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Comparative analysis of fat and muscle proteins in fenofibrate-fed type II diabetic OLETF rats: the fenofibrate-dependent expression of PEBP or C11orf59 protein

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Fenofibrate, an agonist of PPARα, plays an important role in activating many proteins catalyzing lipid metabolism, and it also has a considerable effect on improvement of insulin sensitivity in the diabetic condition. To investigate fenofibrate-dependent expression of peripheral tissue proteins in diabetes, we analyzed whole muscle or fat proteins of fenofibrate-fed OLETF rats, an animal model of type II diabetes, using 2-dimensional gel electrophoresis. We found that many proteins were specifically expressed in a fenofibrate-dependent manner in these diabetic rats. In particular, a functionally unknown C11orf59 protein was differentially expressed in the muscle tissues (about 5-fold increase) in fenofibrate-fed OLETF rats as compared to control rats. Additionally, the signal proteins phosphatidylethanolamine binding protein and IkB interacting protein were differentially regulated in the fenofibrate-treated adipose tissues. We suggest here that these proteins might be involved in controlling lipid or carbohydrate metabolism in diabetes via PPARα activation. [BMB reports 2010; 43(5): 337-343]

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcriptional regulators that control expression of many genes involved in carbohydrate and lipid metabolism. Among them, PPARγ is a key regulator in increasing insulin sensitivity in the diabetic state (1), and PPARα is a lipid modulator, increasing fatty acid oxidation and lowering the level of triglycerides in serum (2, 3). Although PPARα is mainly involved in the regulation of lipid metabolism, it has been implicated in having a pivotal role in the development of diabetes (4). Stimulation of PPARα activity causes the inhibition of type II diabetes spontaneously occurring in Otsuka Long-Evans Tokushima Fatty (OLETF) rats (5, 6). Also, some reports indicate that PPARα activation stimulates insulin sensitivity in peripheral tissues such as skeletal muscle and adipocytes (7, 8).

To date, several agonists against PPARγ and PPARα have been discovered and are clinically used for world-wide treatment of metabolic disorders such as diabetes or hyperlipidemia. Fenofibrate, a PPARα agonist, can specifically bind to PPARα and activate expression of numerous genes participating in fatty acid oxidation, control of triglycerides and cholesterol metabolism (9, 10). Fenofibrate, therefore, was very effective to the nonalcoholic fatty liver diseases by modulating lipid metabolic enzymes such as fatty acid transport protein, fatty acid binding protein, long chain acyl-CoA dehydrogenase and acyl-CoA oxidase (11). The abdominal and skeletal adiposity in the diabetic rats was greatly improved by treatment with fenofibrate (4). Furthermore, fenofibrate has been shown to decrease levels of secretory E-selectin and increase levels of secretory phospholipase A2 associated with cardiovascular disease (12) and improved diet-induced cardiac function by increasing glucose oxidation and decreasing fatty acid oxidation (13).

Since fenofibrate is effective in preventing lipid accumulation, it is additionally associated with improvement of diabetes. In particular, it improves insulin sensitivity in diabetic animals (8, 14). Moreover, fenofibrate has been demonstrated to improve diabetes-associated diseases such as diabetic nephropathy and cardiovascular disease (15, 16).

In this study, we used comparative proteomics analysis to analyze total fat and muscle proteins content of fenofibrate-fed OLETF rats and found that some proteins including PEBP and C11orf59 were relatively changed in a fenofibrate-dependent manner.

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RESULTS

2-D gel analysis of fat and muscle proteins of fenofibrate-fed type II diabetic OLETF rats

OLETF rats spontaneously develop type II-like diabetes around 60 weeks after birth, signaled by a decrease of insulin sensitivity in the peripheral tissues (*e.g.*, muscle or fat) and obesity (5, 6). Therefore, they are used as a type II diabetic animal model. To investigate how fenofibrate influenced expression of overall proteins in the peripheral tissues of these diabetic rats, we fed fenofibrate mixed chow (320 mg/kg) to the OLETF rats until type II diabetes was developed (61 weeks) as described previously (17). The treatment fenofibrate to the diabetic model rats significantly reduced fat content associated with diabetes and body weight (17). We further isolated skeletal muscle and subcutaneous adipose tissue from fenofibrate-fed OLETF rats (n = 7) and control OLETF rats with no fenofibrate feeding (n = 7) from which we analyzed whole proteins extracted from these tissues using 2-dimensional electrophoresis gel (2-D-gel). About 2,000 protein spots were observed in each silver-stained gel as shown in Fig. 1. According to PDQuest analyses from at least three separate gels, several tens of proteins were differentially expressed in muscle or adipose tissues of fenofibrate-fed OLETF rats compared to control OLETF rats that were fed general chow. We identified those up-regulated or down-regulated protein spots in the skeletal muscle and fat tissues using MALDI-TOF mass spectrometry, a total of 5 proteins from muscle and 13 proteins from fat tissue (Table 1). Several cell signaling proteins (Rho GDP dissociation inhibitor, phosphatidylethanolamine binding protein, inhibitor of NF-kB kinase interacting protein, and protein kinase C) in addition to some energy metabolism-related proteins (ATP synthase, group X secretary phospholipase A2, and apolipoprotein) were discovered. Moreover, a couple of proteins involved in oxidative stress such as Cyp2d12 and catalase were identified. In particular, C11orf59 homolog, functionally unknown as of yet, was significantly elevated in fenofibrate-fed OLETF rats (Fig. 1, Table 1). This protein belongs to the UPF0404 family, activating RhoA protein via interacting with CDKN1B. Phosphatidylethanolamine binding protein (PEBP) known as a Raf-1 kinase inhibitor was also decreased in the diabetic condition (Fig. 1, Table 1), suggesting that the MAP kinase pathway might be associated with fenofibrate-induced improvement of insulin sensitivity and reduction of adiposity in diabetes. Furthermore, an inhibitor of NF-kB kinase interacting protein was dramatically increased in the fat tissues of fenofibrate-fed rats. However, tripartite-motif containing protein 32, a RING-domain E3 ligase, was approximately 3-fold down-regulated in the adipose tissue of fenofibrate-treated OLETF rats (Table 1). Two muscle proteins (myosin light polypeptide 3 and myosin regulatory light chain 2) were differentially expressed in muscle tissues in a fenofibrate-dependent manner. Homer protein homolog responding to cell junction and translational initiation factor E1F-2Bα were significantly elevated in the fat tissues of fenofibrate-fed diabetic rats.

Fenofibrate-dependent expression of C11orf59 protein and PEBP

Among proteins differentially expressed in fenofibrate-fed OLETF rats, we further examined the level of expression in different tissues for C11orf59 and PEBP proteins by western blot analy-

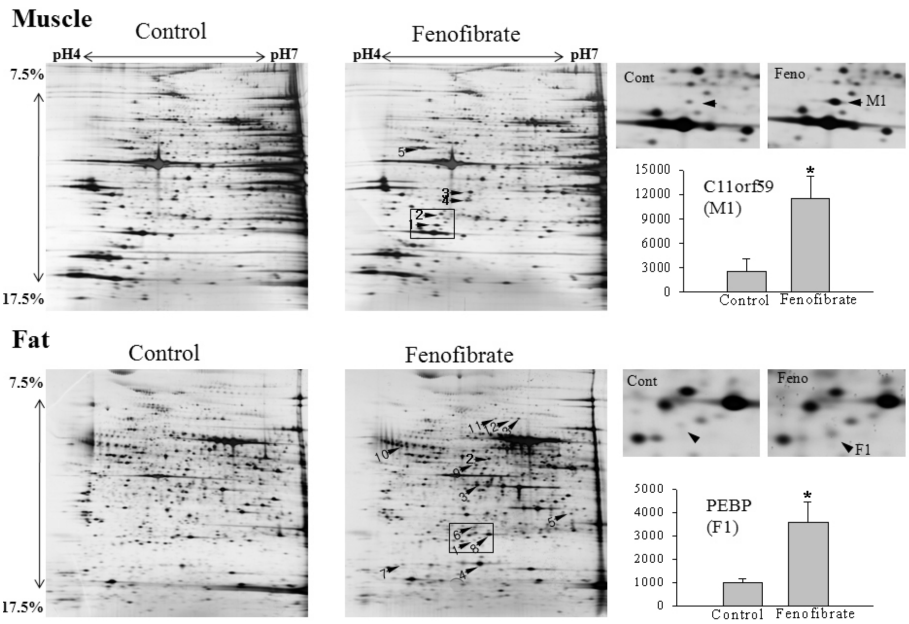

Fig. 1. 2-D gel analysis of total muscle and fat proteins. Total proteins were isolated from muscle or fat tissues of either fenofibrate-treated or control OLETF rats. Proteins were separated using 2-D-gel analyses; isoelectric focusing (pH4-7 strips) and SDS-PAGE (7.5-17.5%). The silver-stained gels are represented in the figure. Numbers in the figure indicate the isolated protein spots (shown in Table 1). The right gel was magnified from a specific area of 2-D-gel (left rectangle). Two proteins (indicated by arrows, C11orf59 (M1), PEBP (F1)) were quantified and represented to the graphic illustration as an average number of at least three independent gels ±SD (right bar graphs). The numbers of each graph are arbitrary values. *Represents $P<0.05$ as compared to control.

Table 1. A list of proteins differentially expressed by fenofibrate in diabetic OLETF rats

Spot #	Accession number	Protein name	MW/pI (Masses matched)	Sequence coverage	Protein function	Change
M1	Q6P791	C11orf59 homolog	17,721/4.9 (30%)	24%	Unknown	+4.7
M2	Q99PT1	Rho GDP dissociation inhibitor alpha	23,408/5.1 (33%)	16%	Signal transduction	−3.5
M3	P16409	Myosin light polypeptide 3	22,025/5.0 (29%)	41%	Muscle contraction	+3.1
M4	P04466	Myosin regulatory light chain2 skeletal muscle isoform	18,970/4.8 (75%)	50%	Muscle contraction	−3.3
M5	P10719	ATP synthase subunit beta, mitochondrial	56,354/5.2 (75%)	30%	ATP synthesis	+2.5
F1	P31044	Phosphatidylethanolaminebinding protein (PEBP)	20,802/5.5 (30%)	30%	Signal transduction	+3.6
F2	P02770	Serum albumin precursor	68,719/6.1 (41%)	24%	Transport	+4
F3	Q9Z2X5	Homer protein homolog	39,892/5.3 (26%)	12%	Cell junction	+5
F4	Q9QZT3	Group X secretary phospholipase A2	17,088/6.2 (25%)	13%	Lipid degradation	+4.7
F5	Q64270	Translation initiation factor E1F-2B alpha	33,678/8.4 (38%)	13%	Protein synthesis	−2.3
F6	Q8BQS4	Protein FAM 102B	36,514/5.6 (44%)	19%	Unknown	+6.5
F7	Q9DBZ1	Inhibitor of NF-kB kinase interacting protein	42,532/5.0 (28%)	15%	Signal transduction	+9
F8	P04639	Apolipoprotein A-I	30,062/5.5 (37%)	38%	Cholesterol transport	+2
F9	Q5T6V5	UPF0553 protein c9orf64	39,029/5.6 (22%)	16%	Unknown	−2.0
F10	B7ZP10	Cyp2d12 protein	51,875/5.9 (33%)	10%	Oxidation reduction	+2.5
F11	B1WBU8	UPF0639 protein	59,055/6.0 (35%)	11%	Unknown	+4.3
F12	116138241	Hypothetical protein RIKEN cDNA 4930430A15 gene	55,080/5.9 (33%)	8%	Unknown	+2.6
F13	P05545	Serine protease inhibitor A3K	46,562/5.3 (33%)	20%	Protease inhibition	+4.9
F14	141794892	Protein kinase c zeta	67,630/5.4 (37%)	12%	Signal transduction	−3
F15	Q8CH72	Tripartite motif-containing protein 32	72,058/6.5 (25%)	8%	Protein degradation	−2.9
F16	P04762	Catalase	59,758/7.1 (21%)	13%	Oxidation reduction	−2.8

The spot numbers begin with M or F indicate proteins isolated muscle or fat tissue, respectively. Plus (+) or minus (−) in the column of change represents increased or decreased fold-values compared with control

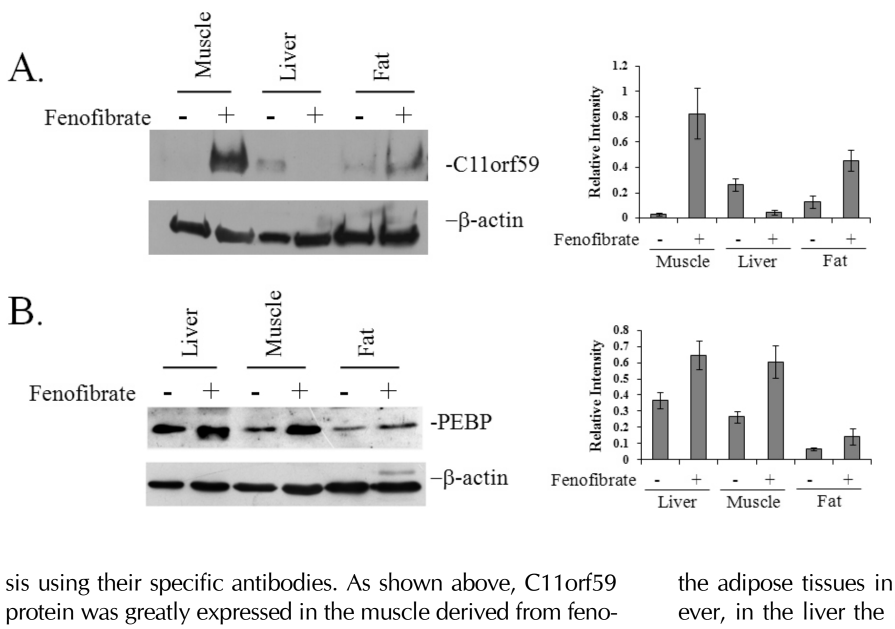

Fig. 2. Expression of C11orf59 and PEBP proteins in tissues. Total proteins extracted from tissues (muscle, fat and liver) of fenofibrate-fed or control OLETF rats were separated on a 10% SDS-PAGE. Protein was detected by western blot using specific antibodies (A) C11orf59 and (B) PEBP. The bar graphs (right) represents the quantification of the western blot result (left). Relative intensity was determined from the intensity values of C11orf59 or PEBP divided by the intensity values of β-actin obtained from at least three independent experiments.

sis using their specific antibodies. As shown above, C11orf59 protein was greatly expressed in the muscle derived from fenofibrate-fed rats (Fig. 2), while we barely observed expression of C11orf59 protein in the muscle tissue of control OLETF rats. We also detected the differential expression of this protein in the adipose tissues in a fenofibrate-dependent manner. However, in the liver the level of C11orf59 protein was very low and its expression in the presence of fenofibrate was relatively decreased. PEBP was also up-regulated in fenofibrate-fed OLETF rats (Fig. 1). All three tissues (liver, muscle and fat) revealed a

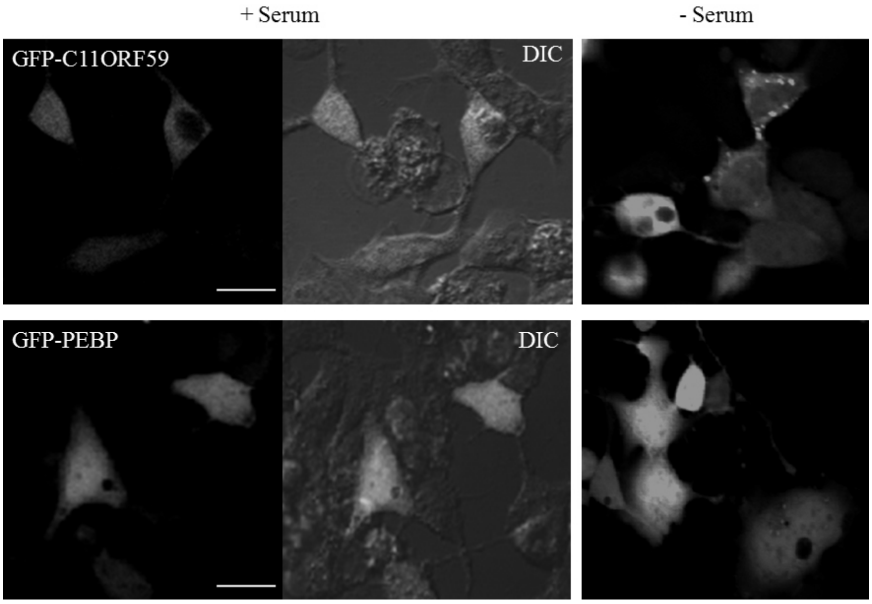

Fig. 3. Localization of C11orf59 and PEBP in cells. GFP-fusion constructs (GFP-C11orf59 or GFP-PEBP) were transfected into 293A cells using Lipofectamine 2000 reagents. After 24 h, expression of GFP-fused proteins was observed under a confocal miscroscope (Olympus, FV-1000). Cells were cultivated under DMEM media plus 10% FBS (+serum), and (−serum) shows cells cultivated under only DMEM. DIC indicates differential interference contrast images. Scale bars represent 20 μm.

similar pattern although liver and muscle showed relatively greater PEBP expression compared to fat (Fig. 2).

We further examined where these two proteins localized in cells using GFP-fused constructs. GFP-C11orf59 proteins were predominantly observed in the cytosol, and they were expressed in small granules such as endosomes (Fig. 3). In particular, we observed several large vesicles in cells in the serum-free medium. Similarly, PEBP was distributed in all cellular compartments including the nucleus (Fig. 3).

DISCUSSION

The incidence rate of type II diabetes, a common metabolic disorder, has been gradually increasing around the world due to heavy-caloric daily diets. This metabolic disease is associated with various environmental issues in addition to genentic factors leading to increased insulin resistance in insulin-targeting tissues such as muscle, fat and liver in an age-dependent manner. In particular, the insulin resistance in type II diabetes is directly related to a significant elevation of free fatty acids in serum due to the increase of adipose tissues in the obese condition. Therefore, control of obesity is one of major targets in type II diabetes. Fenofibrate, a PPARα activator, stimulates expression of many genes involving in lipid catabolism (specifically fatty acid oxidation and degradation of triglycerides), and it has been very effective in improvement of insulin sensitivity in diabetes animal models such as OLETF rats. In the current study, we isolated fat and skeletal proteins that were differentially regulated in fenofibrate-fed diabetic OLETF rats using conventional 2-dimensional gel electrophoresis analysis.

In the adipose tissue, some proteins are increased in a fenofibrate-dependent manner. Group X secretary phospholipase A2 (sPLA2) catalyzes release of fatty acids from phospholipids and plays a role in many cellular responses. It stimulates insulin secretion from the pancreas (18) and it is also found in insulin secretary granules upon glucose stimulation (19), suggesting that sPLA2 participates in insulin secretion in pancreatic islet beta cells. In fact, fenofibrate had significantly increased plasma levels of sPLA2 in diabetic patients (12). Similarly, apolipoprotein A1, a principal apolipoprotein in HDLs, is crucial for maintaining the cholesterol level that eventually controls β-cell function (20). The increased level of apolipoprotein A1 in fenofibrate-fed rats seems to have provided a beneficiary effect not only on preventing the onset of diabetes but also in prevention of adiposity.

Inhibitor of NF-kB kinase interacting protein (IkB kinase interacting protein, IKIP) has been shown to respond to X-ray irradiation in a p53-dependent manner, and promotes cell death (21). In the same report we found that expression of IKIP was largely increased in fenofibrate-fed OLETF rats, thus we can conclude that insulin resistance is associated with IKK/NF-kB pathway in adipose tissue (22). Also, production of adipocytokines (*e.g.*, IL-6, TNFα) by IKK/NF-kB pathway is a key step in triggering insulin resistance in the fat tissues (23). Thus, IKK modulation by IKIP expression might improve insulin sensitivity in the peripheral tissues. Tripartite motif protein 32 (TRIM32), one of the E3 ligases, was relatively decreased in the adipose tissue of OLETF rats fed with PPARα agonist. In fact, mutated TRIM37, a member of the tripartite motif protein family, is directly associated with insulin resistance, fatty liver and glucose tolerance in the type II diabetes (24). PEBP was also highly elevated in the insulin-targeting tissues (liver, muscle and fat) in a fenofibrate-dependent manner (Fig. 1, 2), suggesting a possible role of PEBP activation in the improvement of insulin sensitivity and control of lipid metabolism. Besides the phospholipid binding property of PEBP, this protein has been identified as a natural inhibitor of the ERK pathway via a direct interaction of Raf-1 kinase (25). ERK activation by cytokines in the diabetic condition triggers insulin resistance (26), and its activation also inhibits insulin-induced glucose uptake in muscle cells (27). Furthermore, reduction of ERK activity can improve insulin sensitivity in obese Zucker rats (28). These studies indicate that the ERK pathway is essential for controlling the diabetic condition. Finally, C11orf59, a small protein (161 amino acids, 17,721 Da), is functionally undiscovered yet. However, it could be a RhoA activator according to its sequence similarity. If so, RhoA pathway is connected with diabetes by interfering with insulin signaling (29). As we have shown, this protein is likely localized at the intracellular granules (e.g., endosome). Therefore, it might be involved in controlling vesicle trafficking, associating with insulin secretion or signaling. However, the exact role of this protein in cells relating to diabetes still remains to be discovered.

MATERIALS AND METHODS

Materials

IPG strips (17 cm) of 4-7 were purchased from Bio-Rad (Hercules, CA, USA). Bio-Lyte (pH 4-7) was obtained from Bio-Rad. SDS, acrylamide, methylene-bisacrylamide, TEMED, ammonium persulfate, DTT, urea, tris base, glycine, glycerol, and CHAPS were purchased from Bio-Rad or USB (Cleveland, OH, USA). Silver nitrate, iodoacetamide, and α-cyano-4-hydroxycinnamic acid were purchased from Sigma (St. Louis, MO, USA). Anti-PEBP and C11orf59 antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA. USA).

Protein extraction of diabetic rat tissues

Otsuka Long-Evans Tokushima Fatty (OLETF) rats were kindly donated from Otsuka Pharmaceuticals (Tokushima, Japan) and fed a powder diet containing fenofibrate (0.5% w/w, 320 mg/kg/day) ($n=7$) or no fenofibrate ($n=7$) until 60 weeks of age as described previously (17). Sub-cutaneous fat and skeletal muscle tissues were obtained from these rats used for the other study (17). The isolated tissues were washed with PBS several times. Total proteins were extracted using the mechanical homogenizer in the solution (8 M urea, 4% CHAPS (3-[(3-cholamidopropyl) demethylammonio]-1-propanesulfonic acid), 40 mM Tris, 65 mM dithiothreitol, 0.05% SDS), and by centrifugation at 13,000 rpm for 30 min.

2-D gel proteomics analysis

Proteomic analysis was carried out as described in our previous work (30). IPG gel strips (17 cm-length) were rehydrated in a swelling solution (7 M urea, 2% Chaps, 100 mM DTT, 0.5% IPG buffer, and bromophenol blue) containing 50 μg proteins (silver staining) or 500 μg (Coomassie staining) for 12 h at 20°C. Isoelectric focusing was performed at 20°C in three steps: at 250 V for 15 min, 10,000 V for 3 h, and 40,000 V hours. The scanned gel images were analyzed using a standard protocol in PDQuest software (Biorad). 2-D gel analysis was evaluated by Student's *t*-test. Results are expressed as the mean ± SEM. Differences were considered significant at $P < 0.05$. We obtained peptides from Coomassie blue-stained gels for mass spectrometry based on the PDQuest analyses as described (30). The Voyager TM-DE (delayed extraction) STR biospectrometry workstation was used for MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) mass spectrometry to identify proteins described (30).

Western blot

Total cell proteins extracted from tissues were separated on a 10% SDS-gel electrophoresis and subsequently transferred to a nitrocellulose membrane as described (31). The membrane was blocked for 1 h at room temperature in 5% skim milk/TBST (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 2.7 mM KCl, and 0.1% tween 20) and incubated with primary antibodies in 5% skim milk/TBST for 2 h at room temperature. After washing for 30 min with TBST three times, the membrane was incubated with secondary antibodies conjugated with HRP in 5% skim milk/TBST for 1 h. Specific proteins were detected with an enhanced chemiluminescence system (ECL, PIERCE) after additional washing with TBST three times.

Cell transfection and confocal microscopy analysis

C11orf59 clone (purchased from KRIB) and PEBP gene (obtained from Dr. Kolch, UK) were amplified by PCR using their specific primers containing a *Bgl*II site (forward) and a *Sal*I site (reverse), and subcloned into pEGFP-C (Invitrogen) cleaved by *Bgl*II and *Sal*I restriction enzymes. Both GFP-fused constructs (pEGFP-C11orf59 or pEGFP-PEBP) were transiently transfected into 293A cells cultivated in the medium (DMEM +10% FBS with 100 U/ml of penicillin, and 100 μg/ml of streptomycin) using Lipofectamine2000 reagent (Invitrogen) according to the manufacturer's protocol. Cells were grown for 24 h in a humidified 5% CO_2 incubator at 37°C in a plate with a cover glass. GFP-fused protein expression and localization in cells were visualized under a confocal microscope (Olympus FV-1000).

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