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ORIGINAL ARTICLE

# Novel function of the chromosome 7 open reading frame 41 gene to promote leukemic megakaryocyte differentiation by modulating TPA-induced signaling

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12-*O*-tetradecanoylphorbol-13-acetate (TPA) activates multiple signaling pathways, alters gene expression and causes leukemic cell differentiation. How TPA-induced genes contribute to leukemic cell differentiation remains elusive. We noticed that chromosome 7 open reading frame 41 (C7ORF41) was a TPA-responsive gene and its upregulation concurred with human megakaryocyte differentiation. In K562 cells, ectopic expression of C7ORF41 significantly increased CD61 expression, enhanced ERK and JNK signaling, and upregulated RUNX1 and FLI1, whereas C7ORF41 knockdown caused an opposite phenotype. These observations suggest that C7ORF41 may promote megakaryocyte differentiation partially through modulating ERK and JNK signaling that leads to upregulation of RUNX1 and FLI1. In supporting this, C7ORF41 overexpression rescued megakaryocyte differentiation blocked by ERK inhibition while JNK inhibition abrogated the upregulation of FLI1 by C7ORF41. Furthermore, we found that Y34F mutant C7ORF41 inhibited megakaryocyte differentiation. nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) was the major activator of C7ORF41 that in turn repressed NF-κB activity by inhibiting its phosphorylation at serine 536, while MAPK/ERK was the potent repressor of C7ORF41. Finally, we showed that C7ORF41 knockdown in mouse fetal liver cells impaired megakaryocyte differentiation. Taken together, we have identified the function of a novel gene *C7ORF41* that forms interplaying regulatory network in TPA-induced signaling and promotes leukemic and normal megakaryocyte differentiation.

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## INTRODUCTION

Leukemic cells possess the ability to proliferate indefinitely. These cells stay in certain stage of blood cell development and lose their capacities to undergo terminal differentiation. Forced differentiation has long been proposed to be a rational method to cure leukemia. Indeed, differentiation therapy has been very successful in curing acute promyeloid leukemia (APL), which characteristically bears t(15;17) translocation and produces promyelocytic leukemia–retinoic acid receptor α fusion protein. Combinatory application of all-trans retinoic acid and arsenic trioxide synergistically causes terminal differentiation of APL cells and achieves complete clinic remission in APL patients.[1] Unfortunately, the exquisite sensitivity of APL to all-trans retinoic acid -based differentiation therapy may not be extended to other acute myeloid leukemia subtypes due to the heterogeneous etiology with different underlying molecular genetic aberrations. Extensive study on the molecular basis of forced differentiation in leukemic cells may be critical for the development of rational differentiation therapy for other subtypes of acute myeloid leukemia.

Compared with relative specific therapeutic effect of all-trans retinoic acid/arsenic trioxide on APL cells, phorbol 12-*O*-tetradecanoylphorbol-13-acetate (TPA) has broader effect on cell differentiation. It induces differentiation of multiple types of leukemic cells including HL-60, U937 and K562 cells.[2–4] TPA treatment induces macrophage differentiation in HL-60 cells and megakaryocyte differentiation in K562 cells. It has been shown to be an effective cancer therapeutic reagent in myelocytic leukemia patients and has been indicated as a potential colorectal cancer therapeutic reagent.[5–7] TPA has also been shown to increase white blood cell and neutrophil counts in solid tumor cancer patients.[5] Thus, further study on the molecular basis of TPA-induced leukemic cell differentiation may help to develop more effective and safe differentiation therapy.

Megakaryocyte differentiation in K562 cells induced by TPA treatment has been documented, which serves as a cell model for megakaryocyte differentiation. TPA primarily activates protein kinase C that subsequently activates mitogen-activated protein kinases (MAPK)/ERK, SAPK/JNK and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), whereas inhibits p38 and PI3K/AKT.[8–10] How each downstream signaling pathway may affect TPA-induced megakaryocyte differentiation has not been fully elucidated. The MAPKs are important signal transducers that have a pivotal role in physiological megakaryocyte development or TPA-induced megakaryocyte differentiation by upregulating the expression of several important transcription factors in megakaryopoiesis, including the ETS proteins and the myeloid transcription factor RUNX-1 (refs. 11–14). Activation of NF-κB by TPA also promotes megakaryocyte differentiation.[10] Yet, NF-κB responsive genes have not been reported in this process. The role of p38 MAPKs in megakaryocyte development has been far less studied.[9,15,16] Moreover, the role of JNK is barely known. Noticeably, the MAPK/ERK is activated in various carcinomas and its activation is believed to promote carcinogenesis. In addition, protein kinase C mutations or altered expression has been

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observed in multiple cancers.[17] How an apparent cancer-promoting signaling pathway in carcinoma can turn into a differentiation signaling pathway in leukemic cells remains unsolved. Nevertheless, the mechanism by which TPA may induce megakaryocytic differentiation remains to be further addressed.

In an effort to probe the mechanism of TPA-induced megakaryocyte differentiation, we noticed that a novel gene *C7ORF41* (chromosome 7 open reading frame 41) was upregulated and exhibited a dynamic expression pattern. C7ORF41 is conserved in evolution with little information about its expression, function or protein structure. It was shown to express differentially in human embryo development.[18] On the basis of a hierarchical clustering computational analysis, we predicted that C7ORF41 could function in hematopoiesis.[19] In this study, we found that C7ORF41 was upregulated in human CD34+ cells during the differentiation into megakaryocytes. It likely acted as signaling molecule to enhance ERK and JNK signaling and subsequently promoted megakaryocyte differentiation by upregulating RUNX1 and FLI1. Further supporting C7ORF41 function to promote megakaryopoiesis, C7ORF41 knockdown in mouse fetal liver cells impaired megakaryocyte differentiation. C7ORF41 expression was activated by NF-κB and in turn repressed NF-κB activity. One conserved tyrosine residue mutation completely abolished its function in signaling and megakaryocyte differentiation. Thus, we have identified a novel mechanism by which C7ORF41 functions to participate in TPA-induced megakaryocyte differentiation by modulating MAPK/ERK, SAPK/JNK and NF-κB signaling.

## MATERIALS AND METHODS

### Cell culture

Human erythroid leukemia cell lines K562 and HEL were cultured in a complete 1640 RPMI medium (Gibco BRL, Grand Island, NY, USA) and HEK293T cells were maintained in a complete Dulbecco's modified Eagle medium, both of which were supplemented with 10% fetal bovine serum, streptomycin and penicillin. Human megakaryocyte culture was performed by culturing human CD34+ bone marrow cells (purchased from Fred Hutchinson Cancer Research Center) in StemSpam SFEM media (Stemcell Technologies, Vancouver, BC, Canada) supplemented with penicillin/streptomycin, lipids (40 μg/ml), stem cell factor (100 ng/ml), and TPO (50 ng/ml) for 10 days.

Mouse megakaryocyte culture from fetal liver cells was described previously.[20] Briefly, imprinting control region mice (10 weeks old) were purchased from Hunan SLAC Laboratory Animal Co Ltd. Mating was set up and sperm plug was checked every morning in the following 3 days. Female mice with plug were separated and marked as gestation day 0.5. On day 12.5, pregnant mice were killed and fetal livers were dissected under microscopy. Livers were passed through a 23-gauge needle to obtain a single cell suspension. Red cells were lyzed by incubating the cell in the red cell lysis buffer (NH4Cl 0.15 M, KHCO3 1 mM, EDTA 0.1 Mm, pH 7.2–7.4) at 37 °C for 5 min. Cells were cultured in the expansion media (RPMI supplemented with 10% fetal bovine serum, 1/10 stem cell factor conditional media, 10 ng/ml interleukin-3, 10 ng/ml interleukin-6) overnight. In addition, they are transduced with control or retroviral vector expressing short hairpin RNAs (shRNA) specific for mouse C7ORF41. The transduced cells were selected with puromycin (1 μg/ml) in megakryocyte differentiation media (RPMI supplemented with 10% fetal bovine serum, 1/50 stem cell factor conditional media, 10 ng/ml TPO) for additional 3 days. At the den of culture, total cells (0.5 million) were collected for quantitative RT–PCR to confirm the downregulation of C7ORF41. Animal studies were approved by the Animal Care and Use Committees of Wuhan University.

### Lentivirus or retrovirus infection

Gene overexpression or knockdown was achieved through lentiviral or retroviral transduction as previously described.[21,22] All vectors carried puromycin-resistant gene and the transduced cells were selected with puromycin (1 μg/ml) for a week to obtain stable cell lines. C7ORF41 was fused with a Flag tag in the C-terminal. shRNAs for knocking down human or mouse C7ORF41 were determined by online shRNA searching tool (http://www.sirnawizard.com/design_ advanced.php), and blasted at NCBI to avoid off-target. The sequences of shRNA oligos for human C7ORF41 are as follows: shC7no. 1: 5′-GCACTTCGAAATAGGACATCT-3′; shC7 no. 2: 5′-GGCTCCCAGCATCCAAATTAA-3′. The shRNA oligo sequence for mouse C7ORF41 is shC7: 5′-GACGAGACGGCAGCTTACTTA-3′. Empty plasmids were used as controls.

### RNA isolation and quantitation of gene expression

For quantitative RT–PCR, total RNA was extracted from cells using TRIzol reagent (Invitrogen, Grand Island, NY, USA) and reverse-transcribed into complementary DNA using High-Capacity Reverse Transcription kit (Applied Biosystems, Grand Island, NY, USA). Quantitative RT–PCR was performed in the following conditions: 95 °C for 15 min followed by of 95 °C for 30 s, 60 °C for 30 s and 72 °C 1 min for 40 cycles. The reactions were run in triplicate on the 7900HT Fast Real-Time PCR System (Applied Biosystems). The relative quantitation of real-time PCR product was measured using the comparative $\Delta\Delta C_t$ method (docs.appliedbiosystems.-com/pebiodocs/04303859.pdf.) and presented in bar graph format.

### Flow cytometry analysis of megakaryocyte differentiation

K562 cells were incubated with TPA (10 nM) as times indicated. The resultant cells were washed with phosphate-buffered saline and stained with phycoerythrin-conjugated anti-CD61 or anti-CD41 antibody (BD Biosciences, San Jose, CA, USA) for 30 min. CD61 and CD41 expression levels were measured using flow cytometry (FACSCalibur, BD Biosciences). For mouse megakaryocyte culture, phycoerythrin-conjugated anti-mouse CD41 and antigen-presenting cell-conjugated anti-mouse CD42 antibodies were used. FACS data were analyzed with the FlowJo software (TreeStar, Ashland, OR, USA).

### Immunoprecipitation, immunoblotting and antibodies

Immunoprecipitation and western blot were performed as previous described.[21] Antibodies used in this study included goat anti-GATA-1, rabbit anti-FLI1, mouse anti-RUNX1, mouse anti-HSC70 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit anti-phospho-ERK, anti-phospho-JNK, anti-phospho-p38, anti-phospho-NF-κB (Cell Signaling Technology, Beverly, MA, USA); mouse anti-GAPDH, anti-lamin A/C and anti-HA (ProteinTech Group, Chicago, IL, USA). HSC70, GAPDH and Lamin A/C antibodies served as loading controls. Goat anti-mouse, donkey anti-goat and goat anti-rabbit IgG horseradish peroxidase-conjugated antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA) and mouse monoclonal FLAG antibody was from Sigma (St Louis, MO, USA). Full-length protein of human C7ORF41 expressed in BL21 bacteria was used to raise C7ORF41 rabbit polyclonal antibody.

### Nucleus and cytoplasm extraction

Briefly, $5–10 \times 10^6$ cells were collected and incubated in cytoplasm lysis buffer (20 mM HEPES pH 7.4, 10 mM KCl, 1 mM EDTA and 10% glycerol) with 0.2% NP-40 for 10 min on ice in the presence of protease inhibitors. Cell lysates were centrifuged at 14 000 r.p.m. for 10 min, and the supernatants were collected as cytoplasm extractions. The pellets were washed once and resuspended in nucleus lysis buffer (20 mM HEPES pH 7.4, 10 mM KCl, 1 mM EDTA, 20% glycerol, 350 mM NaCl and protease inhibitors), and nuclear extractions were harvested after centrifugation (16 000 *g*, 10 min).

### Luciferase assay

All luciferase assays were performed in 293T cells and all luciferase reporter constructs expressed firefly luciferase. Briefly, NF-κB reporter (NF-κB-luci), C7ORF41 promoter reporter or CD41α promoter reporter constructs were cotransfected with the same amount of pRL-TK (internal control) expressing a renila luciferase in combination with other plasmids as indicated. The dual luciferase activity was assayed according to the manufacturer's instructions (Dual-Luciferase Reporter Assay System, Promega, Madison, WI, USA). In addition, the promoter activity was presented as relative luciferase activity by normalizing the firefly luciferase activity to the internal control of renila luciferase activity.

### Statistical analysis

All statistical analyses were performed using the Student's *t*-test (two-tailed, unpaired). A *P*-value of 0.05 or less was considered significant.

## RESULTS

### C7ORF41 upregulation concurs with megakaryocyte differentiation

Differential expression of C7ORF41 was observed in human embryo development that was predicted to function in hematopoiesis.[18] As little information about its expression is available, we first profiled C7ORF41 expression in mouse tissues by western blot. C7ORF41 expression was readily detected in the thymus, bone marrow and spleen (Figure 1a). Low levels of C7ORF41 expression were also observed in various leukemic cell lines and carcinoma cell lines. Expression of C7ORF41 appeared relatively higher in leukemic cells including K562 than that of carcinoma cells (Figure 1b). Thus, we used K562 cells as a model to further investigate its function. K562 cells approximate megakaryocyte–erythrocyte progenitor cells with bi-potency to differentiate into megakaryocytes or red cells in response to TPA or cytosine arabinoside treatment, respectively. TPA treatment efficiently induced megakaryocyte differentiation in K562 cells evidenced by increased expression of CD61 and CD41 as previously reported (Figure 1c). It also rapidly upregulated C7ORF41 in 2 h, remained at relatively high level till 24 h, and eventually decreased to basal level in 48 h (Figure 1d). This expression pattern was similar to RUNX1, one essential transcription factor for megakaryocyte differentiation. Transcription factor FLI1 critical for megakaryocyte differentiation was gradually accumulated over time. Interestingly, C7ORF41 expression was constantly upregulated in human CD34 + cells induced to differentiate towards megakaryocytes over 10 days (Figure 1e). In addition, similar TPA-induced C7ORF41 upregulation was observed in an erythrocyte leukemia cell line HEL (Supplementary Figure S1A). These observations suggest a possible correlation of C7ORF41 expression and megakaryocyte differentiation.

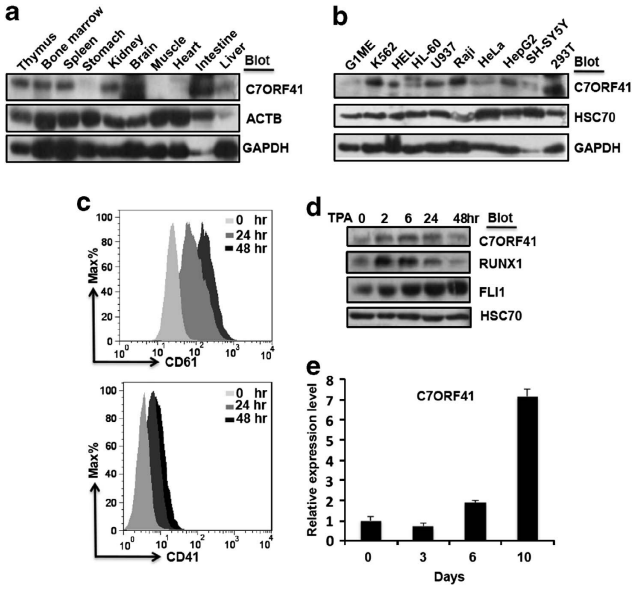


**Figure 1.** C7ORF41 upregulation concurs with megakaryocyte differentiation. The expression of C7ORF41 in mouse tissues (**a**) or cell lines (**b**) was measured using western blot. HSC70, ACTB and GAPDH serve as loading controls. (**c**) K562 treated with TPA for times as indicated. The megakaryocyte differentiation of K562 cells was assayed by measuring CD61 and CD41 expression through staining cells with phycoerythrin (PE)-conjugated anti-CD61 or anti-CD41antibody and was analyzed using flow cytometry. Histogram was representative data from three independent experiments (duplicates) with similar results. (**d**) K562 cells treated with TPA for times as indicated were harvested for western blot to detect C7ORF41, RUNX1 and FLI1 expression. HSC70 serves as a loading control. (**e**) Human CD34 + bone marrow cells were cultured in the presence of TPO for 0, 3, 6 or 10 days. The RNA was isolated for quantitative RT–PCR to measure C7ORF41 mRNA.

### C7ORF41 promotes megakaryocyte differentiation

To explore the effect of C7ORF41 on megakaryocyte differentiation, we overexpressed C7ORF41 in K562 cells through lentivirus transduction (Figure 2a). Ectopic expression of C7ORF41 did not alter the megakaryocyte-specific marker CD61 expression at rest state. Upon TPA stimulation, C7ORF41 overexpression significantly increased CD61 and CD41 expressions compared with control cells (Ctrl; Figure 2b). In addition, C7ORF41 overexpression also enhanced megakaryocyte differentiation in HEL cells (Supplementary Figure S1B). In contrast, C7ORF41 knockdown by lentivirus-mediated shRNA (shC7 no. 1 and shC7 no. 2) significantly decreased CD61 expression (Figures 2c and d). Particularly, we used two different shRNAs to knock down C7ORF41 and obtained similar results. We chose shC7 no. 1 for further experiments in K562 cells. In addition, C7ORF41 knockdown (shC7) in mouse fetal liver cells also dramatically impaired megakaryocyte differentiation evidenced by reduced CD41 and CD42 expression compared with that of control cells (Figure 2e). These observations suggest that C7ORF41 may promote megakaryocyte differentiation under pathological and physiological conditions.

### C7ORF41 enhances ERK and JNK signaling and upregulates RUNX1 and FLI1

TPA treatment primarily activates protein kinase C that stimulates multiple downstream signaling pathways and regulates expression of many TPA-responsive genes including several megakaryocyte-specific genes in K562 cells. C7ORF41 may act on any of these steps to influence megakaryocyte differentiation. To address this, we first determined its subcellular distribution. We fractionated cytoplasm (C) and nucleus (N) extractions from C7ORF41 overexpression cells treated with (+) or without (−) TPA for times as indicated and detected the presence of C7ORF41 by western blot. As controls, GAPDH was only detected in cytoplasm extraction, and Lamin A/C was only present in nucleus extraction (Figure 3a). Apparently, C7ORF41 was only detected in cytoplasm extraction and TPA treatment did not change its distribution (Figure 3a). Thus, we postulated that C7ORF41 likely acted as signaling molecule in the cytoplasm.

To test whether C7ORF41 may affect TPA-induced signaling, we measured activation of several downstream signaling molecules by western blot. As expected, we observed a dynamic pattern of ERK and JNK activation. TPA treatment rapidly stimulated ERK phosphorylation within several hours that was maintained at high level till 24 h. Its phosphorylation gradually decreased almost to the basal level within 48 h (Figure 3b). Interestingly, a two-wave of JNK activation pattern was observed: rapid JNK phosphorylation in 2 h and down in 6 h followed by a constant increase of JNK activation till 48 h (Figure 3b). Noticeably, C7ORF41 overexpression increased both ERK and JNK activation without altering the dynamic patterns, most prominent for ERK phosphorylation at 2–6 h and JNK phosphorylation at 2 h or 24–48 h (Figure 3b). Inhibition of p38 signaling was documented in TPA treatment and we failed to find any significant change (Figure 3c).[9] These results demonstrated that C7ORF41 enhanced ERK and JNK signaling.

Megakaryocyte differentiation is controlled by master transcriptional factors including RUNX1, FLI1 and GATA1/2. C7ORF41 may also promote megakaryocyte differentiation by modulating the expression of these transcriptional factors. Indeed, we observed a dynamic expression pattern of RUNX1 similar to ERK activation. The expression of RUNX1 was rapidly upregulated in 2 h, maintained in high level till 12 h and gradually decreased to basal level in 48 h (Figure 3b). C7ORF41 increased RUNX1 expression

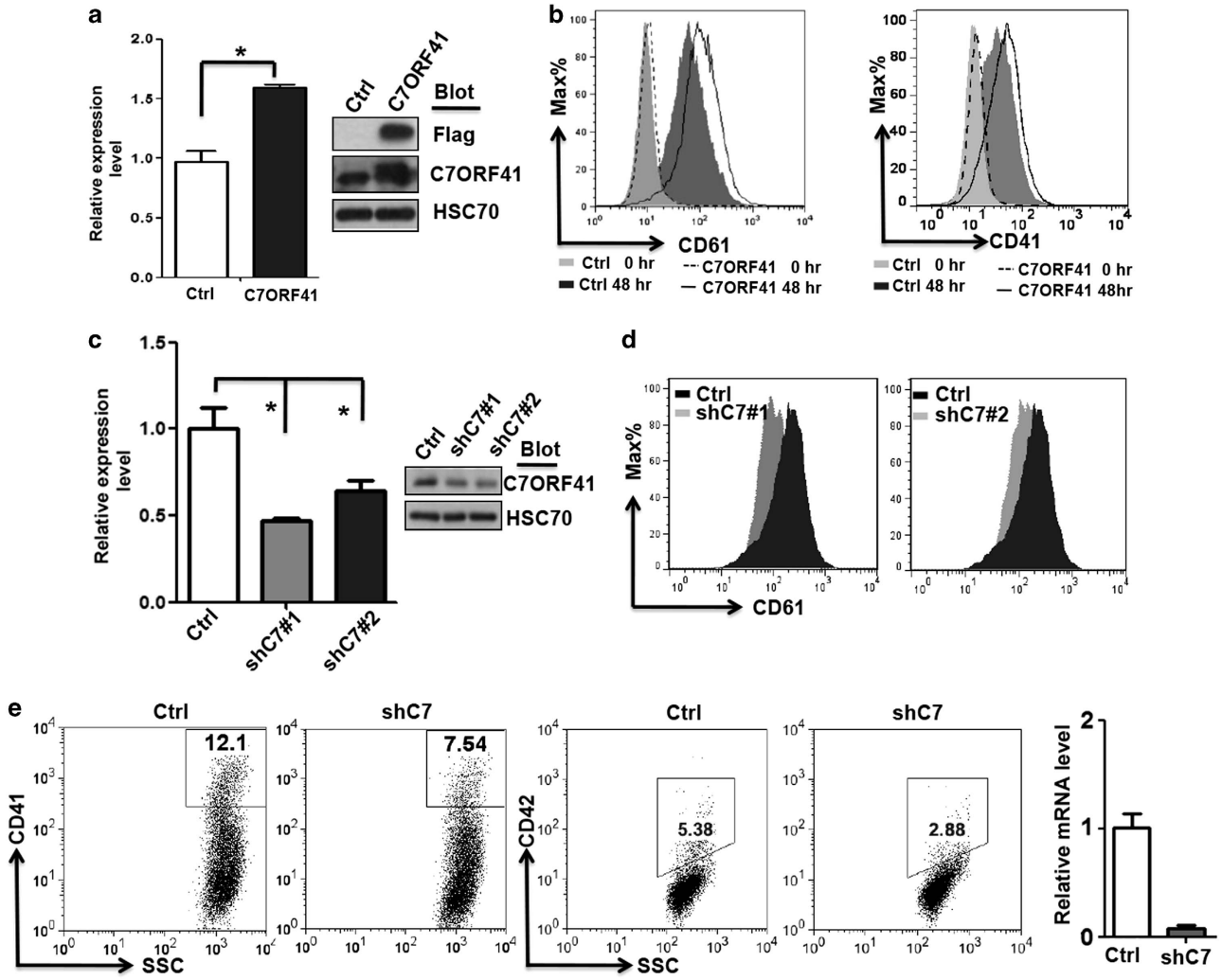


**Figure 2.** C7ORF41 promotes TPA-induced megakaryocyte differentiation. (**a**) K562 cells were transduced with control lentiviral vector (Ctrl) or a lentiviral vector expressing C7ORF41. The relative expression level of C7ORF41 mRNA (left panel) in the resultant cells was measured using quantitative RT–PCR. The endogenous or exogenous C7ORF41 protein in the resultant cells was also detected with western blot using antibody specific for C7ORF41 or antibody specific to Flag tag fused to exogenous C7ORF41 (right panel). HSC70 serves as a loading control. (**b**) The transduced K562 cells were treated with TPA for 48 h (Ctrl 48 h and C7ORF41 48 h) or without TPA (Ctrl 0 h and C7ORF41 0 h). The megakaryocyte differentiation of the treated cells were assayed by staining cells with phycoerythrin (PE)-labeled anti-CD61 and anti-CD41 antibodies and analyzed using flow cytometry. (**c**) K562 cells were transduced with control lentiviral vector (Ctrl) or a lentiviral vector expressing shRNA specific for human C7ORF41 (shC7 no. 1, shC7 no. 2). The downregulation of C7ORF41 mRNA or protein was confirmed using quantitative RT–PCR (left panel) and western blot (right panel), respectively. Data were the statistics of two experiments (triplicates) with similar results. (**d**) The C7ORF41 knockdown cells (shC7 no. 1, shC7 no. 2) and control cells (Ctrl) were treated with TPA for 48 h, and the megakaryocyte differentiation was measured by staining cells with anti-CD61 antibody and analyzed by flow cytometry. Histogram was representative data from three independent experiments (duplicates) with similar results. (**e**) Fetal liver cells isolated from pregnant mice at gestation 12.5 days were transduced with control lentiviral vector (Ctrl) or lentiviral vector expressing shRNA specific for mouse C7ORF41 (shC7). The transduced fetal liver cells were selected with puromycin and cultured in the presence of TPO for additional 3 days. The megakaryocyte differentiation was measured by staining cells with PE-conjugated anti-mouse CD41 and CD42 antibodies and was analyzed by flow cytometry. The total cells (0.5 million) were also collected to make total RNA to confirm C7ORF41 knockdown by quantitative RT–PCR (Bar graph on the right). * indicates significance compared with control ($P < 0.05$).

even without TPA treatment compared with control cells, whereas ERK phosphorylation was not significantly altered by C7ORF41 (Figure 3b). Such upregulation was maintained till 24 h and reduced to the basal level comparable to that of control cells in 48 h (Figure 3b). In addition, FLI1 was accumulated during TPA-induced megakaryocyte differentiation and C7ORF41 drastically increased their expression at 48 h compared with that of control cells (Figure 3c). In contrast, GATA1/2 expression was not significantly changed by C7ORF41 (Figure 3c). These results demonstrated that C7ORF41 upregulated the expression of RUNX1 and FLI1.

To demonstrate the specific effect of C7ORF41 on signaling and megakaryocyte transcription factor expression, we downregulated C7ORF4 and observed opposite phenotype in C7ORF41 knockdown cells: the phosphorylation of ERK and JNK was reduced and the expression of FLI1 was less in these cells compared with control cells (Figures 3d and e). Considering the critical role of ERK signaling and the expression of RUNX1 and FLI1 in megakaryocyte differentiation, these observations suggest that C7ORF41 may promote megakaryocyte differentiation in part by enhancing ERK and JNK signaling as well as upregulating RUNX1 and FLI1 expression.

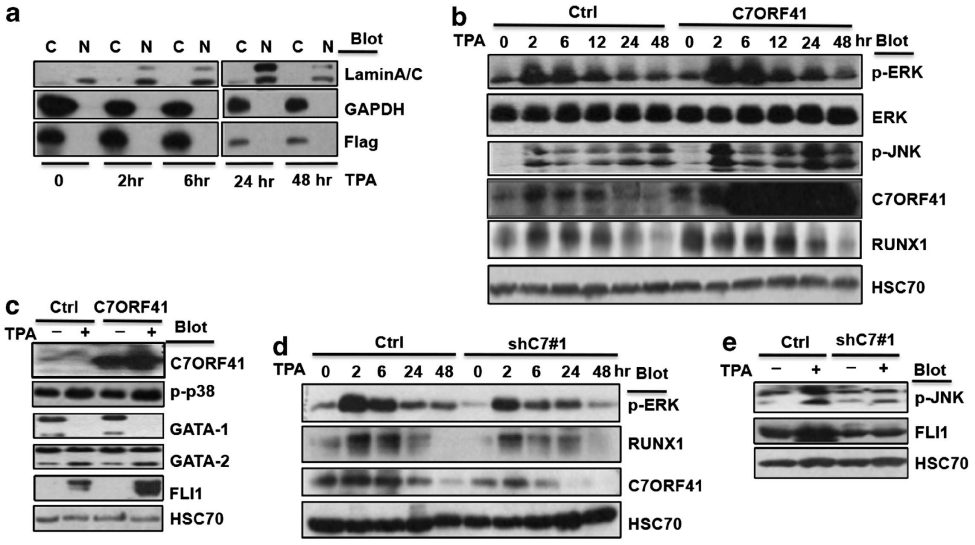


**Figure 3.** C7ORF41 enhances ERK and JNK signaling and upregulates RUNX1 and FLI1. (**a**) C7ORF41-overexpressing K562 cells were treated with TPA as times indicated, and cytoplasmic extracts (C) or nucleus extracts (N) were fractionated from the treated cells. The presence of C7ORF41 was detected using western blot. GAPDH and LaminA/C served as control for cytoplasmic protein or nucleus protein, respectively. Repositioned lanes are from the same blotting. (**b**) Control (Ctrl) or C7ORF41-overexpressing cells were stimulated with TPA for times indicated. The phosphorylation of ERK (p-ERK) and JNK (p-JNK), expression of RUNX1 and C7ORF41 were detected by western blot. (**c**) Control (Ctrl) or C7ORF41-overexpressing cells treated with (+) or without (−) TPA for 48 h were harvested for western blot to detect phosphorylation of p38 (p-p38), or expression of GATA-1, GATA-2 and FLI1 proteins. (**d**) Control (Ctrl) or C7ORF41 knockdown (shC7 no. 1) K562 cells were stimulated with TPA as times indicated. The p-ERK or expression of RUNX1 and C7ORF41 was detected by western blot. (**e**) Control (Ctrl) or C7ORF41-downregulated (shC7 no. 1) cells treated with (+) or without (−) TPA for 48 h were harvested for western blot to detect p-JNK, or the expression of FLI1. All data were representative blots of at least two independent experiments with similar results.

### Critical role of ERK and JNK signaling in C7ORF41 enhanced megakaryocyte differentiation

ERK signaling has a critical role in TPA-induced megakaryocyte differentiation. Pretreatment of K562 cells with PD98059 within 24 h of TPA treatment effectively impaired both the activation of ERK and megakaryocyte differentiation, whereas there was no effect after 24 h as previous studies demonstrated.[23–25] The critical role of sustained ERK signaling in upregulation of RUNX1 in megakaryopoiesis has also been documented.[26] To determine the role of enhanced ERK signaling in C7ORF41 function, we inhibited ERK signaling by PD98059, a highly selective synthetic inhibitor of MAPK kinase 1. Consistent to previous studies, TPA plus PD98059 treatment (Ctr + PD98059) reduced TPA-induced CD61 expression compared with control cells treated with TPA alone (Ctrl; Figure 4a). Overexpression of C7ORF41 overcame the inhibitory effect of PD98059. The megakaryocyte differentiation of the C7ORF41-overexpressing cells treated with TPA plus PD98059 (C7ORF41 + PD98059) was more pronounced than that of control cells treated with TPA plus PD98059 (Crtl + PD98059). The CD61 expression in C7ORF41 + PD98059 cells was restored to the level comparable to that of control cells treated with TPA alone (Ctrl; Figure 4a). Correspondingly, the RUNX1 expression in C7ORF41 + PD98059 cells was higher than that of control cells treated with TPA and PD98059 (Figure 4b). Considering RUNX1, expression was upregulated by C7ORF41 without TPA treatment while ERK phosphorylation was not significantly altered by C7ORF41, upregulation of RUNX1 by C7ORF41 may have both ERK-dependent and -independent mechanisms. These observations demonstrated that ERK signaling in part mediated the effect of C7ORF41 on enhancing megakaryocyte differentiation induced by TPA.

As the JNK activation was also enhanced by C7ORF41, we further tested the role of JNK signaling in the function of C7ORF41. Prolonged JNK inhibitor treatment caused extensive cell death (data not shown) that prevented us from testing its effect on megakaryocyte differentiation. We noticed that JNK activation and accumulation of FLI1 concurred at late stage of megakaryocyte differentiation. Thus, we tested the effect of JNK signaling on FLI1 protein accumulation. After 2 days of TPA treatment, cells were further treated with JNK inhibitor X for additional 12 h. We found that the enhanced expression of FLI1 by C7ORF41 was damped by JNK inhibitor X (Figure 4c). In contrast, PD98059 treatment showed no effect at all (Figure 4c), which is consistent to previous studies.[22–24] These observations suggest an important role of JNK signaling in megakaryocyte differentiation by maintaining FLI1 expression at late stage. In addition, JNK signaling may in part mediate upregulation of FLI1 by C7ORF41. Further supporting this idea, overexpression of a dominant-negative form of JNK (JNK DN) reduced JNK phosphorylation and TPA-induced megakaryocyte differentiation (Figures 4d and e).

Taken together, our observations suggest that C7ORF41 may promote megakaryocyte differentiation in part by enhancing ERK and JNK signaling that subsequently leads to upregulation of RUNX1 and FLI1, two types of transcription factors critical for megakaryocyte differentiation.

### A conserved tyrosine site Y34 is critical for C7ORF41 function

Tyrosine phosphorylation is one of the characters of signaling molecules. We thus tested whether C7ORF41 was tyrosine phosphorylated by performing immunoprecipitation followed by western blot with tyrosine phosphorylation antibody. Indeed, we observed that C7ORF41 was tyrosine-phosphorylated (Figure 5a). Gene conservative usually implies that the gene function is important. And gene alignment can identify conserved tyrosine and/or threonine residues that are critical for the function in various signaling molecules. To reveal this, we performed multiple protein sequence alignment and found that C7ORF41 was fairly conserved in nine species tested, of which four tyrosine residues were highly preserved (Figure 5b). Noticeably, the tyrosine 34 was predicted to be a kinase substrate or protein-binding sites (Supplementary Figure Table S1). Thus, we tested the role of tyrosine 34 in C7ORF41 gene function by testing the function of a

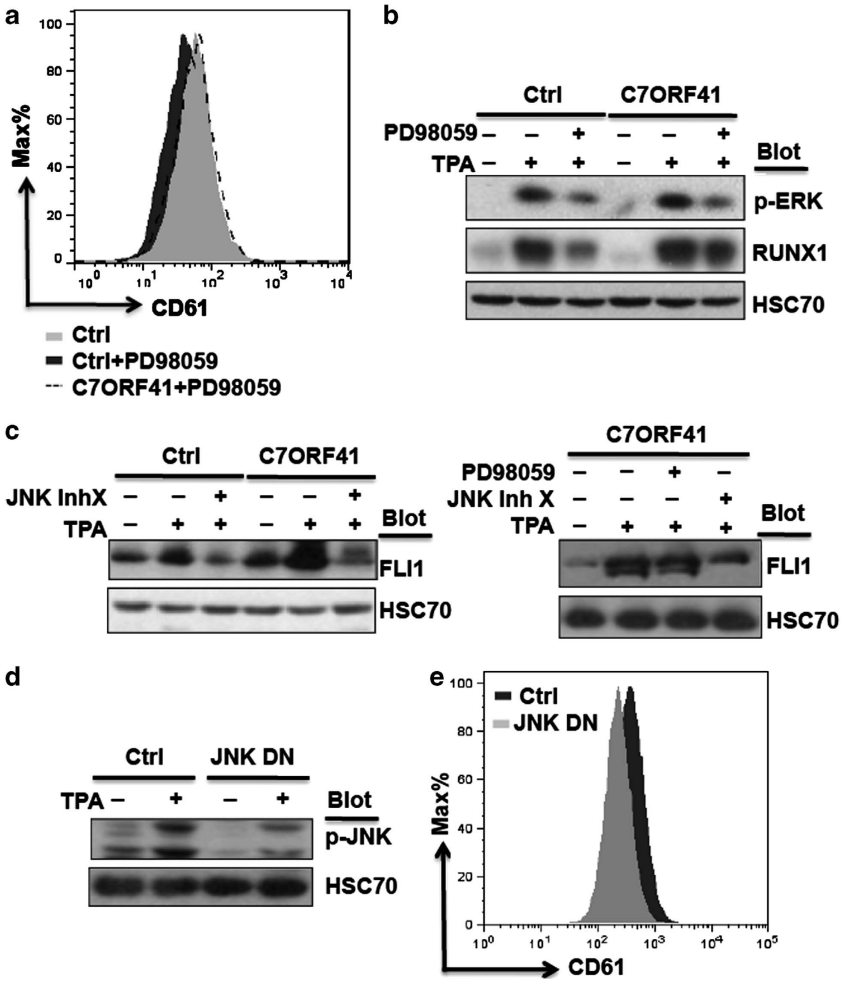


**Figure 4.** Activation of ERK and JNK are essential for C7ORF41 function in promoting megakaryocyte differentiation. (**a**) Control cells (Ctrl + PD98059) and C7ORF41-overexpressing cells (C7ORF41 + PD98059) were treated with TPA and PD98059 for 48 h. Control cells treated with TPA alone (without PD98059, Ctrl) served as control. The megakaryocyte differentiation of treated cells was assayed by staining cells with PE-labeled anti-CD61 antibody and was analyzed using flow cytometry. (**b**) Control cells or C7ORF41-overexpressing cells were treated with (+) or without (−) TPA and PD98059 as indicated for 48 h. The treated cells were harvested for western blot to detect p-ERK and the expression of RUNX1. (**c**) The control (Ctrl) or C7ORF41-overexpressing cells were treated with (+) or without (−) TPA for 48 h and further treated with (+) or without (−) JNK inhibitor X or PD98059 as indicated for additional 12 h. The resultant cells were harvested for western blot to detect the expression of FLI1. (**d**) Control cells and cells expressing a dominant-negative form of JNK (JNK DN) were treated with (+) or without (−) TPA for 48 h. The resultant cells were harvested for western blot to detect the p-JNK. (**e**) Control cells (Ctrl) or dominant negative JNK-overexpressing cells (JNK DN) were treated with TPA for 48 h. The megakaryocyte differentiation of treated cells was assayed by staining cells with anti-CD61 antibody and was analyzed using flow cytometry. All blots and histograms were representative data from three independent experiments (duplicates) with similar results.

mutant form of C7ORF41 in which the tyrosine 34 was mutated to phenylalanine (C7Y34F). Overexpression of C7Y34F lost the ability to promote megakaryocyte differentiation. In fact, C7Y34F blocked megakaryocyte differentiation (Figures 5c and d). Consistently, C7Y34F inhibited CD41$\alpha$ promoter activity (Figure 5e) and impaired ERK and JNK phosphorylation as well as the expression of FLI1 (Figure 5f). Surprisingly, Y34F mutation did not abolish tyrosine phosphorylation of C7ORF41 (Figure 5g), suggesting that C7ORF34 might be phosphorylated at multiple tyrosine sites. These observations suggest that phosphorylation of tyrosine 34 may be critical for C7ORF41 function in promoting megakaryocyte differentiation.

### C7ORF41 is regulated by NF-κB and negatively feeds back to repress NF-κB signaling

TPA treatment activates various signaling pathways including MAPK/ERK, SAPK/JNK and NF-κB. To test which TPA-activated signaling pathways may upregulate C7ORF41 expression, we measured C7ORF41 promoter activity by performing dual luciferase assay. Interestingly, several TPA-activated signaling pathways such as MAPK/ERK and SAPK/JNK did not stimulate C7ORF41 promoter activity. In fact, the constitutively active form of MEK1 or RAS, two specific upstream MAPK/ERK activators, even repressed its activity, although ERK activation seemed important for C7ORF41 function in megakaryocyte differentiation (Figures 4a and 6a). Interestingly, constitutively active PI3K but not AKT repressed C7ORF41, suggesting that PI3K signaling may suppress C7ORF41 through an AKT-independent mechanism as demonstrated in previous studies.[27] Noticeably, NF-κB dramatically increased the C7ORF41 promoter activity (Figure 4a). We confirmed the role of NF-κB in upregulating C7ORF41 by using SC-514, a specific NF-κB inhibitor. SC-514 treatment repressed NF-κB phosphorylation (p-p65) activated by TPA treatment and reduced endogenous C7ORF41 expression at both mRNA and protein levels (Figures 6b and c). It also blocked the upregulation of C7ORF41 in response to TPA

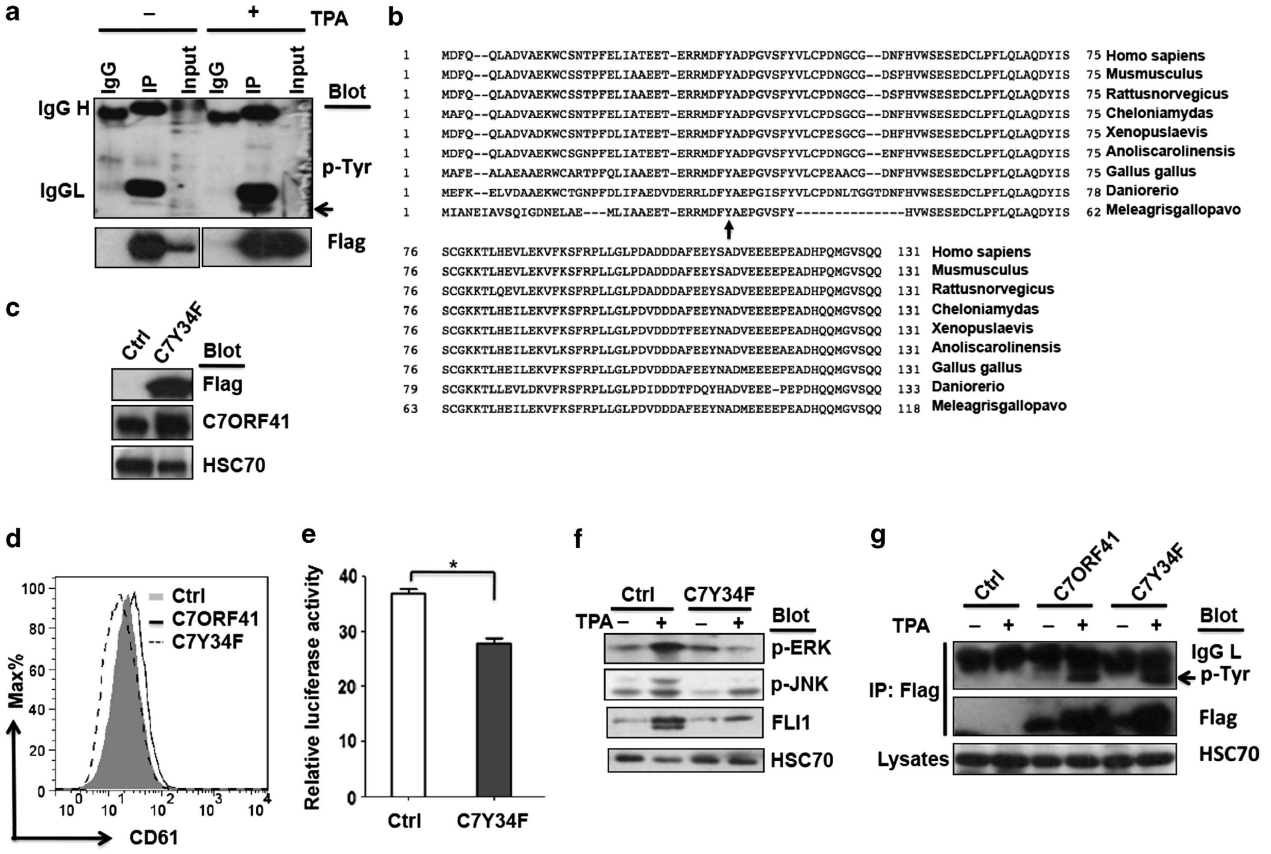


**Figure 5.** Conserved tyrosine 34 is critical for C7ORF41 function. (**a**) C7ORF41-overexpressing cells were treated with (+) or without (−) TPA for 12 h and harvested for immunoprecipitation with an antibody (IP) recognizing Flag that has been fused to exogenous C7ORF41 protein. As control, pre-immuned normal mouse IgG was also used for immunoprecipitation. One percent of cell lysates was used as input. The tyrosine phosphorylation of C7ORF41 was measured by blotting with an antibody that specifically recognized phosphorylated tyrosine (p-Tyr). The presence of exogenous C7ORF41 was confirmed by blotting with anti-Flag antibody. Arrow indicates tyrosine phosphorylation of C7ORF41 below the IgG light chain (IgG L). IgG H indicates IgG heavy chain. (**b**) Sequences from nine species were aligned to show the conservation of C7ORF41. Arrow indicates the conserved tyrosine 34. (**c**) Cells transduced with control vector or a lentiviral vector expressing mutant C7ORF41 with Y34 substitution by phenylalanine (C7Y34F) were used for western blot to detect endogenous or exogenous C7ORF41 protein with C7ORF41 antibody and Flag antibody, respectively. (**d**) Control cells, wild-type C7ORF41-overexpressing or mutant C7ORF41 (C7Y34F)-overexpressing cells were treated with TPA for 48 h. The megakaryocyte differentiation of treated cells was assayed by staining cells with PE-conjugated anti-CD61 antibody and was analyzed using flow cytometry. Histogram was representative data from three independent experiments (duplicates) with similar results. (**e**) The CD41$\alpha$ promoter activity reporter vector was contransfected with control vector (Ctrl) or C7Y34F into 293T cells as indicated. The promoter activity was measured and presented as relative luciferase activity. * indicates significance compared with control ($P<0.05$). (**f**) Control cells or C7Y34F cells were treated with (+) or without (−) TPA for 48 h. The treated cells were harvested for western blot to measure p-ERK and p-JNK or the expression of FLI1. (**g**) Control cells (Ctrl), C7ORF41 or C7Y34F cells were treated with (+) or without (−) TPA for 12 h and used for immunoprecipition with FLAG antibody. The expression of C7ORF41 and C7Y34F was detected by blotting with FLAG antibody, and the tyrosine phosphorylation was measured by blotting with p-Tyr.

treatment (Figures 6b and c). These observations suggest that NF-κB is a critical activator of C7ORF41 in response to TPA treatment.

In contrast to the function of C7ORF41 to enhance TPA-induced ERK and JNK signaling, C7ORF41 seemed in turn to repress NF-κB activity. We found that C7ORF41 overexpression significantly reduced the transcription activity of a NF-κB-responsive promoter, whereas C7ORF41 knockdown by shRNA increased it (Figures 6d and e). Furthermore, C7ORF41 appeared to suppress NF-κB activity by reducing phosphorylation of p65 at serine 536 (Figure 6f), which contributes to NF-κB transactivation and increases p65 transcriptional activity.[28] These observations suggest that C7ORF41 may act as a feedback to inhibit NF-κB activity by suppressing its phosphorylation in response to TPA treatment.

## DISCUSSION

TPA has a broad influence on cell differentiation and apoptosis in leukemic blood cells. How TPA-induced genes may mediate its function remains to be further addressed. In an effort to address the mechanism of TPA-induced megakaryocyte differentiation, we found that C7ORF41 was a TPA-responsive gene that promoted megakaryocyte differentiation through a novel mechanism.

C7ORF41 may function to promote blood cell differentiation. We previously identified C7ORF41 as a differential expression gene in human embryo development.[18] On the basis of a hierarchical clustering computational analysis, C7ORF41 was predicted to function in hematopoiesis.[19] Here, we confirmed C7ORF41 expression in blood cells and demonstrated its upregulation in human megakaryocyte differentiation, suggesting a potential role of C7ORF41 in blood cell differentiation. By using K562 cells as a model, we provided evidence that C7ORF41 enhanced TPA-induced megakaryocyte differentiation. C7ORF41 seemed to have no effect on blood cell proliferation since overexpression or downregulation of C7ORF41 did not change K562 cell proliferation and its overexpression did not affect colony-forming ability in mouse bone marrow cells (Supplementary Figures S2A–C).

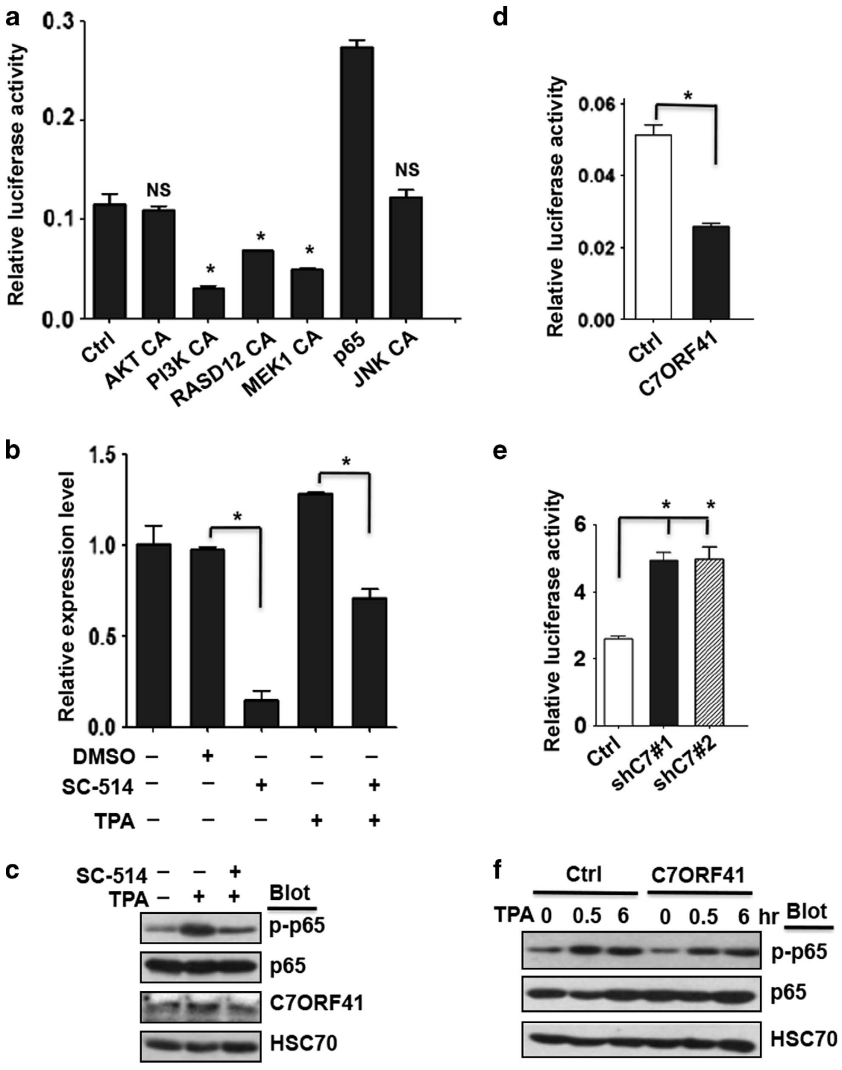


**Figure 6.** NF-κB-activated C7ORF41 negatively feeds back to repress NF-κB signaling. (**a**) C7ORF41 promoter activity reporter construct was transfected into 293T cells in combination with a control vector (Ctrl) or vectors expressing various signaling molecules as indicated. The promoter activity was measured and presented as relative luciferase activity. (**b**) K562 cells treated with TPA and/or NF-κB inhibitor SC-514 as indicated for 12 h. The treated cells were harvested for quantitative RT–PCR to measure C7ORF41 mRNA expression level. (**c**) The treated cells were also harvested for western blot to detect the phosphorylation of p65 (p-p65) and the expression of C7ORF41. (**d**) 293T cells were transfected with NF-κB reporter construct in combination with control (Ctrl) vector or C7ORF41-overexpressing vector (C7ORF41). The NF-κB activity was measured and presented as relative luciferase activity. (**e**) 293T cells were transfected with NF-κB reporter construct in combination with control (Ctrl) vector or C7ORF41 knocking down vector (shC7 no. 1, shC7 no. 2). The NF-κB activity was measured and presented as relative luciferase activity. (**f**) Control or C7ORF41-overexpressing cells were treated with TPA for times as indicated. The phosphorylation of NF-κB (p-p65) and total p65 were measured by western blot. *$P<0.05$ and *NS* indicates no significance compared with control.

However, we did observe that C7ORF41-overexpressing cells were more sensitive to TPA treatment than control cells. C7ORF41 overexpressing cells showed higher CD61 expression and fewer C7ORF41-overexpressing cells survived TPA treatment while C7ORF41 downregulation led to the opposite phenotype (Figure 2 and Supplementary Figures S2D and E). Interestingly, we noticed that C7ORF41 was upregulated when K562 cells were induced to differentiate into erythrocytes by Arabinofuranosyl Cytidine or hemin (Supplementary Figures S3A and B). Indeed, C7ORF41 overexpression promoted erythrocyte differentiation evidenced by increased γ-globin expression and benzidine-staining positive cells (Supplementary Figures S3C–F). Further supporting this, C7ORF41 overexpression in zebra fish through microinjection of C7ORF41 mRNA led to increased numbers of mature red blood cells evidenced by o-dianisidine staining for hemoglobin (Supplementary Figure S3G). In a mouse bone marrow transplantation experiment, C7ORF41-transduced bone marrow progenitor cells failed to engraft into recipient mice (Supplementary Figure S4). The green-fluorescent protein (GFP) positivity in peripheral blood cells from recipient mice transplanted with C7ORF41-overexpressing bone marrow cells was decreased to almost background a month post transplantation (from 36% GFP positivity before transplantation versus ~1.6% GFP positivity 1 month after transplantation), whereas GFP positivity in peripheral blood cells from recipient mice transplanted with control bone marrow cells retained high level comparable to that before transplantation (60% before transplantation and ~49.5% 1 month after transplantation) (Supplementary Figure S4). Altogether, the present evidence points to a potential role of C7ORF41 in blood cell differentiation.

How C7ORF41 may modulate TPA-induced multiple signaling pathways remain unclear. Our study demonstrated that C7ORF41 enhanced TPA-activated ERK and JNK phosphorylation and repressed NF-κB activity. We tested whether C7ORF41 did so by

interacting with any signaling molecules in these pathways. We found that C7ORF41 immunoprecipitation failed to co-immunoprecipitate ERK or JNK (Supplementary Figure S5). Furthermore, we performed a phosphorylation site and protein interaction prediction[29] (http://www.hprd.org/index_html; supplementary Table S1). Src kinase, SHP1 and Crk were suggested to interact with C7ORF41. However, C7ORF41 immunoprecipitation failed to co-immunoprecipitate CRK, SHP1 or FYN (Supplementary Figure S5). Thus, how C7ORF41 may affect downstream signaling remains to be further studied.

C7ORF41 seems to be tightly regulated. Only p65 significantly increased C7ORF41 promoter activity. In supporting this, NF-κB inhibition dramatically impaired C7ORF41 expression (Figure 6). Rapid NF-κB activation at immediate early stage of TPA treatment has been documented and its activation mediates megakaryocyte differentiation.[10] However, the NF-κB-responsive gene that contributes to this process has not been identified. Our study provides evidence that C7ORF41 may serve as a NF-κB-responsive gene to promote megakaryocyte differentiation. Surprisingly, C7ORF41 appeared to repress NF-κB activity potently by inhibiting p65 phosphorylation at serine 536, which was required for optimal p65 activation.[28] The mechanism by which C7ORF41 may suppress NF-κB phosphorylation remains under intensive investigation.

Taken together, we have identified novel function of a new gene *C7ORF41* that may promote leukemic megakaryocyte differentiation through a novel mechanism by which C7ORF41 forms regulatory network in TPA-induced signaling (Figure 7). In this network, initial TPA treatment primes downstream signaling MAPK/ERK, SAPK/JNK and NF-κB. NF-κB activation further upregulates C7ORF41 that may serve to amplify TPA-induced ERK and JNK signaling to ensure megakaryocyte differentiation. On the other hand, C7ORF41 upregulation feeds back to serve as a negative regulator of NF-κB activity that may quench TPA-induced NF-κB signaling. In addition, enhanced ERK signaling in turn acts to damp C7ORF41 upregulation that may tune TPA-induced signaling. Our findings shed light on understanding of forced differentiation in leukemic cells and may provide useful information for development of rational differentiation therapy.

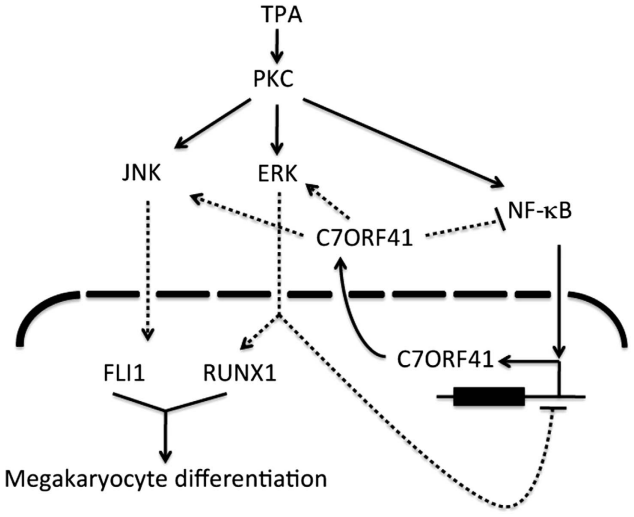


**Figure 7.** The proposed mechanism by which C7ORF41 modulates TPA-induced signaling and megakaryocyte differentiation in K562 cells. TPA-activated NF-κB upregulates C7ORF41 that further enhances TPA-induced ERK and JNK signaling to promote megakaryocyte differentiation by upregulating FLI1 and RUNX1. C7ORF41 upregulation feeds back to suppress NF-κB signaling and C7ORF41 upregulation was restrained by ERK signaling.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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