Differentiated embryo chondrocyte 1 (DEC1) is a novel negative regulator of hepatic fibroblast growth factor 21 (FGF21) in aging mice

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Running Title: DEC1, a transcriptional repressor of FGF21

ABSTRACT

Human differentiated embryo chondrocyte 1 (DEC1) is frequently used as a marker of senescence *in vivo*. Fibroblast growth factor 21 (FGF21), a novel endocrine-like member of the FGF superfamily, is highly expressed in the liver, and FGF21-transgenic mice have extended lifespans. Thus, we hypothesized that FGF21 may play a role in the DEC1-mediated aging process. In this study, DEC1 knockout (KO) mice were used to characterize the mechanism by which FGF21 protects mice from aging. Aging is strongly diminished in DEC1 KO mice, which is reflected by decreased lipid levels and oxidative stress, leading to an amelioration of liver function and structure. The expression of FGF21 decreased with aging in wild-type (WT) mice, whereas ATF4, Phospho-ERK and Phospho-p38 expression was maintained and was accompanied by a compensatory rise of FGF21 mRNA and protein expression in DEC1 KO mice. Over-expression of DEC1 markedly abolished the hepatic expression of FGF21, and siRNA-mediated inhibition of endogenous DEC1 increased the expression of FGF21. DEC1 further diminished the expression of ATF4 in HepG2 cells over-expressing DEC1. The induction of FGF21 and ATF4 at the mRNA and protein levels during the

course of aging supports the view that DEC1 KO mice are able to restore the age-related imbalance of metabolism. Collectively, the data obtained in this study suggest that DEC1 is a novel negative regulator of hepatic FGF21 expression.

Keywords: DEC1, FGF21, aging, liver, hepatic homeostasis

1. Introduction

Human differentiated embryo chondrocyte 1 (DEC1), also known as BHLHE40, Bhlhb2, Stra13 and Sharp-2, one of the clock genes, is a basic helix-loop-helix transcription factor that is involved in regulating circadian rhythms, cell proliferation, differentiation, apoptosis and cellular senescence [1-3]. It has been suggested that disturbances of circadian rhythms may play a role in the induction of various behavioral and physiological dysfunctions, such as diabetes, metabolic syndrome and Alzheimer's disease [4-7]. Therefore, DEC1 has pivotal roles not only in the regulation of circadian rhythms but also in health maintenance. DEC1 is frequently used as a marker of senescence *in vivo*, and it can directly induce senescence in cultured cells [1,8,9]. Dysregulation of the molecular clockwork of aging could, at least in part, be ascribed to this mechanism, whose impact on different tissues is poorly characterized

but is of crucial importance for understanding the process of aging.

Metabolic stresses, including dietary protein restriction [10], endoplasmic reticulum stress [11] and mitochondrial dysfunction [12], either positively or negatively, modulate the expression of fibroblastic growth factor 21 (FGF21) in the liver. The liver integrates a variety of signals into FGF21 gene expression and elicits the systemic regulation of energy metabolism. In mice, FGF21 plays a physiological role in suppressing the rate of lipolysis, functioning as a metabolic regulator of lipid metabolism in concert with growth hormones [13]. FGF21 also regulates energy homeostasis by activating mitochondrial oxidative function that enhances the oxidative capacity of adipose via an AMP-activated protein kinase (AMPK)-dependent pathway [14]. A recent study demonstrated that FGF21 is a target gene of activating transcription factor 4 (ATF4) [15]. FGF21 activates AMPK and up-regulates the expression of genes required for mitochondrial biogenesis and fatty acid β-oxidation [14].

More recently, it was demonstrated that FGF21 is capable of extending the lifespan of mice [16]. Aging is a gradual biological process and induces the dysfunction of normal cells. Increasing evidence indicates that oxidative stress plays very important roles in the process of aging [17,18]. Considering the fact that FGF21 transgenic mice have a significantly extended lifespan, we speculated that FGF21, as a hormone, may

prevent organisms from aging. In order to prove that hypothesis, we used the DEC1 knockout (KO) aging mouse model to investigate the role that FGF21 plays in protecting the liver against aging. DEC1 KO mice have a longer median survival time than wild-type (WT) mice (30 months vs 26 months). Our results demonstrate for the first time that FGF21 has potent protective effects against aging in DEC1 KO mice via its activation of ATF4, which in turn triggers the ERK-mediated pathway in the liver. Our results uncover a critical role for FGF21 in aging that functions to minimize lipid accumulation and the ensuing cellular stress in the liver. FGF21 is not only a potent modulator of glucose/lipid metabolism, but is also a novel and promising hepatoprotective agent.

2. Material and Methods

2.1. Animals

$DEC1^{-/-}$ (DEC1 KO) mice were generated by the inGenious Targeting Laboratory, Inc. (Stony Brook, NY, USA). The 4.7-kb BamHI-BssSI genomic fragment of DEC1, which contains the entire coding region in exons 1 to 5, was replaced with a Neo cassette. The resulting chimeric mice were back-crossed to a C57BL/6J background for three generations. Male DEC1 KO mice (n = 6) and their WT littermates (n = 6) that

were 3 months and 24 months of age were individually housed under specific pathogen free conditions. All procedures were performed in compliance with the standard principles and guidelines for the care and use of laboratory animals of Nihon University School of Dentistry at Matsudo.

2.2. Cell Culture

The human hepatocellular carcinoma HepG2 cell line was a kind gift from Dr. Keiji Tanimoto (Hiroshima University). Cells were cultured in Eagle's Minimum Essential Medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum at 37 ºC in a humidified atmosphere of 95% air and 5% CO_2.

2.3. Antibodies

The antibody against mouse DEC1 was provided by Professor Yukio Kato (Hiroshima University). The anti-FGF21 and anti-β-galactosidase antibodies were purchased from LifeSpan Biosciences, Inc. (Seattle, WA, USA), the anti-ATF4 antibody was from Proteintech Group, Inc. (Chicago, IL, USA), anti-p16, anti-superoxide dismutase 1 (SOD1) and anti-glutathione peroxidase 1 (GTx1) antibodies were from Abcam (Tokyo, Japan), and the anti-ERK, anti-Phospho-ERK, anti-p38, anti-Phospho-p38, and anti-β-actin antibodies were from Cell Signaling Technology (Danvers, MA, USA). The peroxidase-conjugated anti-rabbit secondary antibody was

from Nichirei Biosciences (Tokyo, Japan).

2.4. Quantitative Real Time-PCR (QRT-PCR)

Total RNAs were isolated using an RNeasy Mini kit (Qiagen, Hilden, Germany) and a TURBO DNA-*free*™ Kit (Applied Biosystems, Tokyo, Japan) was used to remove contaminating DNA from the RNA preparations. First-strand cDNAs were synthesized from 1 μg total RNA using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems). Real-time PCR was carried out in 96-well plates using the ViiA™ 7 Real-Time PCR System (Applied Biosystems). All reactions were done in triplicate. The TaqMan probes (Mm00478593_m1 for DEC1, Mm00840165_g1 for FGF21, Mm00515325_g1 for ATF4 and Mm02619580_g1 for β-actin) were obtained from Applied Biosystems. Cells transfected with an empty plasmid without reverse transcriptase were used as a negative control.

2.5. Short interference RNA (siRNA)

siRNAs against DEC1 were synthesized by Qiagen. The sequences for the sense and antisense DEC1 siRNAs were 5′-r (CCAAAGUGAUGGACUUCAA) d (TT)-3′ and 5′-r (UUGAAGUCCAUCACUUUGG) d (GA)-3′, respectively. We also used another siRNA against DEC1 (DEC1 siRNA-2). The sequences for the sense and antisense DEC1 siRNA-2 were 5'-r (GAAGCAUGUGAAAGCACUA) d (TT)-3' and 5'-r

(UAGUGCUUUCACAUGCUUC) d (AA)-3', respectively. The negative control (scrambled) siRNA sequences were 5’-r (UUCUCCGAACGUGUCACGU) d (TT)-3’ and 5’-r (ACGUGACACGUUCGGAGAA) d (TT)-3’, respectively. For the siRNA transfection, HepG2 cells were seeded at $5x10^4$ cells per 35-mm dish. The siRNAs were transfected into the cells using the lipofectamine RNA iMAX reagent (Invitrogen, Carlsbad, CA, USA), and the cells were incubated for 48 h and then subjected to various analyses.

2.6. Over-expression of DEC1

Transient transfections with the expression plasmids for human DEC1 pcDNA were performed using Lipofectamine LTX (Life Technologies, Tokyo, Japan) in HepG2 cells. The cells were incubated for 48 h and then subjected to various analyses.

2.7. Western blotting

Cells were lysed in RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein concentrations were determined using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). DEC1, FGF21, ATF4, ERK, Phospho-ERK and β-actin were used as primary antibodies. The anti-rabbit secondary antibody was used at a dilution of 1:2000. Bound antibodies were visualized by chemiluminescence using the ECL Plus Western Blotting Detection System (Amersham, Uppsala, Sweden), and

images were analyzed using a Luminescent Image Analyzer (LAS-4000 mini; GE Healthcare UK Ltd, Buckinghamshire, England).

2.8. Immunohistochemistry

Liver specimens from DEC1 KO mice and from WT mice were subjected to antigen retrieval with sodium citrate buffer, pH 6.0 (Abcam) for 20 min. After an overnight incubation with the primary antibodies at 4 °C, the specimens were incubated at room temperature for 30 min with the secondary antibody conjugated to peroxidase. After rinsing with PBS, all specimens were color-developed with diaminobenzidine (DAB) solution (DAKO, Tokyo, Japan) and were counterstained with hematoxylin. The immunostaining of all specimens was performed simultaneously to ensure the same antibody reaction and DAB exposure conditions. The stained cells were photographed and the positive cells were assessed in four randomly chosen fields per specimen.

2.9. Statistical analysis

Student's two-tailed t-test was used to determine p values. A $p<0.05$ is considered to be statistically significant.

3. Results

3.1. Opposing functions of DEC1 and FGF21 in mouse liver aging

To investigate the changes in senescence expression that occur during aging of the liver, liver tissues of young adult (3 months old) as well as older (24 months old) WT and DEC1 KO mice were studied using immunohistochemistry for the presence of β-gal, DEC1, and p16 proteins. Fig. 1 shows a significant increase of β-gal, DEC1, and p16 expression in older WT mice. No similar increases were seen in older DEC1 KO mice for either protein. To explore how oxidative stress mediates aging-induced liver dysfunction, we performed immunohistochemistry for SOD1 and GTx1. In aged WT mice, SOD1 was mainly detected in hepatocytes from the centrolobular region and within endothelial cells of the capillary and arteriolar branches of the hepatic artery in the portal spaces (Fig. 2). The distribution of SOD1 in the mouse liver upon aging in WT mice showed an increased immunostaining for GTx1 in the same areas as well as an additional staining in midzonal hepatocytes (Fig. 2). No similar dramatic increase in levels of SOD1 or GTx1 was seen in aged DEC1 KO mice. We also assessed whether the increased senescence and oxidative stress in the mouse liver correlates with increased hepatic lipid levels. Aged WT mice accumulated significantly more hepatic lipids (Fig. 2) than aged DEC1 KO mice, which is likely related to higher senescence and oxidative stress. In young WT samples, positive staining for FGF21, ATF4, ERK and Phospho-ERK was usually noted along the peri-sinusoidal regions and within the

hepatocytes (Fig. 3). In young DEC1 KO liver specimens, hepatocyte staining for those markers was more evident in the periportal regions and was quite heterogeneous, with some cells having more intense staining than others (Fig. 3). Staining was also prominent at the inter-hepatocytic junctions where bile canaliculi are situated (Fig. 3). There was occasional staining of intra- and extra-hepatocytic lipid particles, arterial walls, and lymphatic walls and lumens in aged WT mice (Fig. 3). No similar dramatic increase in levels of lipid was seen in aged DEC1 KO mice. Our data provide the first evidence that there is an inverse expression pattern between DEC1 and FGF21 protein in the mouse liver upon aging.

3.2. FGF21 attenuates liver damage in DEC1 KO mice

To investigate whether FGF21 can protect DEC1 KO mice against aging-induced hepatic dysfunction, we determined the expression of FGF21 and ATF on the ERK and p38 pathway in the mouse liver. FGF21 protein and mRNA expression levels were maintained during the course of aging in DEC1 KO mice (Fig. 4A). In contrast, the expression of FGF21 was also high in young WT mouse liver, but was robustly decreased in old WT mice. Strikingly, p38 phosphorylation was found higher in the liver of old WT mice than in young counterparts. The expression of ATF4, Phospho-ERK, and Phospho-p38 was maintained (Fig. 4A) and was accompanied by a

compensatory rise of FGF21 mRNA and protein expression in DEC1 KO mice.

3.3. DEC1 negatively regulates FGF21 and ATF4

To investigate a direct correlation between the levels of DEC1 protein and FGF21, we over-expressed pcDNA and DEC1 pcDNA in HepG2 cells. Over-expression of DEC1 repressed both FGF21 and ATF4 proteins as well as their mRNA expression levels (Fig. 4B). Next, we performed knockdown assays for DEC1, to estimate how the DEC1 pathway contributes to FGF21 expression. Transfection of a specific siRNA targeting DEC1 in HepG2 cells induced both FGF21 and ATF4 expression compared to the nonspecific siRNA control, suggesting that the knockdown of DEC1 reversed the repression of FGF21 and ATF4 (Fig. 4B).

4. Discussion

Despite intensive research about the metabolic functions of FGF21, its role in aging has not been explored. This study provides novel evidence that a deficiency of FGF21 causes a marked exacerbation of aging and increases the mortality of WT mice, suggesting that FGF21 is a physiological protector against aging.

The accumulation of oxidative stress resembles natural aging in animals. Oxidative stress has been recognized to play major roles in aging and age-associated diseases. It

has been reported that oxidative stress damage reduces the expression of exogenous FGF21 [19] and chronic over-expression of FGF21 extends the lifespan of mice [20]. The results of the present study confirm that aging results in the increased generation of free radicals followed by oxidative damage in the liver of old WT mice. There is a possible explanation for the loss of FGF21 expression in the aging liver. Physical hypoxia (1% oxygen) and chemical inducers of hypoxia (such as cobalt chloride treatment) can decrease both FGF21 mRNA and secreted FGF21 protein levels [19]. It is known that hypoxia-related genes, including DEC1, are active in the hypoxic microenvironment where low levels of oxygen may contribute to the loss of FGF21 in the liver during aging.

In order to understand the mechanism by which FGF21 prevents aging, we developed the DEC1 KO aging model. FGF21 was observed to reverse the decrease of SOD activity, the endogenous antioxidant enzyme that can scavenge ROS or prevent their formation [21], which supports our finding that FGF21 can prevent the aging of DEC1 KO mice via the suppression of oxidative stress.

Targeting the liver signaling axis involving lipid accumulation would be a novel approach for the treatment of aging patients. FGF21 regulates lipid metabolism [22], which suggests that the deletion of FGF21 leads to altered lipid metabolism and the

ectopic accumulation of fat in the liver of mice lacking FGF21. Our histological and immunologic analyses demonstrate that the depletion of FGF21 in WT mice causes a markedly increased lipid accumulation, which can be reversed by the replenishment of DEC1 KO mice, suggesting that FGF21 is able to inhibit one or more key pathogenic events of DEC1-mediated aging. To our knowledge, this is the first report demonstrating the pharmacology and mechanism of FGF21 action in this species.

It is well documented, and the present study confirms, that aging is characterized by a decrease of DEC1-dependent FGF21 expression. The over-expression of DEC1 markedly abolishes hepatic FGF21 expression, and siRNA-mediated inhibition of endogenous DEC1 increases FGF21 expression. Since ATF4 is mainly involved in regulating FGF21 expression, DEC1 further diminishes the expression of ATF4 in DEC1 over-expressing HepG2 cells. Collectively, the data obtained in this study suggest that DEC1 is a novel negative regulator of hepatic FGF21 expression. We also checked if DEC1 was involved in the induction of FGF21 expression in mouse models of aging. Old DEC1 KO mice showed an increased expression of FGF21 in the liver, agreeing with the in vitro data reporting the elevation of DEC1 in old WT mice.

Published data suggest that FGF21 regulates AMPK through an interaction with LKB1 [14], and LKB1/AMPK activation plays a critical role in controlling Cyclin D

expression [23]. We recently reported that DEC1 negatively regulates AMPK activity via LKB1 [24]. Our previous data clearly showed that DEC1 binds to the E-box of the Cyclin D1 gene and suppresses its activity and expression [25]. FGF21 signaling is also mediated by ERK phosphorylation [26]. Injection of mice with FGF21 was shown to induce ERK1/2 phosphorylation and immediate-early gene expression in the liver [22], suggesting that at least some direct effects may occur at pharmacologic concentrations. Moreover, ERK1/2 signaling promotes cell proliferation by regulating cell cycle progression during liver regeneration in mice [27]. Our data indicate that aging in WT mice reduces the basal level of ERK phosphorylation and induces p38 phosphorylation. The basal level of phosphorylation of p38 is increased in aged rat livers, suggesting an age associated increase in p38 activity in aged tissues [28]. DEC1 KO mice are sufficient to enhance ERK phosphorylation, but the aged mice cannot further stimulate that phosphorylation, which is probably due to the fact that the basal phosphorylation of these molecules is saturated under this condition. There were no appreciable changes in the phosphorylation of c-Jun NH_2-terminal kinase (JNK) in the liver of WT and DEC1 KO mice (data not shown).

Another positive effect of FGF21 is a life-altering activity, because it has been reported that mice over-expressing FGF21 have an extended lifespan [16]. Because

DEC1 KO mice exhibit a better health status and also an improved survival, it is reasonable to conclude that these mice benefit from the systemic secretion of FGF21 protein.

One important remaining question in this study is the mechanism by which DEC1 reduces FGF21 gene expression. The presence of a canonical E-box (-51 to -45) in the regulatory region of the mouse FGF21 promoter [29] suggests that this gene may be a direct target of DEC1 that suppresses the E-box. The possible regulation of FGF21 degradation by DEC1 is intriguing because studies have shown the E-box regulation by DEC1 in several tissues [24,25,30]. We surmise a number of possibilities that could explain this situation in this study, and luciferase reporter and chromatin immunoprecipitation assays will be required to define the mechanism of repressor regulation by DEC1. However, other transcription factors and/or indirect mechanisms (e.g. miRNA pathways) may also contribute to the regulation of FGF21 expression by DEC1.

Our data provide convincing evidence that the reduced expression of DEC1 provides a more parsimonious explanation for the observed increases in FGF21. DEC1 KO mice have rescued the age-related imbalance of metabolism and have increased expression levels of FGF21, both most probably contributing to the improved health

status at an advanced age. This finding indicates the importance of FGF21 in liver aging and suggests its potential application to promote hepatic homeostasis in liver aging in humans.

Conflict of interest

None declared.

Acknowledgments

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Figure Legends

Fig. 1. Effect of aging on β-galactosidase (β-gal) and DEC1 expression in mouse liver. Livers isolated from young (3-month-old ; 3M) and from old (24-month-old ; 24M) wild type (WT) and DEC1 KO mice were immunostained for β-gal, DEC1, and p16. Increased numbers of β-gal and p16-positive cells are seen in the liver within

hepatocytes of aged WT mice compared to DEC1 KO mice. Similar differences relative to the DEC1 KO group are seen in DEC1 immunostaining. Cells stained brown are positive for β-gal, DEC1 and p16. Scale bars = 100 μm.

Fig. 2. WT mice accrue hepatic lipid and oxidative stress during aging. Photomicrographs show representative liver sections from WT mice and from DEC1 KO mice stained with H-E. Lipid accumulation is visible as unstained droplets (top panel). Middle and bottom panels show aged WT mice with increased immunostaining of SOD1 and GTx1 in centrolobular hepatocytes and hepatocytes close to preterminal hepatic venules as well as an additional staining in midzonal hepatocytes. Scale bars = 20 μm.

Fig. 3. DEC1-induced senescence and oxidative stress reduce expression of FGF21 in old WT mice. Immunohistochemical analysis of FGF21, ATF4, total ERK and Phospho-ERK in mouse liver reveals that those proteins are distributed in punctate deposits throughout the cytoplasm within hepatocytes, and increased staining intensity is observed in the periportal regions of young WT mice. Scale bars = 100 μm.

Fig. 4A. FGF21 protects DEC1 KO mice against aging-induced hepatic dysfunction. Expression of FGF21 is maintained during the course of aging in DEC1 KO mice. In contrast, FGF21 protein and RNA expression are increased in young WT mouse liver,

and are robustly decreased in old WT mice. Values represent means ± SE (bars) from three independent experiments. *$p<0.05$, as determined using the t-test.

Fig. 4B. DEC1 negatively regulates FGF21 and ATF4. HepG2 cells were transfected with DEC1-inserted pcDNA and DEC1 siRNA. The pcDNA and scramble siRNA were used as each control. At 48 h post-transfection, total RNAs and proteins were prepared and subjected to real-time PCR and Western blot analysis for DEC1, FGF21, ATF4 and β-actin. One representative sample from at least three independent experiments with similar results is shown. Values represent means ± SE (bars) from three independent experiments. *$p<0.05$, as determined using the t-test.

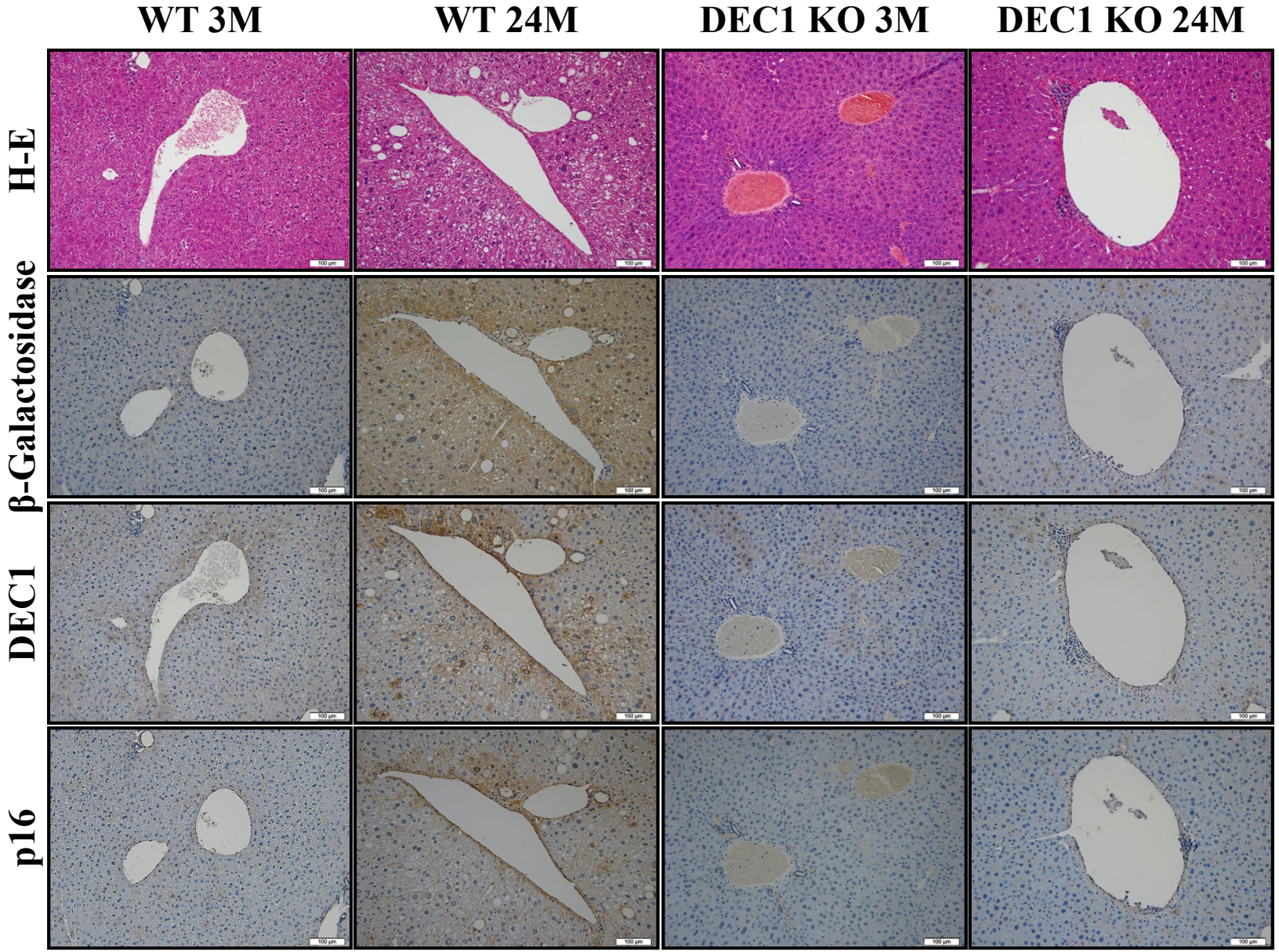


Figure 1

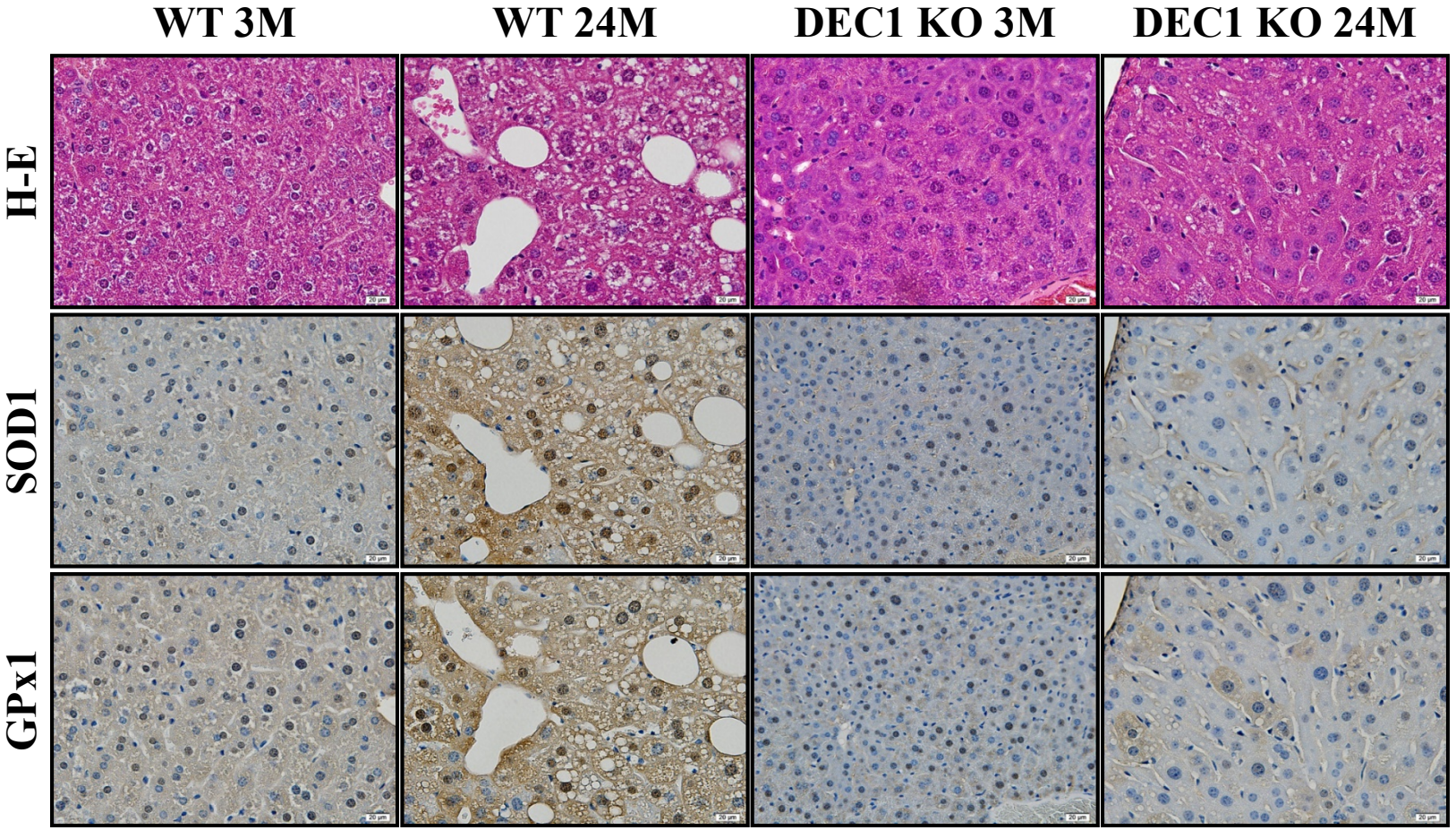


Figure 2

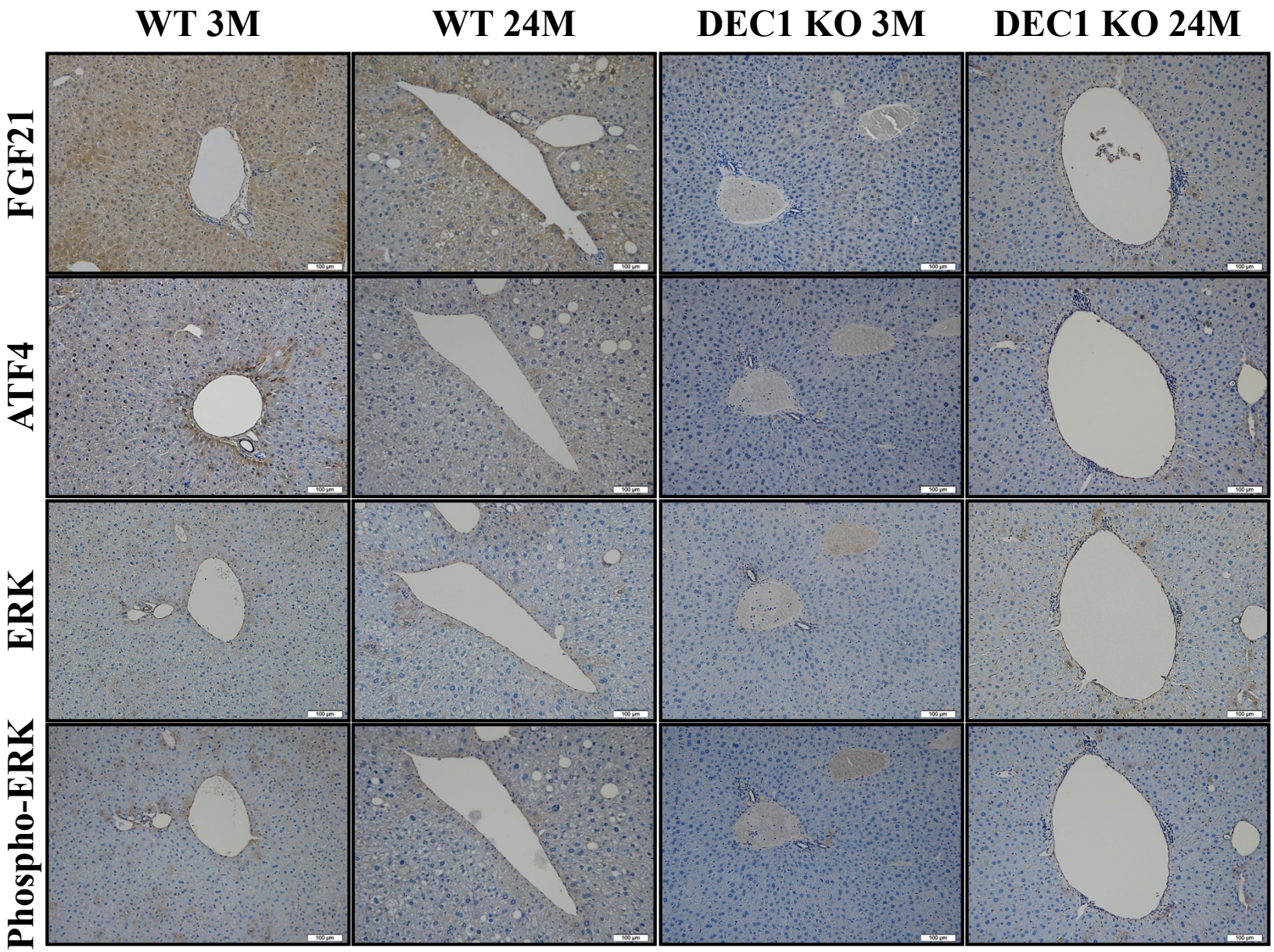
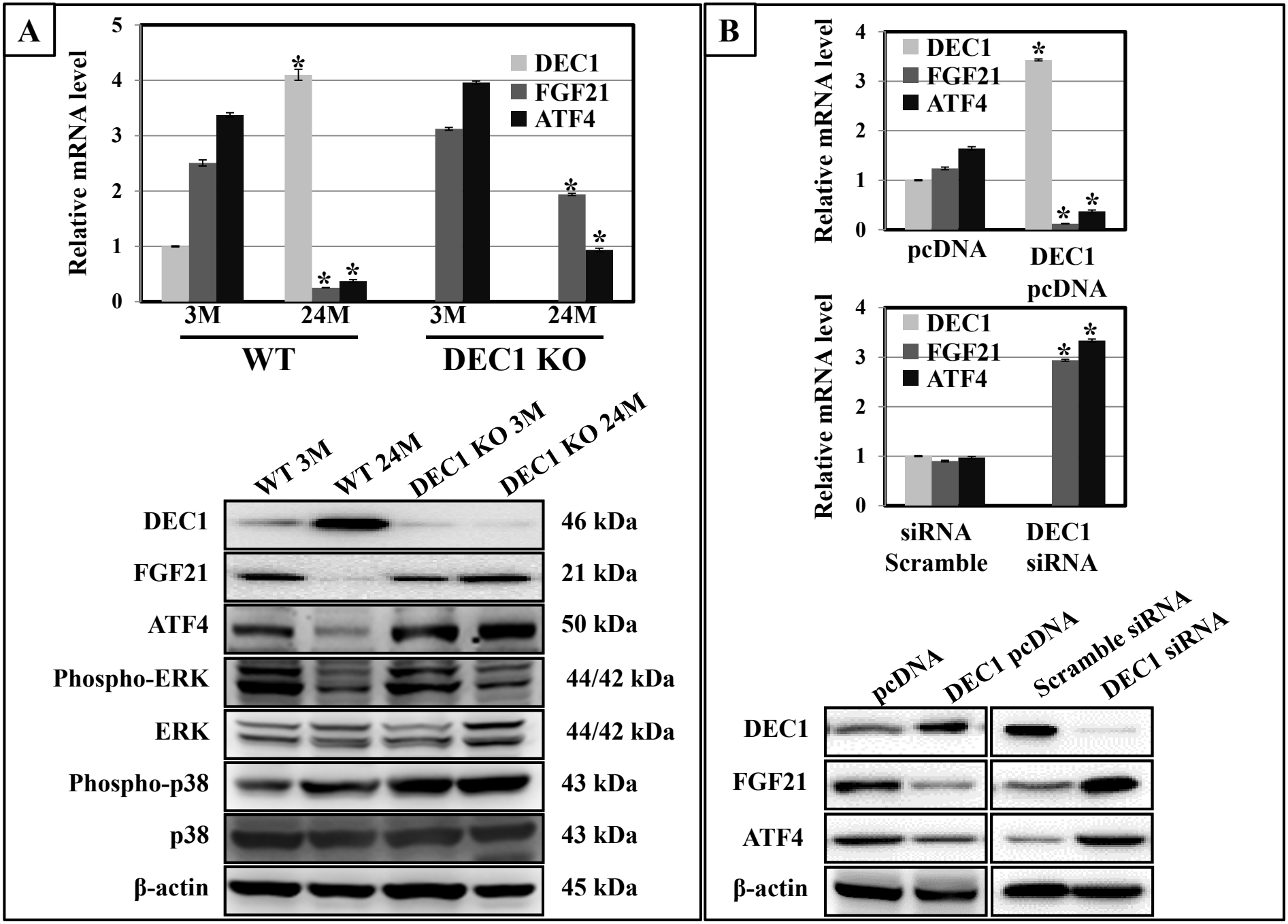


Figure 3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DEC1
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3M
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WT
DEC1 KO
WT 3M
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DEC1 KO 24M
DEC1
FGF21
ATF4
Phospho-ERK
ERK
Phospho-p38
p38
β-actin
46 kDa
21 kDa
50 kDa
44/42 kDa
44/42 kDa
43 kDa
43 kDa
45 kDa
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Relative mRNA level
DEC1
FGF21
ATF4
pcDNA
DEC1 pcDNA
siRNA Scramble
DEC1 siRNA
pcDNA
DEC1 pcDNA
Scramble siRNA
DEC1 siRNA
DEC1
FGF21
ATF4
β-actin

Figure 4