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DEC1 negatively regulates AMPK activity via LKB1

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Abstract

Basic helix-loop-helix (bHLH) transcription factor DEC1 (bHLHE40/Stra13/Sharp2) is one of the clock genes that show a circadian rhythm in various tissues. AMP-activated protein kinase (AMPK) activity plays important roles in the metabolic process and in cell death induced by glucose depletion. Recent reports have shown that AMPK activity exhibited a circadian rhythm. However, little is known regarding the regulatory mechanisms involved in the circadian rhythm of AMPK activity. The aim of this study is to investigate whether there is a direct correlation between DEC1 expression and AMPK activity. DEC1 protein and AMPK activity showed a circadian rhythm in the mouse liver with different peak levels. Knocking down DEC1 expression increased AMPK activity, whereas overexpression of DEC1 decreased it. Overexpressing the DEC1 basic mutants had little effect on the AMPK activity. DEC1 bound to the E-box of the LKB1 promoter, decreased LKB1 activity and total protein levels. There was an inverse relationship between DEC1 expression and AMPK activity. Our results suggest that DEC1 negatively regulates AMPK activity via LKB1.

Keywords

DEC1; AMPK; LKB1; Clock gene

1. Introduction

bHLH transcription factor differentiated embryo-chondrocyte 1 (DEC1), one of the clock genes, plays an important role in the regulation of circadian rhythms, hypoxia response, epithelial mesenchymal transition (EMT), apoptosis and differentiation [1–7]. In mammals, DEC1 mRNA exhibit a circadian pattern of expression in the suprachiasmatic nucleus

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Conflict of interest

None declared.

(SCN), peripheral tissues and human mesenchymal stem cells (hMSCs) [2,8–10]. We previously reported that CLOCK and BMAL1 binding to the E-box of the DEC1 promoter results in the production of DEC1 protein [1,2]. The protein products of DEC1 then suppress CLOCK and BMAL1 transactivation through the E-box of *Dec1* and *Per1* [1,2,11]. This negative feedback regulation plays an important role in circadian rhythms. It has been suggested that disturbance of circadian rhythms may play a role in inducing various behavioral and physiological dysfunctions such as depression, a short sleep cycle, diabetes, metabolic syndrome, Alzheimer's disease and cancer [12–20]. Therefore, clock genes have pivotal roles not only in the regulation of circadian rhythms but also in health maintenance. It has also been reported that abnormalities in DEC1 expression affect various phenomena. Hypoxia, insulin, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β and paclitaxel induced DEC1 expression, whereas cisplatin decreased it; altered DEC1 expression correlated with the phase shift of other clock genes, with immune disorders and with tumor progression [3–6,21–24]. DEC1 overexpression delayed the circadian phase of *Per1*, *Dec2* and *Dbp* [22]. *Dec1* and *Dec2* double-mutant mice displayed altered sleep patterns and an impaired working memory [25]. DEC1 has a pro-apoptotic function in human breast cancer MCF-7 cells, but it induces EMT in human pancreatic cancer Panc-1 cells [4–6]. However, the role of DEC1 in the metabolic process remains unclear.

AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that activates the metabolic process in response to energy demands, such as exercise, heat shock and a low glucose level [26,27]. AMPK has three subunits, α, β and γ; and the α-subunit plays a crucial role in catalytic activity [28,29]. Phosphorylation of Thr 172 in AMPK (pAMPK) is required for AMPK activation, and serine/threonine kinase liver kinase B1 (LKB1) directly mediates this event [30,31]. AMPK activity regulates the function of peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), acetyl-Co A carboxylase (ACC1 and ACC2), glucose-transporter type 4 (GLUT4) and tuberous sclerosis complex (TSC)-2 in the metabolic process, cell growth and tumor progression [32]. A recent report showed that AMPK activity showed a circadian rhythm in the hypothalamus and peripheral tissues and that AMPKα knockout mice displayed a shorter period of free running and disruption of the circadian pattern of *Clock*, *Bmal1* and *Per2* gene expression [33]. It was recently reported that AMPK activity regulates CRY1 phosphorylation and degradation and SIRT1 activity [34,35]. Therefore, AMPK activity is closely associated with clock and clock control genes. In this study, we investigated the relationship between DEC1 expression and AMPK activity. We showed that peak circadian level of DEC1 protein in the mouse liver was inversely correlated with that of AMPK activity. We demonstrated that DEC1 negatively regulated AMPK activity via LKB1. These findings will contribute to a better understanding of the biological function of DEC1 protein and AMPK activity in the circadian rhythm and the metabolic process.

2. Material and methods

2.1. Animals

Six-week-old male C57BL/6 mice were housed as described previously [3,8]. The mice were maintained in a room where the lights were turned on at 8:00 AM (ZT0) and turned off

at 8:00 PM (ZT12). Tissue samples from three mice were extracted from the livers and hearts for western blotting and immunohistochemistry. All animal care and use procedures were approved by the Wakayama Medical University Institutional Animal Care and Use Committee (Wakayama Medical University Permit Number: 660).

2.2. Cell culture and treatment

Cells of the WI-38 human fibroblast cell line, the MCF-7 human breast cancer cell line, and the U2OS human osteosarcoma cell line were obtained from the American Type Culture Collection (ATCC, VA, USA). These cells were cultured in Dulbecco's modified Eagle's medium (Sigma Chemical Co., MO, USA), supplemented with 10% fetal bovine serum and maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.3. DEC1 transfection

FLAG-DEC1 wild type (WT), FLAG-DEC1 Δbasic or FLAG-DEC1R65A expression vectors, the latter of which has point mutation in the basic domain of DEC1, were used for transient transfection, which was performed as described previously [1]. These plasmids were transfected using Fugene HD reagents (Roche Applied Science). DEC1 siRNA was synthesized by Life Technologies. The sequence of the sense and anti-sense DEC1 siRNA were 5′-r (GUAGUGAUUAGCUUACUAA)-3′ and 5′-r (UUAGUAAGCUAAUCACUAC)-3′, respectively. For the siRNA transfection experiments, the cells were seeded at 5×10^4 cells per 35-mm well. After 24 h, the siRNA were transfected into the cells using the Lipofectamine iMAX reagent (Life Technologies). After transfection, the cells were cultured for 24–72 h and then were subjected to western blotting analyses.

2.4. Antibodies

The following commercial antibodies were purchased: actin (Millipore, MA, USA), DEC1 (Novus Biologicals Inc., CO, USA), pAMPK, AMPK, pLKB1 and LKB1 (Cell Signaling Technology, Inc., MA, USA). A mouse monoclonal antibody directed against FLAG was produced in house.

2.5. Western blotting

The cells treated with a siRNA or plasmid were lysed using 0.5% NP-40 lysis buffer for western blotting. The total cell lysates were run on 12.5% SDS-polyacrylamide gels, followed by western blotting, using standard procedures. The WesternBright ECL and Western Bright Sirius kits (BioExpress, UT, USA) were used for antibody detection. The detection was performed using Image Saver 5 for Ez-Capture MG (ATTO, Osaka, Japan), and the exposure times for detection were at 1, 2 and 5 min.

2.6. ChIP assay

A ChIP assay was performed using a kit purchased from the IMGENEX Corporation (CA, USA) according to the manufacturer's instructions. Primers were designed to amplify a fragment of the LKB1 promoter containing the E-box element. Their sequences were as follows: human LKB1-F1, 5′-TTCTGTCTGTAATGGTCACC-3′ and R1, 5′-

CTGTACTGATTGCTGGCTCA-3′. Primers were also designed to amplify-fragments of the LKB1 promoter lacking an Ebox. Their sequences were: human LKB1-F2, 5′-GGGACCTACCGATGCCAATT-3′, R2, 5′-TGCCACTGCACTCCAGCTTG-3′, human LKB1-F3, 5′-CCAGCTAAACAACCTGGCGC-3′ and R3, 5′-GGAAGCCTCAGGACAGCGCT-3′.

2.7. Real-time PCR

We prepared three independent RNA samples (n = 3) from MCF-7 cells for real-time PCR. The total RNA was isolated and first-strand cDNAs were synthesized as previously described [4]. Real-time PCR was performed using SYBR-Green Master Mix (Thermo Fisher Scientific, MA, USA). The sequences of the primers for LKB1 amplification were as follows: LKB1-F, 5′-CATTTGGTGCCAAGAATGGGA-3′ and R, 5′-AGGCTCCGAAGCTCATTGC-3′. The amplified LKB1 product contained 77 bp.

2.8. Immunohistochemistry

DEC1 expression and AMPK activity in mouse liver and heart tissues were evaluated using serial deparaffinized sections. The sections were incubated overnight at 4 °C with anti-DEC1 and pAMPK antibodies and then treated with an HRP-conjugated secondary antibody. Labeling was detected using a Dako EnVision kit/HRP (DAB) (DakoCytomation, Kyoto, Japan). The sections were counterstained using Mayer's hematoxylin.

2.9. Data quantification

The intensities of the bands obtained from western blotting were quantified using Image J software.

3. Results

3.1. Circadian rhythms of DEC1 protein and AMPK activity in the mouse liver

To investigate whether the levels of DEC1 protein and AMPK activity showed circadian rhythms *in vivo*, we performed western blotting and immunohistochemical analyses using samples prepared from mouse livers and hearts at the indicated ZTs. The levels of DEC1 protein and AMPK activity showed circadian rhythms, in which the peak level of DEC1 protein in the liver was observed at ZT6, whereas that of AMPK activity was observed at ZT18. A high level of total AMPK was detected in the liver at every time point (Fig. 1A). Strong positive staining for DEC1 was observed in both the nuclei and cytoplasm of hepatocyte and myocardial cells at ZT6, whereas faintly positive staining for pAMPK was observed in the cytoplasm of hepatocyte and myocardial cells (Fig. 1B).

3.2. DEC1 negatively regulated AMPK activity and LKB1

To investigate a direct correlation between the levels of DEC1 protein and AMPK activity, we knocked down DEC1 expression in MCF-7, U2OS and WI-38 cells using siRNA. Knocking down DEC1 expression for 48 and 72 h increased both AMPK and LKB1 activity as well as the total LKB1 level (Fig. 2). In addition, we overexpressed DEC1-WT and Δbasic and R65A mutants in MCF-7 cells. Overexpressing DEC1-WT decreased both

AMPK and LKB1 activity as well as the total LKB1 level, whereas overexpressing the Δbasic and R65A DEC1 mutants had little effect on either AMPK or LKB1 activity or the total LKB1 level (Fig. 3A). Overexpressing DEC1-WT decreased the LKB1 mRNA level, whereas overexpressing the DEC1 mutants had little effect on that level (Fig. 3B).

3.3. DEC1 bound to the E-box of the LKB1 promoter

To explore how DEC1 regulates LKB1, we performed a ChIP assay. We designed three sets of primers to amplify three different regions of the LKB1 promoter (Fig. 4A). Endogenously and exogenously expressed WT-DEC1 protein bound to the E-box of the LKB1 promoter, whereas the exogenously expressed mutant DEC1 proteins did not bind tightly to this E-box (Fig. 4B). Overexpressing the Δbasic or R65A DEC1 mutant had little effect on their binding to the LKB1 promoter. These results suggest that DEC1 suppresses LKB1 by directly binding to the E-box of the LKB1 promoter.

4. Discussion

A circadian relationship between AMPK activity and clock genes has been reported [33,34]. AMPK activity showed circadian rhythms in the mouse hypothalamus and liver, and the adipose tissue in AMPKα knockout mice disrupted circadian rhythms of *Per2, Bmal1* and *clock* [33,34]. It has also been reported that AMPK activity regulated CRY1 phosphorylation [34]. However, how the circadian rhythm of AMPK activity is regulated is unclear. Generally, DEC1 negatively regulates the expression of target genes through an E-box [1,2,11]. We found the gene for *LKB1*, which is central upstream factor of AMPK activity, has an E-box in its promoter and speculated that DEC1 may regulate AMPK activity via LKB1.

Much is known about the circadian rhythm of DEC1 mRNA levels [2,8–10,22,36–38]. However, the circadian rhythm of DEC1 protein is not well understood. At first, we investigated the relationship between the levels of DEC1 protein and AMPK activity over time. We observed rhythmic levels of DEC1 protein and AMPK activity in the mouse liver and found that their peak levels occurred at different times. Using immunohistochemistry, the level of DEC1 protein in the liver and heart at ZT 6 was found to be inversely correlated with the level of AMPK activity. These results suggested that the circadian rhythm of the DEC1 protein level and the level of AMPK activity in peripheral tissues may be inversely correlated. To examine whether DEC1 directly regulated AMPK activity, we performed DEC1-knockdown and-overexpression experiments. Knocking down DEC1 expression increased the level of AMPK activity, whereas DEC1 overexpression decreased it. This evidence demonstrated that DEC1 negatively regulated AMPK activity. We further examined how DEC1 regulated AMPK activity. LKB1 is a central upstream factor of AMPK activity [30]. We found that the *LKB1* gene has an E-box in its promoter and examined whether DEC1 binds to this E-box. DEC1 bound to the E-box of the LKB1 promoter and decreased the level of LKB1 activity and the total LKB1 levels. These results suggested that DEC1 negatively regulated AMPK activity via LKB1 and that DEC1 is an upstream factor of LKB1 and AMPK activities. In addition, overexpressing WT DEC1 decreased the level of LKB1 mRNA expression. This result indicated that DEC1 regulated the level of LKB1

activity via its transcriptional levels. Furthermore, the Δbasic and R65A DEC1 mutations had little effect on the binding of DEC1 to the E-box of the LKB1 promoter or on the AMPK activity and LKB1 levels. We previously reported that these mutant proteins could not bind to the E-box of the DEC1 promoter [1,11]. Other groups also showed that the Δbasic DEC1 mutant could not bind to the E-box [39,40]. These results definitely support our findings.

In this study, we showed for the first time that the levels of DEC1 protein expression patterns inversely correlated with AMPK activity levels in mice tissues. We demonstrated that DEC1 negatively regulated AMPK activity via LKB1. It is possible that DEC1 regulates aspects of factors downstream of AMPK activity, such as those of PGC-1α, ACC1, ACC2, GLUT-4 and TSC-2.

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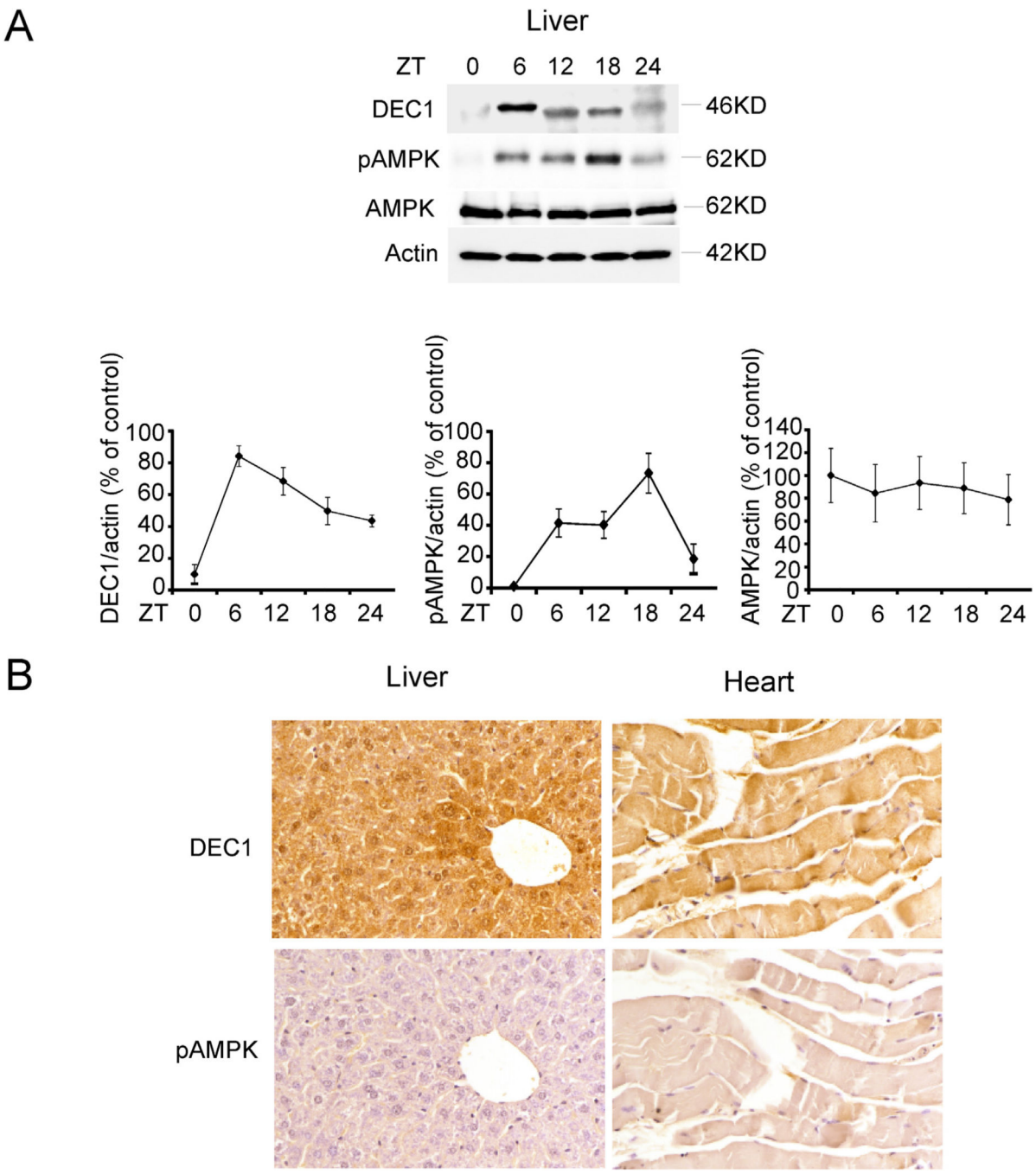

Fig. 1.

Circadian rhythms of the DEC1 protein and AMPK activity levels in mice. (A) Mice were housed under the light–dark conditions described in the Material and methods section. Liver samples were collected from the mice and subjected to western blotting analyses for DEC1, pAMPK, AMPK and actin levels. One representative sample from at least three independent experiments with similar results is shown. The intensities of the DEC1, pAMPK and AMPK bands were quantified and divided by that of actin. Each value is the mean value ± SE (bars) from three independent experiments. (B) Liver and heart samples were collected from mice

at ZT 6 and were subjected to immunohistochemical analysis of the DEC1 and pAMPK levels. One representative sample from at least three independent experiments with similar results is shown.

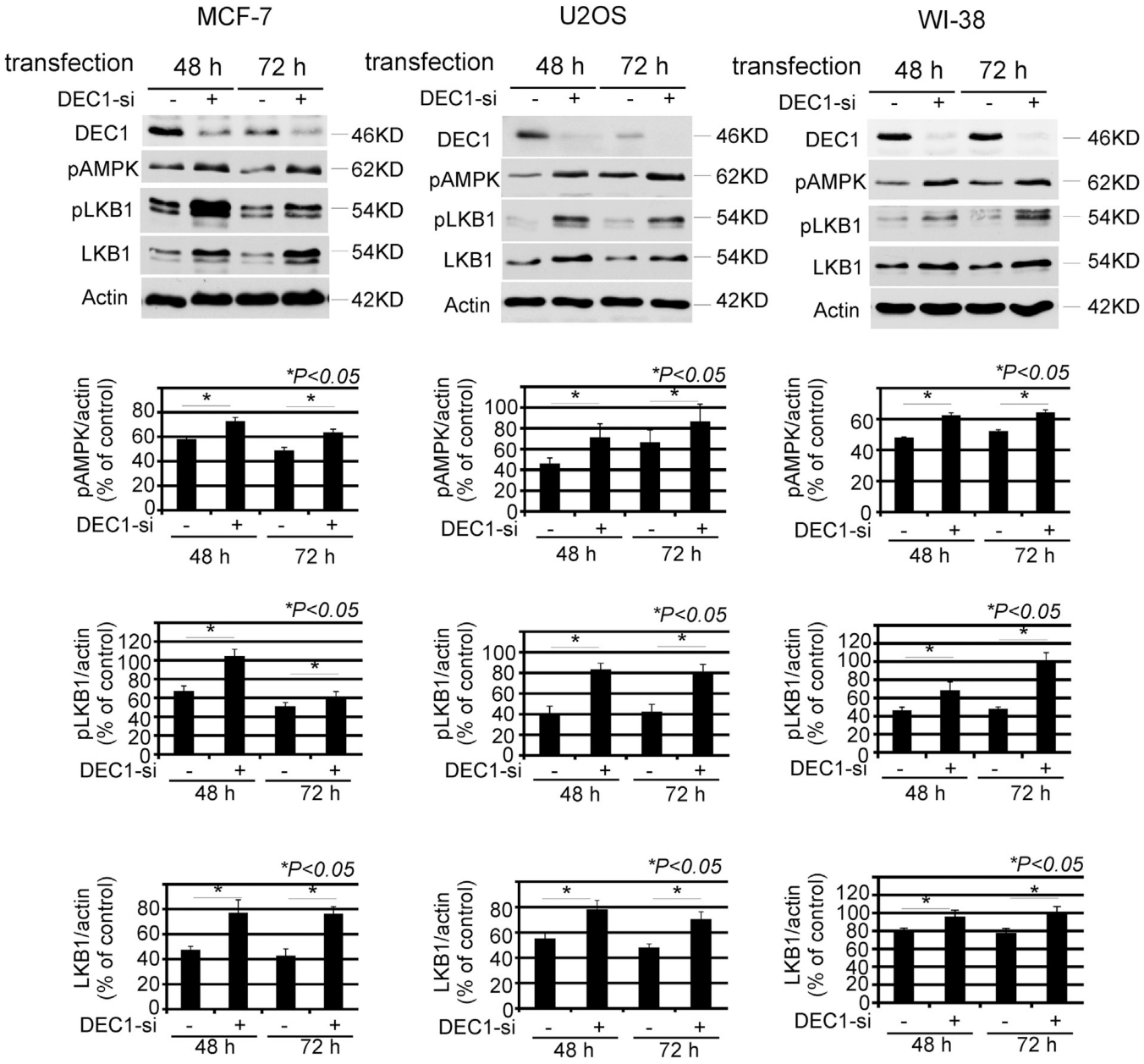

Fig. 2.

DEC1 knockdown increased AMPK activity and LKB1. MCF-7, U2OS and WI-38 cells were transfected with control or DEC1 siRNA for 48 and 72 h, and the lysates were subjected to western blotting analyses for DEC1, pAMPK, pLKB1, LKB1 and actin levels (top panel). One representative sample from at least three independent experiments with similar results is shown. The intensities of the DEC1, pAMPK, pLKB1 and LKB1 bands were quantified as described above (bottom panels). Each value is the mean value ± SE (bars) from three independent experiments. $*P < 0.05$, as determined using the t-test.

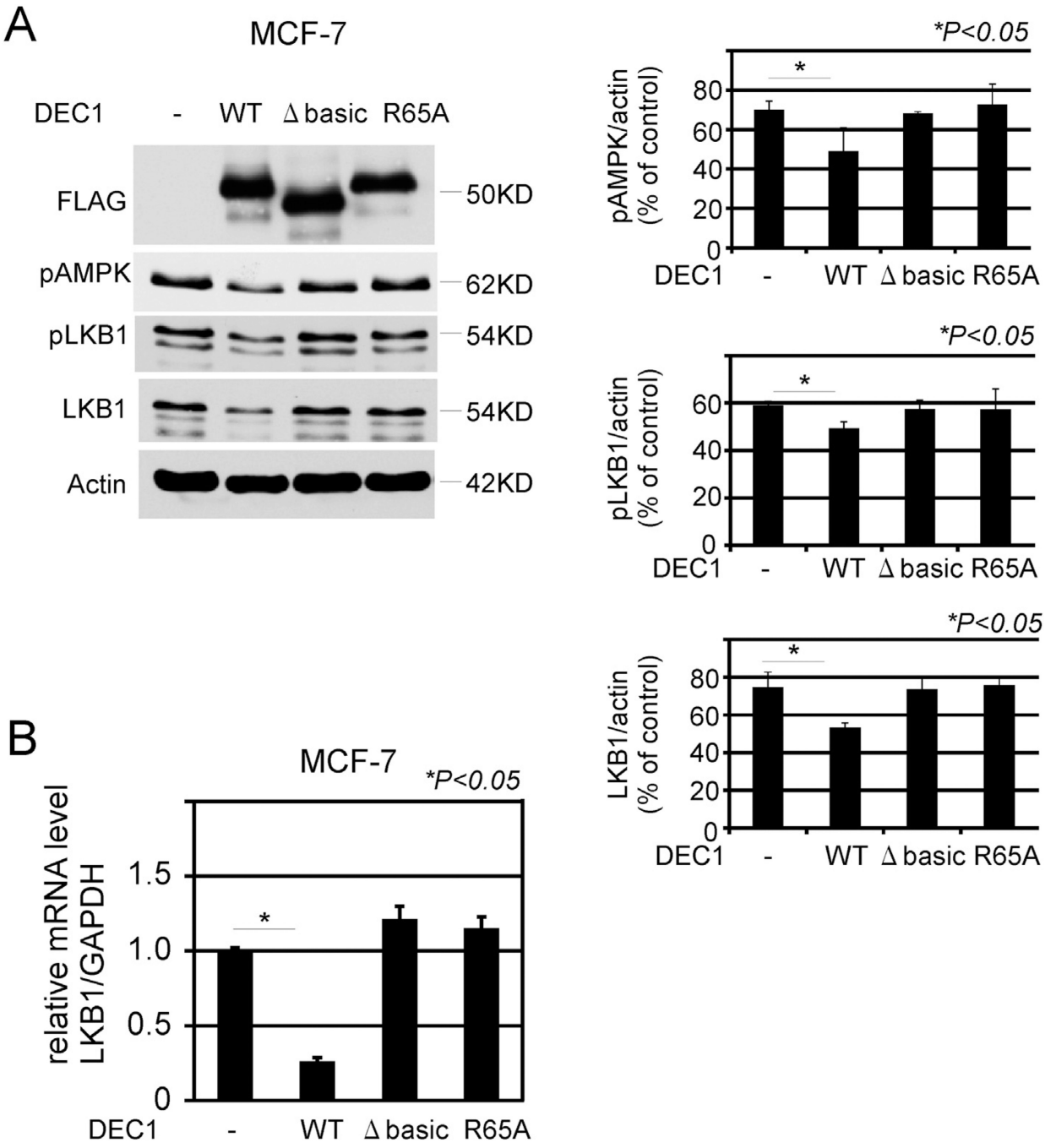

Fig. 3.

DEC1 overexpression decreased AMPK activity and LKB1. (A) MCF-7 cells were transfected with pcDNA, DEC1-WT, DEC1Δbasic or DEC1R65A. At 24-h post-transfection, cell lysates were prepared and were subjected to western blotting analyses for FLAG, pAMPK, pLKB1, LKB1 and actin levels (top panel). One representative sample from at least three independent experiments with similar results is shown. The intensities of the pAMPK, pLKB1 and LKB1 bands were quantified as described above (right panels). Each value is the mean value ± SE (bars) from three independent experiments. **P* < 0.05, as

determined using the t-test. (B) MCF-7 cells were transfected with pcDNA, DEC1-WT, DEC1Δbasic or DEC1R65A as described above, and the total RNAs were prepared and subjected to real-time PCR analysis of the LKB1. Each value is the mean value ± SE (bars) from three independent experiments. *$P < 0.05$, as determined using the t-test.

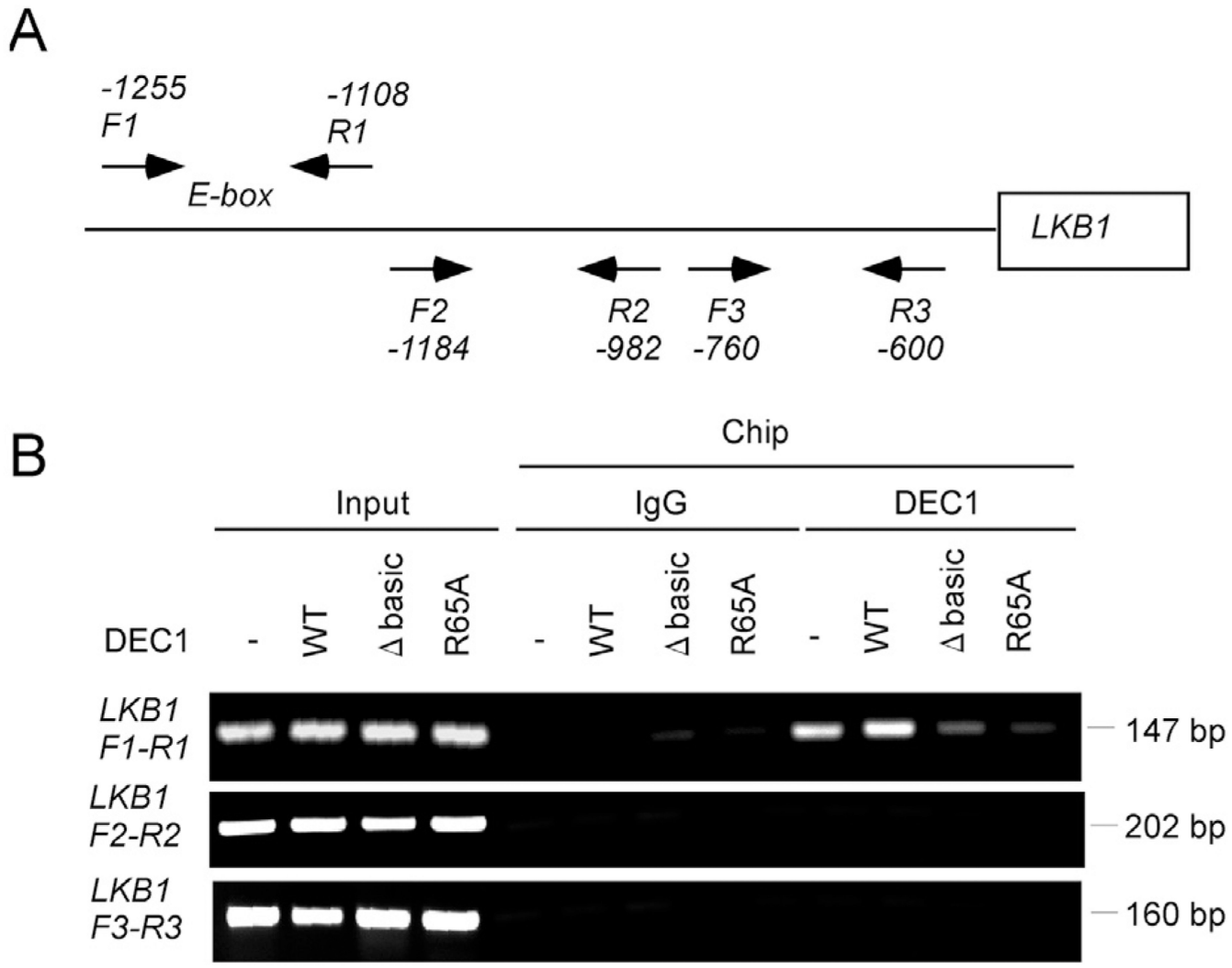

Fig. 4.

DEC1 bound to the E-box of the LKB1 promoter. (A) Primer design scheme for the ChIP assay. The F1 and R1 product included the E-box of the LKB1 promoter, whereas the F2 and R2, or F3 and R3 products did not. (B) A ChIP assay was performed using MCF-7 cells that had been transfected as described above. The eluted DNA fragments were subjected to PCR analysis. Anti-rabbit IgG was used as an immunoprecipitation control. One representative sample from at least three independent experiments with similar results is shown.