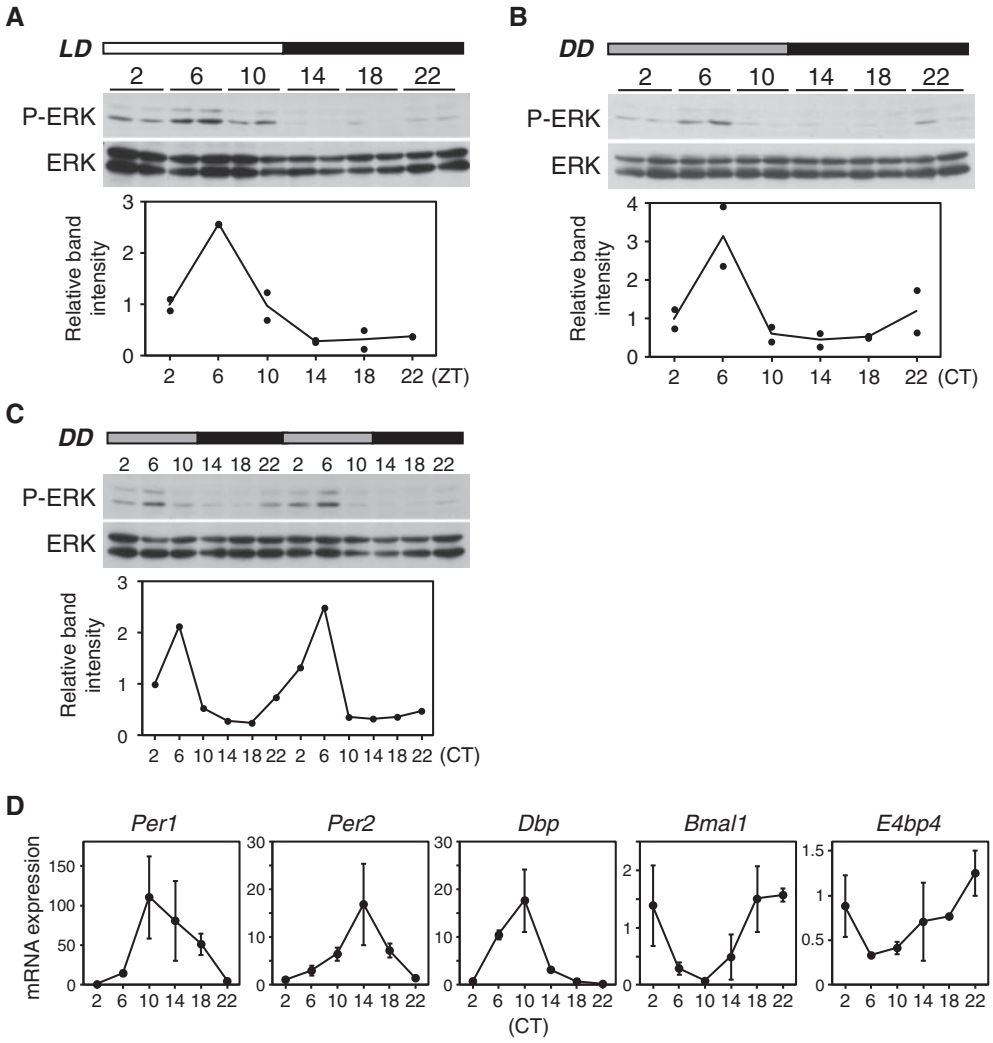

Fig. 1. Circadian oscillation of ERK phosphorylation in mouse liver. (A) Circadian oscillation of ERK phosphorylation in LD conditions. Mice were sacrificed at the indicated time points and liver extracts were analyzed by immunoblotting with the indicated antibodies. Liver samples from two mice were analyzed at each time point. The graph shows relative band intensities of phospho-ERK that were normalized with band intensities of total ERK. The black dot indicates the value of each band and the solid line indicates the means. The mean value at ZT2 was set to 1. (B) Circadian oscillation of ERK phosphorylation in DD conditions. ERK phosphorylation was analyzed as in (A) on the first day in DD conditions. (C) Circadian oscillation of ERK phosphorylation lasts two circadian cycles in DD conditions. ERK phosphorylation was analyzed as in (A) on the first two days in DD conditions. Data from one mouse at each time point were shown. (D) Circadian rhythms of clock gene expression in liver in DD conditions. Mice were sacrificed and mRNA was collected from livers at each time point. The mRNA expression levels of the indicated clock genes were evaluated by real-time quantitative PCR. Each value was normalized to *GAPDH* and is presented as the means ± SD ($n = 3$).

Although the molecular mechanism underlying the circadian oscillation of ERK phosphorylation is unknown, our data strongly suggest that the time-dependent activation of Ras produces ERK phosphorylation rhythms. Given that multiple extracellular signals such as growth factors and cytokines can stimulate Ras activation in a context-dependent manner, circadian oscillations of circulating humoral factors may lead to rhythmic Ras activation in liver. Another possibility is the circadian regulation of proteins that regulate Ras activity. In the SCN, a circadian oscillatory protein SCOP has been proven to be a negative regulator of Ras.[28] Thus, it is possible that, like SCOP in the SCN, there is a Ras-regulating factor whose expression is controlled by the circadian clock in liver.

In the SCN, the circadian oscillation of *Mkp-1* expression has been reported to be controlled by

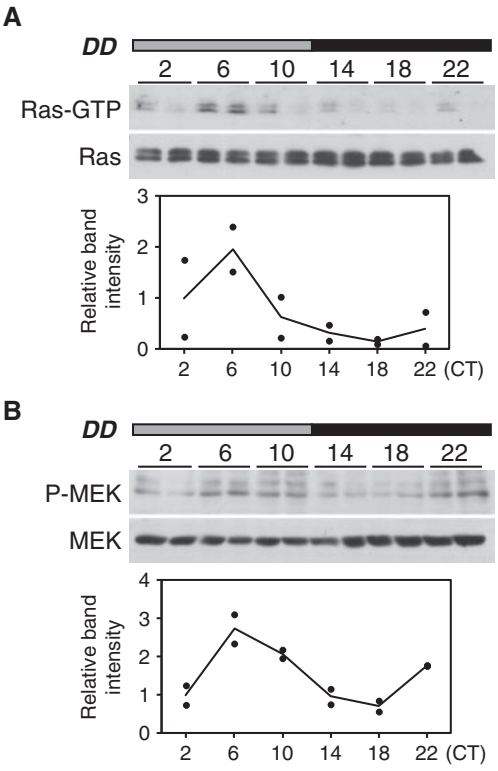

Fig. 2. Circadian oscillation of Ras activation and MEK phosphorylation in mouse liver. (A) Circadian oscillation of Ras activation in DD conditions. The GTP-bound active form of Ras was detected by immunoblotting. The graph shows relative band intensities of Ras-GTP that were normalized with band intensities of total Ras. The graph is presented as in Fig. 1A. (B) Circadian oscillation of MEK phosphorylation in DD conditions. The graph shows relative band intensities of phospho-MEK that were normalized with band intensities of total MEK. The graph is presented as in Fig. 1A.

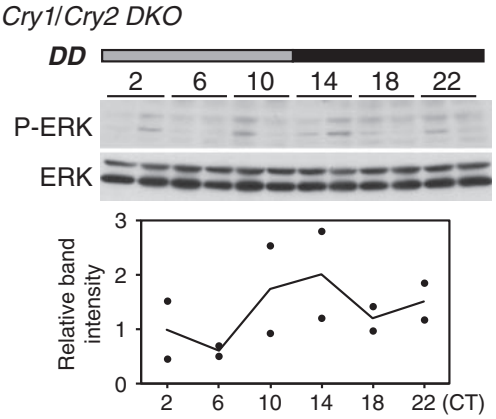

Fig. 3. ERK phosphorylation rhythms are lost in liver of *Cry1/Cry2* double-knockout mice. *Cry1/Cry2* double-knockout mice were sacrificed at the indicated time points in DD conditions and liver extracts were analyzed by immunoblotting with the indicated antibodies. ERK phosphorylation was analyzed as in Fig. 1A.

BMAL1-CLOCK heterodimer through an E-box element in its promoter region.[27] We found that expression rhythms of *Mkp-1* are in antiphase to ERK phosphorylation rhythms in liver and thus may contribute to strengthening the amplitude of ERK phosphorylation rhythms.

Our results show that ERK is phosphorylated in the daytime in mouse liver. The peak time of ERK phosphorylation is similar to that observed in the SCN and the hippocampus.[14),23] In contrast, it has been reported that phosphorylation of MAP kinase occurs in the nighttime in the chick pineal grand.[16] It has also been reported that light stimulation induces dephosphorylation of MAP kinase in the chick pineal grand, whereas light induces phosphorylation of ERK in the SCN.[14),16] These differences might be related to the distinct regulation of MKP-1 expression between in mouse and in chicken; While the expression levels of MKP-1 show circadian oscillation with the peak time at night in mouse liver and the SCN, they are constant throughout the day in the chick pineal grand.[18),27] The detailed mechanisms underlying the distinct regulation of ERK phosphorylation and MKP-1 expression between in mouse and in chicken remain to be elucidated.

The physiological significance of ERK phosphorylation rhythms in liver remains elusive. Previous studies have reported that ERK activity is required for maintaining the circadian clock in the SCN and in mouse embryonic fibroblasts.[29),30] It has also been reported that ERK phosphorylates BMAL1, CRY1, and CRY2 and may regulate their functions.[31),32] Therefore, the circadian activation of ERK might be involved in the regulation of the core circadian clock machinery. It is also possible that ERK functions as an output of the circadian clock function in liver. Taking into consideration that ERK plays an important role in liver physiology and pathology, the circadian control of ERK activity may contribute to the appropriate regulation of liver functions and the maintenance of liver homeostasis.

Materials and methods

Animals. C57BL/6CrSlc wild-type mice and *Cry1/Cry2* double-knockout mice were kept under 12 h light: 12 h dark cycle for 2 weeks prior to the start of experiments. Male mice 11 to 13 weeks of age were used. Animal care was in accordance with institutional guidelines. At indicated time points,

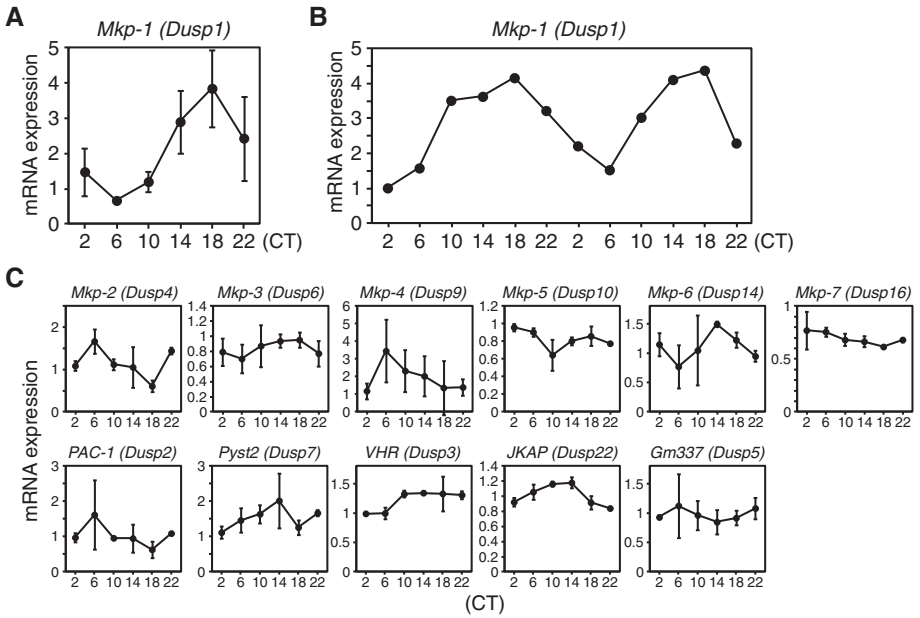

Fig. 4. Expression of *Mkp-1* shows circadian oscillation in mouse liver. (A) Circadian rhythms of mRNA expression of *Mkp-1* in DD conditions. Mice were sacrificed and mRNA was collected from livers at each time point on the first day in DD conditions. The mRNA expression levels of *Mkp-1* were evaluated by real-time quantitative PCR. Each value was normalized to *GAPDH* and is presented as the means ± SD ($n = 3$). (B) Circadian oscillation of *Mkp-1* expression lasts two circadian cycles in DD conditions. The mRNA expression levels of *Mkp-1* was analyzed as in (A) on the first two days in DD conditions. Data from one mouse at each time point were shown. (C) Time profiles of the mRNA expression levels of several dual-specificity phosphatases in DD conditions. The mRNA expression levels of the indicated genes were evaluated by real-time quantitative PCR. Each value was normalized to *GAPDH* and is presented as the means ± SD ($n = 3$).

tissues were harvested, immediately frozen in liquid N_2 and stored at −80°C.

Antibodies. The antibodies utilized in this study included anti-ERK1/2 (Santa Cruz), anti-phospho-ERK1/2 (Cell Signaling), anti-MEK1/2 (Santa Cruz), anti-phospho-MEK1/2 (Santa Cruz), anti-Ras (Upstate).

RNA extraction and real-time quantitative PCR. Total RNA was extracted from mouse liver with the RNeasy Mini kit (QIAGEN) and subjected to cDNA synthesis with random hexamer primers and M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Real-time quantitative PCR was performed with FastStart Universal SYBR (Roche) and Light Cycler (Roche). The PCR primers employed in the present study are as follows: *Per1*, 5′-CAG GCT AAC CAG GAA TAT TAC CAG C-3′ and 5′-GGA CAC AGC CAC AGA GAA GGT GTC C-3′; *Per2*, 5′-CAG TGA TGC CAA GTT TGT GGA GTT C-3′ and 5′-TGA GTG AAA GAA TCT AAG CCG CTG C-3′; *Dbp*, 5′-AAT GAC CTT TGA ACC TGA TCC CGC T-3′ and 5′-GCT CCA GTA CTT CTC ATC CTT CTG T-3′; *Bmal1*, 5′-GTA GTC CCA GTA ACG ATG AGG CAG-3′ and 5′-CTA CAG CGG CCA TGG CAA GTC ACT A-3′; *E4bp4*, 5′-GCT GAA CTC TGC CTT AGC TG-3′ and 5′-CGT CTT TCT TCT CGT CCG GA-3′; *Mkp-1*, 5′-AGG ACA ACC ACA AGG CAG AC-3′ and 5′-GAG GTA AGC AAG GCA GAT GG-3′; *Mkp-2*, 5′-GCT GTC ATC GTC TAC GA-3′ and 5′-AAG CAG GCA GAT GTC CGT-3′; *Mkp-3*, 5′-AGT TTT TCC CTG AGG CCA TT-3′ and 5′-CAT CGT TCA TGG ACA GGT TG-3′; *Mkp-4*, 5′-GCC AAA GAA GAG TGG GAT-3′ and 5′-TGG AAC TTG CTG AAG CCA-3′; *Mkp-5*, 5′-ACA GGG TAG TAG TGG CAC-3′ and 5′-TGG CAA GGA CAG GAG CGT-3′; *Mkp-6*, 5′-GTC ATC AAT GCC ACC ATC-3′ and 5′-GGC CAC AGT GTC AAA GTA-3′; *Mkp-7*, 5′-CAG CGA GAT GTC CTC AAC AA-3′ and 5′-GAA GTC AGG CTT TGG ACA GG-3′; *PAC-1*, 5′-ACT GTC CGG ATC TGT GCT CT-3′ and 5′-CAG GTA GGG CAA GAT TTC CA-3′; *Pyst2*, 5′-CAC TGG AGC CAG AAC CTC TC-3′ and 5′-CTA GGC AGT GCA CCA AGA CA-3′; *VHR*, 5′-GGA TTC TGG CAT CAC CTA-3′ and 5′-AGC ACC CGG CCA TTT TTA-3′; *JKAP*, 5′-GTT GAG CAG GAA CAA GGT-3′ and 5′-TTG AGA TGG TGT

GTC TGC-3′; *Gm337*, 5′-TGC ACC ACC CAC CTA CAC TA-3′ and 5′-AGG ACC TTG CCT CCT TCT TC-3′; *GAPDH*, 5′-CAT CCA CTG GTG CTG CCA AGG CTG T-3′ and 5′-ACA ACC TGG TCC TCA GTG TAG CCC A-3′.

Immunoblotting. Mouse liver was homogenized and lysed with an SDS sample buffer (50 mM Tris-HCl (pH 6.8), 10% glycerol, and 1% SDS), and the resultant whole liver extracts were subjected to immunoblotting with the indicated antibodies. The blots were treated with a horseradish peroxidase-conjugated secondary antibody (Invitrogen) and were developed with enhanced chemiluminescence ECL (GE Healthcare).

Detection of the GTP-bound active form of Ras. GTP-bound active form of Ras was quantified by detecting the amount of Ras capable of binding to the Ras-binding domain (RBD) of Raf-1, basically as described previously.[33] Briefly, a Ras-binding domain of cRaf1 (amino acids 1–149) was fused to GST. GST-RBD was expressed in *Escherichia coli* and purified on glutathione-Sepharose beads. Mouse liver was homogenized and lysed in incubation buffer (25 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM NaF, 1 mM sodium pyrophosphate, 1% Nonidet P-40, 10% glycerol, 20 µg/mL aprotinin, 4 µg/mL leupeptin, 1 mM sodium orthovanadate), and the GTP-bound form of Ras was precipitated with GST-RBD coupled to glutathione-Sepharose beads for 1 h at 4°C. The precipitates were then washed three times with the incubation buffer. Proteins were separated by SDS-PAGE and analyzed by immunoblotting with anti-Ras antibody.

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