

# Fat-specific FUS-DDIT3-transgenic mice establish PPAR $\gamma$ inactivation is required to liposarcoma development

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**FUS-DDIT3** is a chimeric oncogene generated by the most common chromosomal translocation t(12;16)(q13;p11) associated to liposarcomas. The application of transgenic methods and the use of primary mesenchymal progenitor cells to the study of this sarcoma-associated **FUS-DDIT3** gene fusion have provided insights into their *in vivo* functions and suggested mechanisms by which lineage selection may be achieved. These studies indicate that **FUS-DDIT3** contributes to differentiation arrest acting at a point in the adipocyte differentiation process after induction of peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) expression. To test this idea within a living mouse, we generated mice expressing **FUS-DDIT3** within aP2-positive cells, because aP2 is a downstream target of PPAR $\gamma$  expressed at the immature adipocyte stage. Here, we report that **FUS-DDIT3** expression was successfully induced at the aP2 stage of differentiation both *in vivo* and *in vitro*. aP2-FUS-DDIT3 mice do not develop liposarcomas and exhibit an increase in white adipose tissue size. Consistent with *in vivo* data, mouse embryonic fibroblasts (MEFs) obtained from aP2-FUS-DDIT3 mice not only were capable of terminal differentiation but also showed an increased capacity for adipogenesis *in vitro* compared with wild-type MEFs. Taken together, this study provides genetic evidence that the presence of **FUS-DDIT3** in an aP2-positive cell is not enough to cause liposarcoma development and establishes that PPAR $\gamma$  inactivation is required for liposarcoma development.

## Introduction

Myxoid/round cell liposarcoma is the most common subtype of liposarcoma, accounting for ~40% of all cases. The tumor cells are characterized by the chromosomal aberration t(12;16)(q13;p11), which creates the **FUS-DDIT3** oncogene (1–4). This fusion oncogene has not been found in tumor types other than myxoid/round cell liposarcoma (4,5). Due to the absence of a direct link between a cell carrying the cytogenetic abnormality and a test of whether this cell has the capacity to maintain the disease *in vivo*, the nature of the intimate association between the **FUS-DDIT3** oncogene and the phenotype with which it is associated is pending to understand (6–8). *In vitro* the transforming effects of **FUS-DDIT3** have been demonstrated in fibroblasts (9), but curiously not in 3T3-L1 cells under conditions expected to yield oncogenic effects (10), suggesting that the function of **FUS-DDIT3** is influenced by cell environment. Consistent with this notion, transgenic mice engineered to express **FUS-DDIT3** under the

**Abbreviations:** BAT, brown adipose tissue; MEF, mouse embryonic fibroblast; PBS, phosphate-buffered saline; PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$  WAT, white adipose tissue.

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control of the ubiquitous E1F $\alpha$  promoter, which has found to be functional in mesenchymal progenitor/stem cells (11), developed liposarcomas that resemble their human counterpart (9,12,13). In agreement with this view is the genomic analysis carried out in human myxoid liposarcoma (14), which is compatible with the genetic program of a primitive target cell from which myxoid liposarcoma could arise. Further support to this idea comes from the studies showing that the expression of **FUS-DDIT3** in primary mesenchymal progenitor cells give rise to myxoid liposarcoma-like tumors (11,15). Taken together, these data indicate that **FUS-DDIT3**-liposarcoma develops from uncommitted progenitor cells (7,8). However, the observation that **FUS-DDIT3** has been previously reported to block terminal differentiation of pre-adipocytes *in vivo* and *in vitro* (10,16) calls this hypothesis into question. These reports lead to the hypothesis that liposarcoma develops from pre-adipocytes carrying **FUS-DDIT3** that are incapable of terminal differentiation.

While the precise nature of the developmental defect in liposarcoma is not yet clear, it is likely **FUS-DDIT3** ultimately leads to the inactivation or antagonism of one or more adipocyte transcriptional regulatory proteins. The proposed effect of **FUS-DDIT3** on peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) activity (9,12,13,16) close to the absence of PPAR $\gamma$  induction in mesenchymal progenitor/stem cells expressing **FUS-DDIT3** (11) has suggested that **FUS-DDIT3** contributes to differentiation arrest acting at a point in the adipocyte differentiation process after induction of PPAR $\gamma$  expression. In order to test this idea and because aP2 is a downstream target of PPAR $\gamma$  (17) which is expressed at the immature adipocyte stage (18,19), we have generated mice targeting **FUS-DDIT3** expression to aP2-positive cells.

## Materials and methods

### Generation of transgenic mice

The cDNA for human **FUS-DDIT3** was cloned under the control of the 5.4 kb aP2 gene promoter for fat-specific **FUS-DDIT3**-transgenic mice (20) to give rise to aP2-FUS-DDIT3 cassette. Linear DNA fragments for microinjection were obtained and injected by the University of Salamanca Transgenic Facility into CBA  $\times$  C57BL/6J fertilized eggs. We identified transgenic mice by Southern analysis of tail snip DNA after HindIII digestion as described (21). We used the human **DDIT3** cDNA for detection of the transgene. Similar phenotypic features were seen in all assays for both the aP2-FUS-DDIT3-transgenic lines generated.

### Reverse transcription–polymerase chain reaction

To analyze expression of human **FUS-DDIT3** in mouse tissues and mouse embryonic fibroblasts (MEFs), reverse transcription was performed according to the manufacturer's protocol in a 20  $\mu$ l reaction containing 50 ng of random hexamers, 3  $\mu$ g of total RNA and 200 U of Superscript II RNase H<sup>−</sup> reverse transcriptase (Gibco BRL, Madrid, Spain). The sequences of the specific primers were as follows: FUS-F1: 5'-GGTTATGGCAATCAAGACCAG-3' and DDIT3-B1: 5'-CTTGCAGGTCCTCATACCAGG-3'. This oligo pair amplifies specifically the fusion region. The thermocycling parameters for the polymerase chain reaction and the sequences of the specific primers were as follows: 30 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. The polymerase chain reaction products were confirmed by hybridization with specific probes. Amplification of aP2 and 36B4 served as a control to assess the adipose tissue and the quality of each RNA sample, respectively.

### Histological analysis

Mice included in this study were subjected to standard necropsy. All major organs were closely examined under the dissecting microscope, and samples of each organ were processed into paraffin, sectioned and examined histologically. All tissue samples were taken from homogenous and viable portions of the resected sample by the pathologist and fixed within 2–5 min of excision. Hematoxylin- and eosin-stained sections of each tissue were reviewed by a single pathologist (T.F.). For comparative studies, age-matched mice were used (wild-type versus aP2-FUS-DDIT3 mice).

### Preparation of primary MEF

Primary embryonic fibroblasts were harvested from 13.5 day post-coitum embryos. Head and organs of day 13.5 embryos were dissected; fetal tissue was rinsed in phosphate-buffered saline (PBS), minced and rinsed twice in PBS. Fetal tissue was treated with trypsin/ethylenediaminetetraacetic acid and incubated for 30 min at 37°C and subsequently dissociated in medium. After removal of large tissue clumps, the remaining cells were plated out in a 175 cm<sup>2</sup> flask. After 48 h, confluent cultures were frozen down. These cells were considered as being passage 1 MEFs. For continuous culturing, MEF cultures were split 1:3. MEFs and the  $\phi$ MX ecotropic packaging cell line were grown at 37°C in Dulbeccos-modified Eagle's medium (Boehringer Ingelheim, Madrid, Spain) supplemented with 10% heat-inactivated fetal bovine serum (Boehringer Ingelheim). All the cells were negative for mycoplasma (MycoAlert™ Mycoplasma Detection Kit, Cambrex, Madrid, Spain).

### Adipocyte differentiation

Wild-type and aP2-FUS-DDIT3 MEFs were cultured at 37°C in standard D-MEM:F12 medium (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Madrid, Spain), 100 U/ml penicillin (BioWhittaker, Madrid, Spain) and 100 µg/ml streptomycin (BioWhittaker, Madrid, Spain). Cells (10<sup>6</sup>) of each genotype were plated to 10 cm plastic dishes and propagated to confluence. Two days after confluence, the adipocyte differentiation program was induced by feeding the cells with standard medium supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (Sigma, Madrid, Spain), 1 µM dexamethasone (Sigma) and 5 µg/ml insulin (Sigma) for two days, and then with standard medium supplemented with 5 µg/ml insulin for 6 days. This medium was renewed every 2 days. After 8 days, the appearance of cytoplasmic lipid accumulation was observed by Oil-Red-O staining. Briefly, cells were washed with PBS, and then fixed with 3.7% formaldehyde for 2 min. After a wash with water, cells were stained with 60% filtered Oil-Red-O stock solution [0.5 g of Oil-Red-O (Sigma) in 100 ml of isopropanol] for 1 h at room temperature. Finally, cells were washed twice in water and photographed. To prepare RNA for northern blotting, cells were harvested at days 0, 2, 4 and 8 of differentiation.

### RNA extraction

Total RNA was isolated in two steps using TRIzol (Life Technologies, Grand Island, NY) followed by Rneasy Mini-Kit (Qiagen, Valencia, CA) purification following the manufacturer's RNA clean-up protocol with the optional on-column Dnase treatment. The integrity and the quality of RNA were verified by electrophoresis and its concentration was measured.

### Northern blot analysis

Total cytoplasmic RNA (10 µg) of aP2-FUS-DDIT3 MEFs harvested at days 0, 2, 4 and 8 of differentiation and three tumor tissues developed in E1Fa-FUS-DDIT3-transgenic mice were glyoxylated and fractionated in 1.4% agarose gels in 10 mM Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.0). After electrophoresis, the gel was blotted onto Hybond-N (Amersham, Madrid, Spain), UV cross-linked and hybridized to <sup>32</sup>P-labeled DDIT3 and aP2 probes, respectively. Loading was monitored by reprobing the filter with a mouse 36B4 probe.

### Tumorigenicity assay

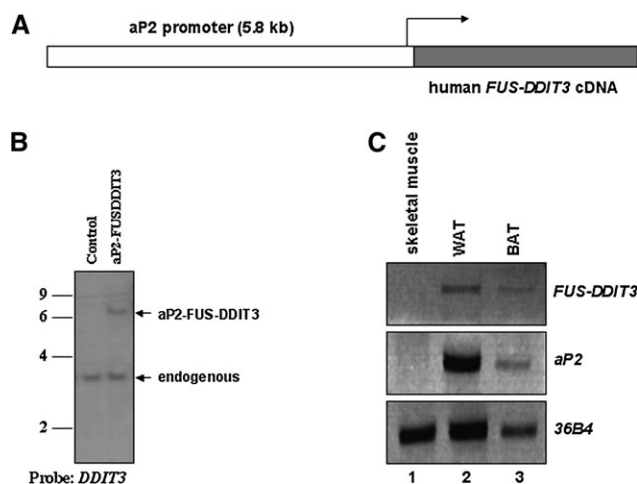
To test the tumorigenicity of control and aP2-FUS-DDIT3 MEFs at day 2 after hormonal induction, 4- to 6-week old athymic (nude) male mice were injected subcutaneously on both flanks with 10<sup>6</sup> cells re-suspended in 200 µl of PBS. The animals were examined for tumor formation every week.

### Western blot analysis

Western blot analysis of white adipose tissue (WAT) was carried out. Extracts were normalized for protein content by Bradford analysis (Bio-Rad Laboratories, Melville, NY) and Coomassie blue gel staining. Lysates were run on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to a polyvinylidene difluoride membrane. After blocking, the membrane was probed with the following primary antibodies: PPARγ (H-100 and E-8, Santa Cruz Biotechnology, Madrid, Spain), RXRα (D-20, Santa Cruz Biotechnology), C/EBPβ (C-19, Santa Cruz Biotechnology), C/EBPδ (M-17, Santa Cruz Biotechnology), C/EBPα (14AA, Santa Cruz Biotechnology) and actin (I-19, Santa Cruz Biotechnology). Reactive bands were detected with an enhanced chemiluminescence system (Amersham).

## Results and discussion

The mouse 5.4 kb promoter fragment of the *aP2* gene (20) was used to drive aP2-directed expression of a human *FUS-DDIT3* transgene in C57BL/6 × CBA mice (aP2-FUS-DDIT3 mice) (Figure 1A and B). This promoter is well characterized, and the fragment used contains all the necessary elements to recapitulate endogenous *aP2* gene ex-



**Fig. 1.** aP2-FUS-DDIT3 mice: transgene construct and expression in adipose tissues. (A) Schematic representation of the aP2-FUS-DDIT3 vector used in this study. (B) Identification of transgenic mice by Southern analysis of tail snip DNA after HindIII digestion. We used the cDNA for human *DDIT3* for detection of the transgene. (C) Expression of the aP2-FUS-DDIT3 transgene was demonstrated by reverse transcription-polymerase chain reaction. Expression of *FUS-DDIT3* was analyzed by reverse transcription-polymerase chain reaction in tissues derived of aP2-FUS-DDIT3 mice. 36B4 and aP2 were used to check cDNA integrity and loading and adipose tissue identification, respectively.

pression (20). Thus, by using the aP2 promoter, any possibility that embryonic expression of *FUS-DDIT3* might interfere with development was minimized. The *FUS-DDIT3* over-expressing animals have normal gestation, birth and litter sizes, and they were viable. A number of founders were generated and two independent lines were obtained. Both lines were used to examine the phenotype further. Polymerase chain reaction with reverse transcriptase of messenger RNA for the human transgene confirmed that expression was largely confined to aP2-positive mouse tissues [brown adipose tissue (BAT) and WAT] with no ectopic expression in aP2-negative tissues (Figure 1C). The phenotype described here is therefore due primarily to expression of the transgene in aP2-positive cells.

Cohorts of transgenic mice were generated to analyze the effect of the *FUS-DDIT3* gene. aP2-FUS-DDIT3-transgenic mice developed healthy were fertile and none of the aP2-FUS-DDIT3-transgenic mice developed liposarcomas in up to 24 months of observation. This result contrasts with our observations in the *FUS-DDIT3*-expressing mice, under the control of the ubiquitous E1Fα promoter, which develop liposarcomas (9). Although liposarcomas did not develop in the aP2-FUS-DDIT3-transgenic mice, the total body weight of adult aP2-FUS-DDIT3 mice ( $34.3 \pm 1.7$  g) was increased compared with age-matched control mice ( $29.7 \pm 1.2$ ). We investigated whether the *FUS-DDIT3* expression in aP2-positive cells altered WAT development in these mice. We analyzed WAT mass in aP2-FUS-DDIT3 mice. aP2-FUS-DDIT3 mice showed a large increase in WAT weight (Table I). In addition, food intake was similar in wild-type ( $2.9 \pm 0.4$  g per mouse per day) and aP2-FUS-DDIT3 mice ( $2.8 \pm 0.5$  g per mouse per day). This overall increase in adipose tissue in aP2-FUS-DDIT3 mice was observed in males and females (Table I). Although white fat is a non-malignant tissue, it has the capability to quickly proliferate and expand (22,23). Thus, *FUS-DDIT3* expression under aP2 control regions is inducing those effects. In contrast to WAT, other tissues including the interscapular BAT and kidney had similar weights for wild-type and aP2-FUS-DDIT3 mice (Table I).

To further characterize the phenotype of adipose tissue, we examined histological sections of WAT and BAT (Figure 2A). We observed no difference between the wild-type and aP2-FUS-DDIT3 mice in the BAT and WAT tissues. The histological analyses of the WAT in aP2-FUS-DDIT3 mice did not evidence any pathological change within the terminally differentiated adipocytes. On the contrary, aP2-FUS-DDIT3



mice had a normal architecture of the tissue and we did not observe any shift in the WAT toward immature in the aP2-FUS-DDIT3 mice. Some aP2-FUS-DDIT3 animals presented adipocytic accumulation in liver (Figure 2B). The above results support the hypothesis that *FUS-DDIT3* expression modulates adipose tissue size. To further explore the molecular basis through which FUS-DDIT3 expression in aP2-positive cells impairs the development of fat tissue, we examined the expression levels of the proteins responsible for WAT development.

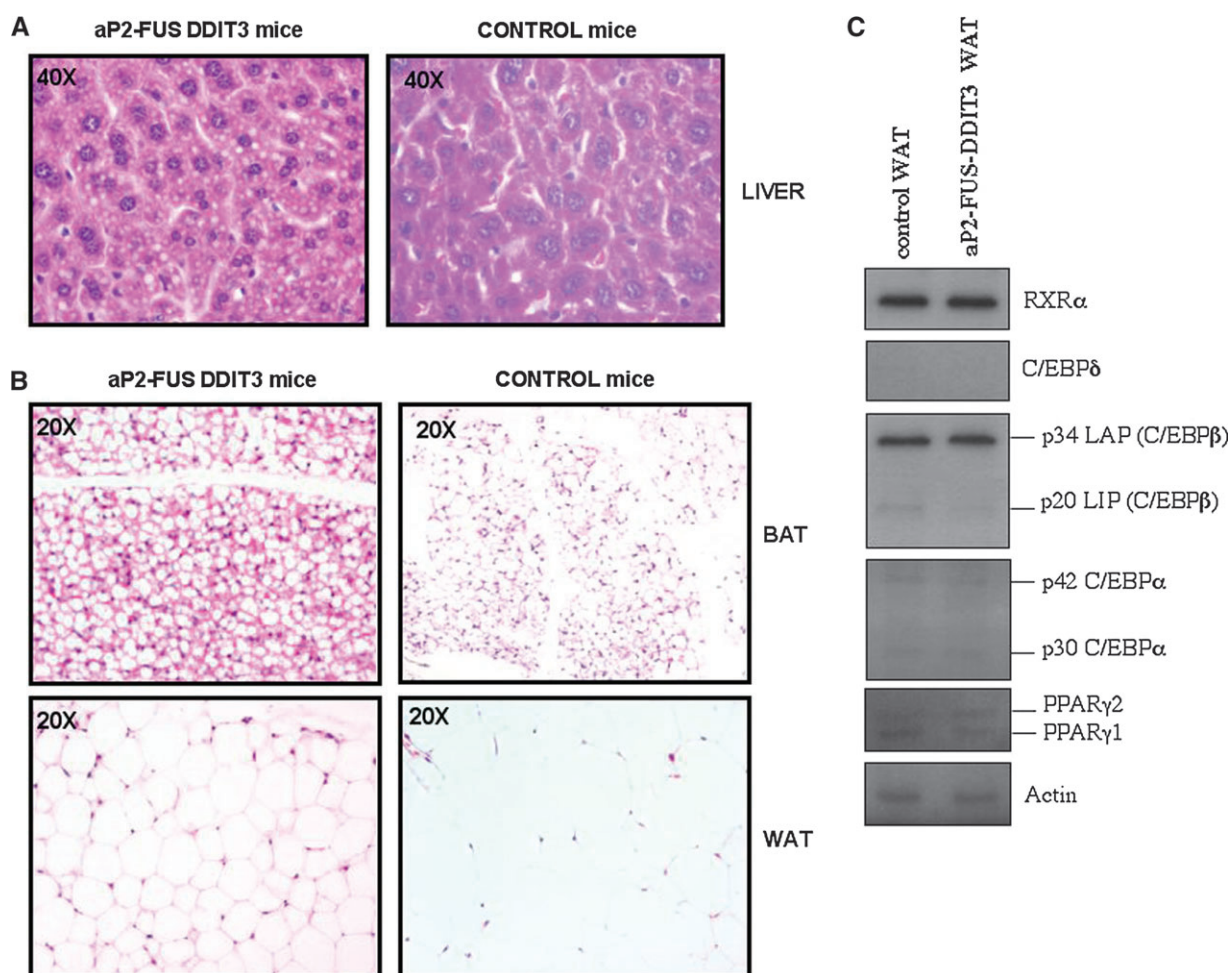
**Table I.** Adipose tissue mass in aP2-FUS-DDIT3 mice

	Kidney	Reproductive fat pad	Inguinal fat pad	Retroperitoneal fat pad
Male				
Control	0.147 ± 0.009	0.78 ± 0.13	0.56 ± 0.10	0.30 ± 0.08
aP2-FUS-DDIT3	0.147 ± 0.010	1.89 ± 0.19	1.58 ± 0.15	0.87 ± 0.09
Female				
Control	0.143 ± 0.007	not determined	0.55 ± 0.07	0.38 ± 0.09
aP2-FUS-DDIT3	0.144 ± 0.009	not determined	1.71 ± 0.13	1.09 ± 0.16

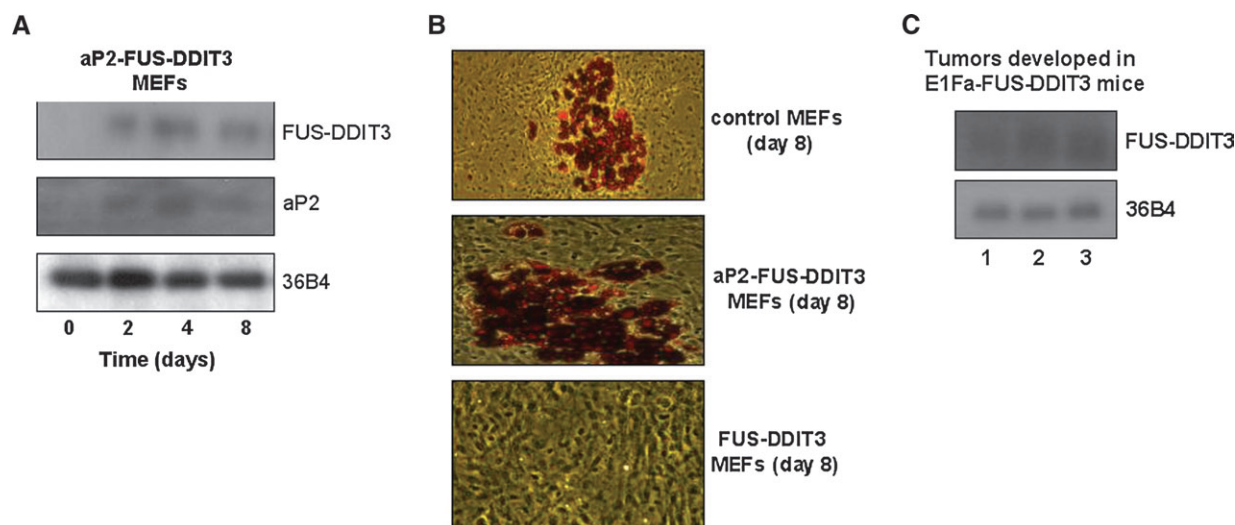
Mice were 6 months old. Weights are given in grams. Values are means ± SEMs from five mice in each group. Differences between control and aP2-FUS-DDIT3 were statistically significant ( $P < 0.01$ ) as determined by Mann–Whitney's test.

The expression of RXR $\alpha$ , C/EBP $\delta$ , C/EBP $\beta$ , PPAR $\gamma$  and C/EBP $\alpha$  was not affected (Figure 2C).

The adipogenesis of MEFs by hormonal induction is a well-established model system for the study of adipocyte differentiation *in vitro* (19). To further examine the contribution of FUS-DDIT3 to adipogenesis, we isolated MEFs from day 13.5 of aP2-FUS-DDIT3 and control embryos (Figure 3). *FUS-DDIT3* expression is not present in uncommitted progenitor cells before differentiation treatment (Figure 3A). However, the amount of *FUS-DDIT3* mRNA was apparent within 2 days and increased in abundance in parallel to aP2 expression (Figure 3A). These results indicate that aP2-FUS-DDIT3 is tightly controlled temporally and spatially during differentiation of uncommitted mesenchymal cells. At day 8 after hormonal induction, there is lipid accumulation in control MEFs (20–30%). However, there was extensive accumulation in aP2-FUS-DDIT3 MEFs (45–55%) (Figure 3B). The amount of *FUS-DDIT3* mRNA in aP2-FUS-DDIT3 was similar to the expression of *FUS-DDIT3* in tumors of E1Fa-FUS-DDIT3 mice (Figure 3C). To test the putative malignant nature of these cells,  $1 \times 10^6$  aP2-FUS-DDIT3 MEFs at day 2 after hormonal induction (when the amount of *FUS-DDIT3* mRNA was apparent) were injected subcutaneously into 40-day-old nude mice. Mice injected resulted in no tumors. These results show that adipocyte differentiation was not blocked in MEFs derived from aP2-FUS-DDIT3-transgenic mice treated with adipogenic hormones and confirm that interference with the normal process of differentiation contributes to the oncogenic potential of FUS-DDIT3 fusion protein (9).



**Fig. 2.** Histologic appearance of adipose tissues in aP2-FUS-DDIT3-transgenic mice. (A) Hematoxylin–eosin-stained sections of the liver tissues coming from control and aP2-FUS-DDIT3 mice. Liver of aP2-FUS-DDIT3 mice shows hepatic steatosis. (B) Hematoxylin–eosin-stained sections of interscapular BAT and reproductive WAT from control and aP2-FUS-DDIT3 mice. The size of cells in aP2-FUS-DDIT3 WAT was similar to control cells. (C) Western blot analyses of regulators of adipocyte function in WAT of control mice and aP2-FUS-DDIT3 mice.



**Fig. 3.** Altered lipid accumulation in aP2-FUS-DDIT3 MEFs. (A) Time course of *FUS-DDIT3* expression during adipocyte differentiation in aP2-FUS-DDIT3 MEFs. MEF cells, incubated for the indicated times after the onset of exposure to inducers of differentiation, were subjected to northern blot analysis. 36B4 was used to check loading. (B) Primary embryonic fibroblasts from control and aP2-FUS-DDIT3 mice and EF-FUSCHOP-transgenic mice (9) were cultured in the presence of standard adipose differentiation induction medium. At day 8 after induction of adipocyte differentiation, cells were fixed and stained for neutral lipids with Oil-Red-O and the morphological differentiation is shown. The original magnification is  $\times 20$ . This experiment was repeated three times using cells prepared from all lines and from different embryos and similar results were obtained. (C) *FUS-DDIT3* expression in three tumor tissues developed in E1Fa-FUS-DDIT3-transgenic mice by northern blot analysis. 36B4 was used to check loading.

In conclusion, this study provides genetic evidence that the presence of *FUS-DDIT3* in an aP2-positive cell is not enough to cause liposarcoma development and these cells are capable of terminal differentiation, underscoring the relevance of relationship between *FUS-DDIT3* and the cell environment. Moreover, although it has been suggested that *FUS-DDIT3* transforms at a point in the differentiation process after induction of PPAR $\gamma$  expression, our findings further establish that PPAR $\gamma$  inactivation is required for liposarcoma development. The precise mechanism whereby *FUS-DDIT3* contributes to PPAR $\gamma$  inactivation and differentiation arrest, however, remain to be elucidated.

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*Conflict of Interest statement:* None declared.

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