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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and Epidermal Growth Factor Cooperatively Suppress Peroxisome Proliferator-Activated Receptor-γ1 Stimulation and Restore Focal Adhesion Complexes during Adipogenesis: Selective Contributions of Src, Rho, and Erk Distinguish These Overlapping Processes in C3H10T1/2 Cells

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ABSTRACT

Stimulation of PPARγ1 and adipogenesis in multipotential C3H10T1/2 cells by the combination of dexamethasone and 3-isobutyl-1-methylxanthine (DM) is suppressed by 2,3,7,8 tetrachlorodibenzodioxin (TCDD) (10 nM). This suppression requires sustained activation of extracellular signal-regulated kinase (Erk)1/2. We show that it arises from an effect of TCDD on epidermal growth factor (EGF) signaling. DM initiates an early loss of cell adhesion that is reversed by this TCDD/EGF synergy. Src kinase activity was completely essential for adhesion restoration, sustained Erk activation, and suppression of peroxisome proliferator-activated receptor (PPAR)γ1. MEK/Erk activity did not contribute, however, to TCDD-induced adhesion. Stimulation of adhesion may therefore precede elevation of Erk. Adhesion is produced by interaction of αβ integrins with extracellular matrix proteins and subsequent Src-mediated phosphorylation of focal adhesion kinase (FAK, Tyr576/577) and paxillin (Tyr118). TCDD enhanced the steady state Src-mediated phosphorylation of FAK but not of paxillin. Protein tyrosine phosphatase (PTPase) inhibition by orthovanadate (OVA) showed that this Src activity is highly restricted by PTPases. Partial inhibition of PTPases by OVA mimicked TCDD in producing EGF- and Src-dependent effects on cell adhesion and PPARγ1 suppression. TCDD may therefore induce a protein that enhances Src effectiveness at adhesion sites. Rho kinase (ROCK) inhibition blocked TCDD/EGF stimulation of clustered focal adhesion complexes without affecting either sustained Erk activation or suppression of PPARγ1. Thus, this ROCK-mediated clustering of integrin complexes is not needed for the effects of TCDD on Erk and PPARγ1. A minimal cholesterol depletion with β-methylcyclodextrin attenuated TCDD effects on PPARγ1 and Erk activation. TCDD intervention is therefore linked to extracellular proteins. It indicates that TCDD-enhanced stimulation of EGF signaling to Erk may derive from the initial αβ integrin complexes.

Extensive studies on adipocyte differentiation have used mouse embryonic fibroblastic cell models, including 3T3-L1 and 3T3–422A, which are already committed to the adipocyte lineage. C3H10T1/2 (10T1/2), a pluripotent progenitor cell type that is similar to mouse embryo fibroblasts, has also been used (Alexander et al., 1998). This cell type develops into fat, bone, or muscle cells according to the stimulus (Ntambi and Young-Cheul, 2000). In general, differentiation of 3T3-L1 and 10T1/2 cells is stimulated by a hormonal mixture (IDM) composed of insulin, dexamethasone (Dex), and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), which is administered to confluent cells along with serum renewal (Cowherd et al., 1999). Over approximately 48 h in 10T1/2 cells, IDM cooperatively directs several

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ABBREVIATIONS: Dex, dexamethasone; IBMX, 3-isobutyl-1-methylxanthine; IDM, insulin/dexamethasone/3-isobutyl-1-methylxanthine; C/EBP, CCAAT/enhancer-binding protein; PPAR, peroxisome proliferator-activated receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; DM, dexamethasone/3-isobutyl-1-methylxanthine; URS, unrenewed serum condition; MAP, mitogen-activated protein; OVA, sodium orthovanadate; ECM, extracellular matrix; FAK, focal adhesion kinase; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; Y27632, *N*-(4-pyridyl)-4-(1-aminoethyl)cyclohexanecarboxamide dihydrochloride; MCD, β-methylcyclodextrin; PBS, phosphate-buffered saline; MEK, mitogen-activated protein kinase kinase; Erk, extracellular signal-regulated kinase; PD98059, 2′-amino-3′-methoxyflavone; ROCK, Rho kinase.

sequential waves of gene responses. The earliest increases include c-myc, c-fos, and jun-b, followed by C/EBPδ and C/EBPβ, which then stimulate transcription of PPARγ1, a key regulator of commitment to triglyceride synthesis. Ligand activated PPARγ1 and C/EBPβ stimulate increases in C/EBPα and also PPARγ2, a larger form derived from an alternative promoter and first exon (Lazar, 2005). The additional 30 amino acids at the N terminus cause differences in coactivator recognition. The two PPARγ forms and C/EBPα combine to mediate the insulin induction of lipogenic genes. We have shown that only Dex and IBMX are needed to generate insulin-responsive lipogenesis and that a serum change can be omitted (Cho and Jefcoate, 2004). The present studies use this simplified protocol with C3H10T1/2 cells.

The adipocyte differentiation process is inhibited by the environmental contaminant, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a potent activator of the aryl hydrocarbon receptor (AhR). AhR is a member of the helix-loop-helix/periodicity-aryl hydrocarbon receptor nuclear translocator–simple-minded (PER/ARNT/SIM) nuclear receptor family that heterodimerizes with a related protein ARNT when activated by TCDD (Safe, 2001). This activation induces a select few genes in 10T1/2 cells including CYP1A1 and CYP1B1 (Hanlon et al., 2005b). In mice treated with higher doses of TCDD than that for inducing CYP1A1, an overall loss of body fat and weight (wasting syndrome) accompanied by increased circulating triglycerides is seen (Liu and Matsumura, 1995). Previous work has characterized a TCDD-mediated inhibition of adipocyte differentiation in 3T3-L1 preadipocytes (Phillips et al., 1995). TCDD prevents the initial stimulation of PPARγ and later regulators but does not affect the expression of early response genes, including C/EBPβ (Phillips et al., 1995; Chen et al., 1997).

10T1/2 cells differentiate rapidly without cell division and without the mitotic stimuli provided by serum renewal or insulin (Cho and Jefcoate, 2004). We have used this characteristic of the 10T1/2 cells to completely separate gene expression associated with mitotic regulation of 10T1/2 cells from the regulation of differentiation (Hanlon et al., 2005a). In this study, we have focused on simple stimulation of the combination of dexamethasone and 3-isobutyl-1-methylxanthine (DM) without serum renewal (DM/URS).

Our previous studies have shown that TCDD synergizes with growth factors, including EGF and fibroblast growth factor in the suppression of PPARγ1 expression (Hanlon et al., 2003). This synergy was proportional to the activation of Erk and completely dependent on this activity. We tested these adipogenic responses for their sensitivity to TCDD and EGF. Using microarray analysis, 30 to 40 of the most robust adipogenic responses are, like PPARγ, blocked cooperatively by the combination of TCDD and EGF treatments (Hanlon et al., 2005a) (Table 1).

EGF activation of Erk through the MAP kinase pathway is typically transient because of the activation of the opposing MAP kinase phosphatase (Camps et al., 2000). We have found that TCDD enhances cell adhesion (Hanlon et al., 2005a). Cell adhesion has been linked to the prolonged activation of MAP kinase that seems necessary for TCDD suppression of PPARγ1. Cell adhesion has been strongly implicated in the regulation of adipogenesis. In addition to the cell rounding in the first 24 h of adipogenesis (Spiegelman and Farmer, 1982; Hausman et al., 1996), enhanced adhesion of 3T3-L1 cells on fibronectin diminishes differentiation (Castro-Munozledo et al., 1987; Kamiya et al., 2002). The actin-binding protein enc1 is involved in both morphology changes and differentiation during adipogenesis (Zhao et al., 2000). In fat tissue, extensive deposition of ECM is also evident (Nakajima et al., 1998).

Previous work has shown that TCDD action on adipogenesis depends on Src kinase. It is noteworthy that responses are diminished in Src−/− mice and mouse embryonic fibroblasts derived from them (Vogel and Matsumura, 2003). αβ

TABLE 1

Microarray analysis of EGF cooperation in TCDD-induced transcriptional changes during adipocyte differentiation in C3H10T1/2 cells

Affymatrix array data are compiled from Hanlon et al. (2004, 2005).

	IDM	Changes Produced with Presence of IDM		
		E	T	ET
Direct response of TCDD				
ALDH3A1	N.S.	N.S.	7.6	34.4
Glypian1	N.S.	N.S.	3.7	5.2
NQD1	N.S.	1.5	3.5	3.5
CYP1B1	1.3	N.S.	2.3	3.0
Indirect TCDD responses produced through inhibition of IDM				
Lipogenic				
ADRP	5.3	−2.0	−3.8	−6.0
DAG-*O*-Acyl transferase	5.2	−1.4	−2.3	−3.2
Isocitrate DH	3.0	−1.6	−1.3	−2.6
Secreted				
Cervloplasmin	−4.9	1.5	1.4	2.2
CCN5	−2.8	2.6	2.5	7.2
IGFBP5	20.1	1.9	−2.6	−8.4
Visvatin	5.2	−2.5	−1.8	−3.7
TIMP1	−1.6	N.S.	N.S.	1.7
Nuclear factors				
PPARγ	2.5	2.5	−1.8	−3.7
KLF4	−7.6	1.8	1.6	2.8
C/EBPα	2.6	N.S.	N.S.	−2.8
PPARγ ligands				
CEH	21.8	−4.3	−9.8	−24.9

IDMB, insulin + Dex + IBMX + BRL; I, IDMB; IE, IDMB + EGF; IT, IDMB + TCDD; IET, IDMB + EGF + TCDD; N.S., not significant.

Integrin heterodimers initiate cell adhesion signaling through interaction with extracellular matrix proteins which recruit focal adhesion kinase (FAK) and adaptor proteins such as paxillin and CAS, which provide connections initiated by vinculin to F-actin stress fibers (Turner, 2000a). Src is a crucial mediator for these integrin complexes and intracellular signaling to the nucleus. More extended focal adhesion complexes associated with cell spreading derive from extensive clustering of $\alpha\beta$ integrin complexes with FAK, paxillin, and vinculin. This clustering is produced by Rho kinase-mediated cross-linking of F-actin stress fibers with myosin 2 (Defilippi et al., 1999).

This research addresses two general hypotheses. First, that focal adhesion signaling plays a key inhibitory role in the regulation of PPARγ1 synthesis. Second, that TCDD activation of AhR induces proteins that enhance the coupling of growth factor receptors to adhesion processes. The data provides evidence for a process in which a TCDD-induced protein enhances Src participation in ECM/integrin-mediated adhesion, with Erk-activation and generation of a PPARγ1 suppressor in subsequent steps.

Materials and Methods

Materials. MEK inhibitor U0126 was from Promega (Madison, WI). Src inhibitor PP2 and Rho kinase inhibitor Y27632 were from Calbiochem (San Diego, CA). IBMX, dexamethasone, sodium orthovanadate, β-methylcyclodextrin (MCD), and EGF were from Sigma Chemical (St. Louis, MO). Dulbecco's modified Eagle's medium/Ham's F-12 medium was from Invitrogen (Auckland, NZ). Fetal bovine serum was from Atlanta Biologicals (Norcross, GA). Trypsin (0.25%) and PBS were from Mediatech (Herndon, VA). The PPARγ antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The mouse monoclonal paxillin antibody was purchased from BD Biosciences PharMingen (San Jose, CA). The anti-phospho (Tyr118) paxillin antibody, anti-phospho (Tyr416) Src antibody, total Src antibody, anti-phospho (Tyr576/577) FAK antibody, total FAK antibody, and anti-phospho (Thr202/Tyr204) Erk were purchased from Cell Signaling Technology (Danvers, MA). The Alexa Fluor 488 phallotoxin, the Rhodamine Red-X goat anti-mouse IgG, and the ProLong Antifade Kit were purchased from Invitrogen.

Cell Culture. Mouse pluripotent C3H10T1/2 cells with passage number less than 15 were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium containing 10% fetal bovine serum and penicillin-streptomycin (10 IU/ml and 10 μg/ml, respectively). Cells were cultured in six-well plates with initial plating density of 3×10^5 cells/well. For differentiation, cells were induced with 1 μM Dex and 0.5 mM IBMX under the unrenewed serum condition (URS). TCDD (10 nM) was added to cells 48 h before induction and replaced with fresh TCDD at time 0 when Dex and IBMX were added. In the experiments regarding TCDD and EGF cooperation, EGF (10 nM) was added at time 0. For the experiments testing the role of Src inhibitor PP2 (1 μM) and MEK inhibitor U0126 (10 μM), these two inhibitors were also added to cells at time 0 during differentiation.

Western Blotting. Cells were treated as desired, and then the media was removed and cells were washed twice with PBS. Total proteins were extracted using radioimmunoprecipitation assay lysis buffer (150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1% Nonidet P-40, 0.1% SDS, 50 mM Tris base, pH 7.4, 0.25% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and 1% protease inhibitor cocktail from Sigma. Total cell lysates were heated at 100°C for 5 min. Total protein (60–70 μg), determined by BCA kit from Pierce (Rockford, IL) was loaded on 10% SDS-polyacrylamide gels and separated by electrophoresis, then were transferred to a nitrocellulose membrane. After blocking, this membrane was incubated with the primary antibody of interest at 4°C overnight. The target proteins were then

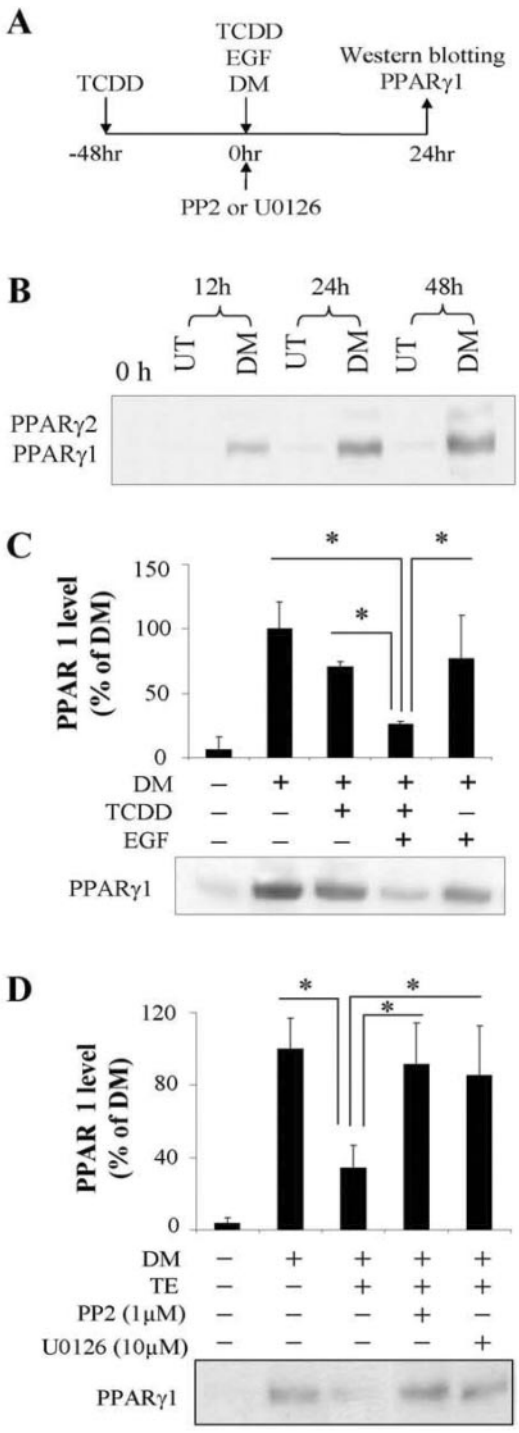

Fig. 1. TCDD and EGF cooperatively suppress the DM-induced PPARγ expression in a Src and Erk dependent manner. A, experimental scheme. B, time course of DM-induced PPARγ expression. C3H10T1/2 cells were either untreated (UT) or treated with Dex and IBMX (DM) as described under *Materials and Methods* for 12, 24, and 48 h. PPARγ1 and -γ2 expression was analyzed by Western blotting using PPARγ antibody; results shown are representative of at least two experiments. C, TCDD and EGF cooperatively suppress DM-induced PPARγ1 expression. Cells were treated with TCDD (10 nM) or EGF (10 nM) alone, and TCDD+EGF in combination (TCDD/EGF) in the presence of DM for 24 h. D, the suppression of PPARγ1 by TCDD/EGF is reversed by the inhibition of Src and Erk. Src inhibitor PP2 (1 μM) and MEK inhibitor U0126 (10 μM) were added with TCDD/EGF, and the expression of PPARγ1 was analyzed 24 h later. In C and D, the relative level of PPARγ1 versus that of DM induction was expressed as mean ± S.E.M. from triplicate samples. *, $p < 0.05$, Student's t test.

detected by horseradish peroxidase-conjugated secondary antibodies, and signals were visualized by adding ECL Western Blotting Detection Reagents from GE Healthcare (Little Chalfont, Buckinghamshire, UK). The intensities of bands films were quantified using software ImageQuant 5.2. The statistical analysis of significance was performed by Student's *t* test.

Immunostaining Methods. Cells were cultured on sterile cover slips that were placed in six-well plates under the unrenewed serum condition (Hanlon et al., 2005a). Upon reaching confluence, cells were induced with 1 μM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine for 24 h, then fixed by 4% paraformaldehyde for 15 min at room temperature. 0.15 M glycine was applied to the fixed cells on coverslips for 10 min at room temperature, followed by penetration with 0.2% Triton X-100 (in PBS) for 10 min at room temperature. After rinsing with PBS, blocking solution containing 1% goat serum and 1% fatty-acid free bovine serum albumin in PBS was added, and the coverslips were incubated for 30 min at 37°C. After blocking, the paxillin antibody (1:100 dilution) and Alexa Fluor 488 phallotoxin (5 units/ml) were added to coverslips, which were then incubated for 30 min at room temperature. After rinsing, Rhodamine Red X secondary antibody (1:400 dilution) was added and incubated for 30 min at room temperature. Finally, the coverslips were mounted with antifade solution for subsequent analysis using fluorescent microscope (AXIOSCOP 20; Zeiss, Welwyn Garden City, UK).

Cell Treatment with MCD. MCD with different concentrations (0.1, 1, or 4 mM) was added to cells 5 h before differentiation induction. At time 0, C3H10T1/2 cells were induced to differentiation as described above with replacement of fresh MCD in the medium. Cells were treated for 24 h, and the total proteins were harvested to study the effect of MCD on PPARγ1 expression and Erk phosphorylation.

Src Kinase Assay. After cells were treated with TCDD and EGF under URS/DM conditions, the kinase activity of Src was assayed using a Universal Tyrosine Kinase Assay kit from Takara Biotech, Inc. (Shiga, Japan). In brief, the Src kinase was first immunoprecipitated by the Src antibody and diluted. Then the diluted samples were added to a 96-well plate which was precoated with synthesized peptide substrate (poly Glu-Tyr). The reaction was started by adding ATP-2Na, and the phospho-tyrosine was detected by an anti-phosphotyrosine (pY20)-HRP antibody provided by the kit. Then, the substrate of HRP was added to each reaction, and the kinase activity was measured by the absorbance at 450 nM with the μQuant plate reader from Bio-Tek Instruments (Winooski, VT).

Statistical Analysis. In the experiments of triplicate samples, the result was shown by mean ± S.E.M. Statistical significance between different treatments was analyzed by Student's *t* test.

Results

TCDD and EGF Cooperatively Inhibit the Expression of PPARγ1 When Stimulated by Dex/Mix under URS Condition; MEK and Src Are Key Mediators. In previous studies, we have shown that PPARγ1 expression

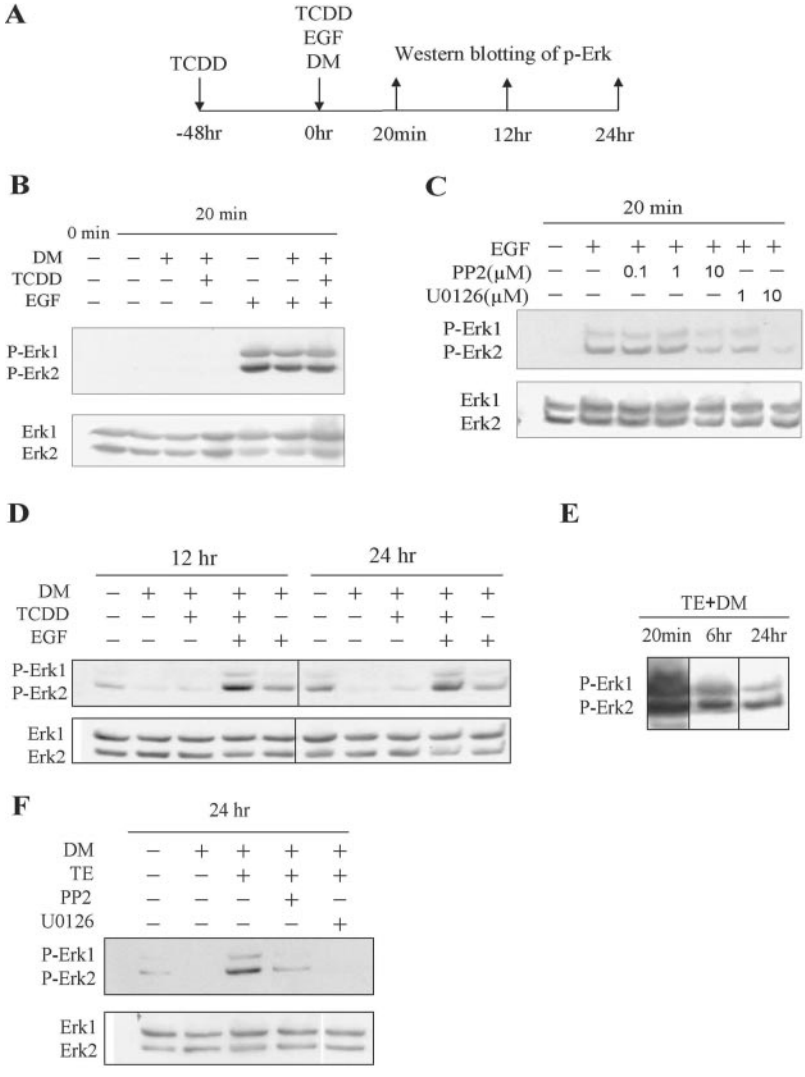

Fig. 2. Effects of Src inhibition on acute Erk1/2 activation by EGF and sustained Erk1/2 activation by TCDD/EGF. A, experimental scheme. B, the presence of TCDD (10 nM) or DM does not alter the acute Erk activation by EGF (10 nM). C, the acute EGF-induced Erk1/2 activation is sensitive to MEK inhibition but not to Src inhibition to the same degree. Cells were pretreated with either the MEK inhibitor U0126 (1 and 10 μM) or the Src inhibitor PP2 (0.1, 1, and 10 μM) for 20 min, then EGF was added for 20 min. D, TCDD/EGF causes a sustained Erk1/2 activation at 12 and 24 h after treatment in the presence of DM, which was not observed when cells were treated with either TCDD (10 nM) or EGF (10 nM) alone. E, the level of TCDD/EGF-induced sustained Erk1/2 activation is low compared with the acute Erk1/2 activation at 20 min. F, the sustained Erk1/2 activation induced by TCDD/EGF at 24 h is suppressed by PP2 (1 μM) and U0126 (10 μM). In the above experiments, cells were treated as indicated, and the total proteins were harvested at the indicated time points, and phosphorylation of Erk1/2 (Thr202/Tyr204) is analyzed by phosphospecific antibody using Western blotting described under *Materials and Methods*. The results shown above are representative of at least two experiments.

and subsequent adipogenesis are fully induced by DM without insulin and also that omission of the serum renewal (URS) with this addition does not lessen these responses (Cho and Jefcoate, 2004). These two changes completely remove any initial stimulation of the MEK/Erk pathway and mitogenesis but maintain a modest increase in DNA synthesis.

In this study, we focus on the effects of TCDD and EGF using these DM/URS conditions (Fig. 1A) to avoid additional effects of insulin and serum stimulation on the MEK/Erk pathway. The additional 30 amino acids at the N terminus of PPARγ2 lead to an appreciably lower mobility on SDS polyacrylamide gels. Our previous studies have shown that in 10T1/2 cells, PPARγ1 increases after approximately 12 h of stimulation and before PPARγ2, which typically requires several days of insulin stimulation (Hanlon et al., 2003; Cho and Jefcoate, 2004). In Fig. 1B, we show that in absence of serum renewal, DM substantially advances the stimulation of PPARγ1 over untreated cells within 24 h. This response comes 12 h earlier than with the standard IDM conditions (Cho and Jefcoate, 2004), whereas PPARγ2 protein was barely detectable at 48 h. Figure 1C shows that after DM/URS stimulation, TCDD pretreatment produces only approximately 20% suppression of PPARγ1 expression. Based on our previous findings with IDM stimulation (Hanlon et al., 2003), we tested whether EGF would enhance the inhibitory effect of TCDD on DM induction of PPARγ1. EGF alone produced approximately 20% suppression of PPARγ1 but, in combination with TCDD (TCDD/EGF), produced approximately 80% suppression, which is close to unstimulated levels (Fig. 1C).

The cooperation between TCDD and EGF in this suppression was completely reversed by the MEK inhibitor U0126 (10 μM) (Fig. 1D) at levels that completely inhibit the phosphorylation of Erk (see Fig. 2). MEK inhibitors at higher concentrations can bind to AhR (Andrieux et al., 2004). Elsewhere, we have shown that inhibition of MEK/Erk by either U0126 or PD98059 does not change the parallel TCDD induction of CYP1B1, a marker protein for AhR activation (Hanlon et al., 2003) (X. Liu and C. Jefcoate, unpublished data). EGF also does not enhance the stimulation of an AhR-selective xenobiotic response element-luciferase reporter under these conditions (Hanlon et al., 2005b) even though MAP kinases directly enhance ARNT/AhR activity in some cell types (Tan et al., 2004). In addition, we have shown previously that inhibitor effects on Erk phosphorylation closely match the reversal of PPARγ1 synthesis and of subsequent adipogenesis (Hanlon et al., 2003). Thus, the reversal of TCDD/EGF effects by U0126 comes from the interaction with MEK rather than from a displacement of TCDD from AhR.

Several reports have implicated Src kinase as a participant in TCDD activity (Vogel and Matsumura, 2003; Vogel et al., 2003; Hoelper et al., 2005). In Fig. 1D, we also show that a low level of a selective Src kinase inhibitor PP2 (1 μM) also

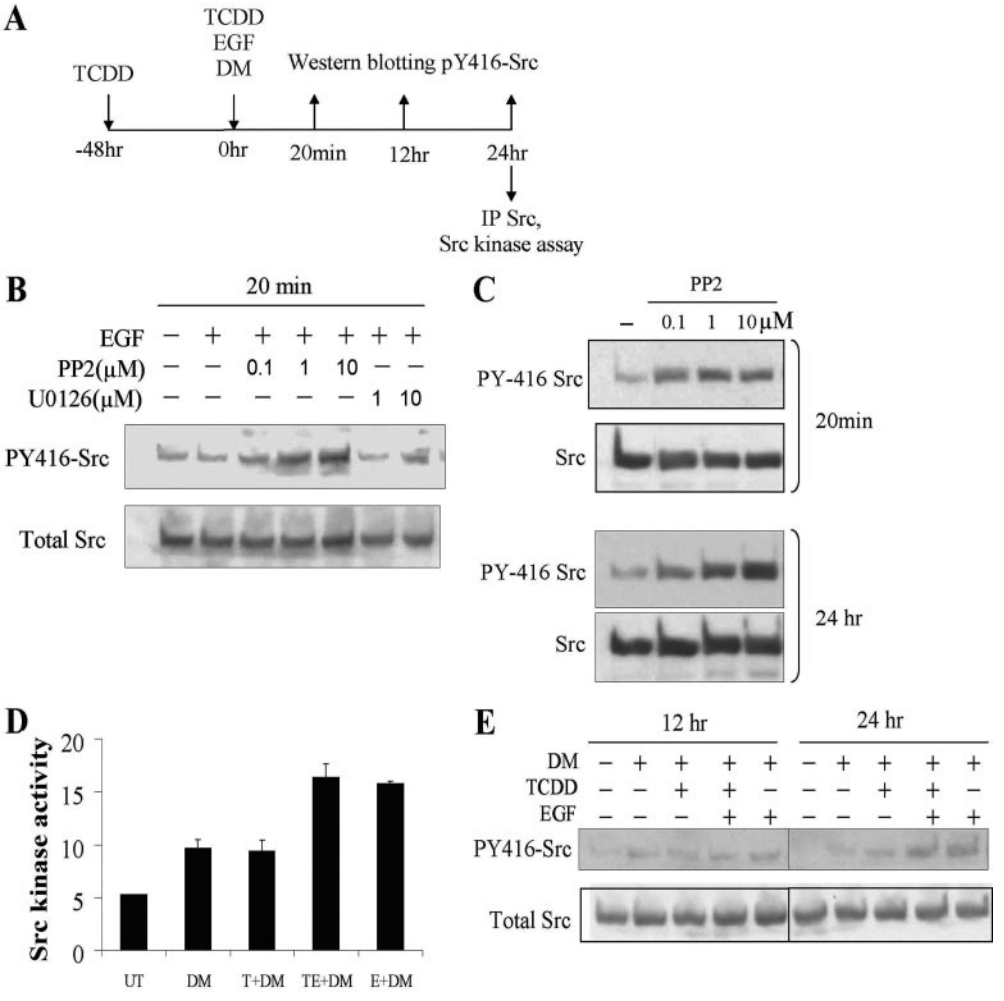

Fig. 3. Effect of TCDD treatment on Src activation. A, experimental scheme; B, Src phosphorylation on Tyr416 is enhanced by PP2 during the acute EGF treatment. Cells were pretreated with either the MEK inhibitor U0126 or the Src inhibitor PP2 for 20 min; EGF was then added for 20 min. The phosphorylation of Src Tyr416 was analyzed using phosphor-specific antibody as described under *Materials and Methods*. C, PP2 alone causes an increase of Src Tyr416 phosphorylation. Cells were treated with PP2 (0.1, 1, and 10 μM) as in B, or for 24 h, without EGF. The phosphorylation of Src Tyr416 was analyzed as described in B. D, EGF increases Src kinase activity at 24 h in the presence or absence of TCDD. After the treatments indicated in the chart, Src kinase activity was analyzed as described under *Materials and Methods;* results shown are from duplicated samples and representative of the trend from other experiments. E, EGF increases the phosphorylation of Src Tyr416 at 24 h in the presence or absence of TCDD. Cells were treated as indicated; the phosphorylation of Src Tyr416 at 12 and 24 h was analyzed by the same method in B.

completely prevented the inhibitory effect of the TCDD/EGF combination.

Acute Erk Activation by EGF Is Not Sensitive to Inhibition of Src with PP2 (1 μM), Which, However, Blunts the Sustained-Erk Activation by the Combination of TCDD and EGF. Src functions at the plasma membrane to enhance the activity of dimeric growth factor receptors and integrin receptors, particularly with respect to activation of the MEK/Erk MAP kinase pathway (Tice et al., 1999). EGF very rapidly stimulates Erk phosphorylation. DM or TCDD (Fig. 2B) did not affect this acute stimulation. The activation was, as expected, completely blocked by U0126 but was not sensitive to 1 μM PP2 (Fig. 2C). A higher concentration of PP2 (10 μM) partially inhibited Erk phosphorylation, consistent with a tightly coupled participation of Src in EGF receptor *trans*-phosphorylation (Tice et al., 1999).

We have shown previously that delayed inhibition of Erk activation even after the initial 6 h is fully effective in preventing TCDD suppression of PPARγ1 and adipogenesis (Hanlon et al., 2003). This suggests that TCDD activity depends on prolonged Erk activity rather than the high transient Erk stimulation that is reversed after 20 min by stimulation of the MAP kinase-activated phosphatase (Camps et al., 2000). We therefore focused on sustained Erk phosphorylation, which maintains a steady state between 6 and 24 h (Fig. 2E). In contrast to the early EGF-stimulated peak, TCDD substantially stimulated this *p*-Erk steady-state level up to approximately 10% of the 20-min peak (Fig. 2D). In the presence of DM, EGF could sustain *p*-Erk at only approximately the basal level, which was otherwise completely suppressed by DM. In contrast to the acute stimulation, 1 μM PP2 substantially blocked this stimulation of sustained Erk phosphorylation by TCDD/EGF (Fig. 2F). The time period for this Erk activity is consistent with the delayed period (6–12 h) required for Erk mediation of the PPARγ1 suppression (Hanlon et al., 2003; Cimafranca et al., 2004). The high sensitivity to PP2 of the sustained pErk also matches the sensitivity of the TCDD/EGF suppression of PPARγ1 (Fig. 1).

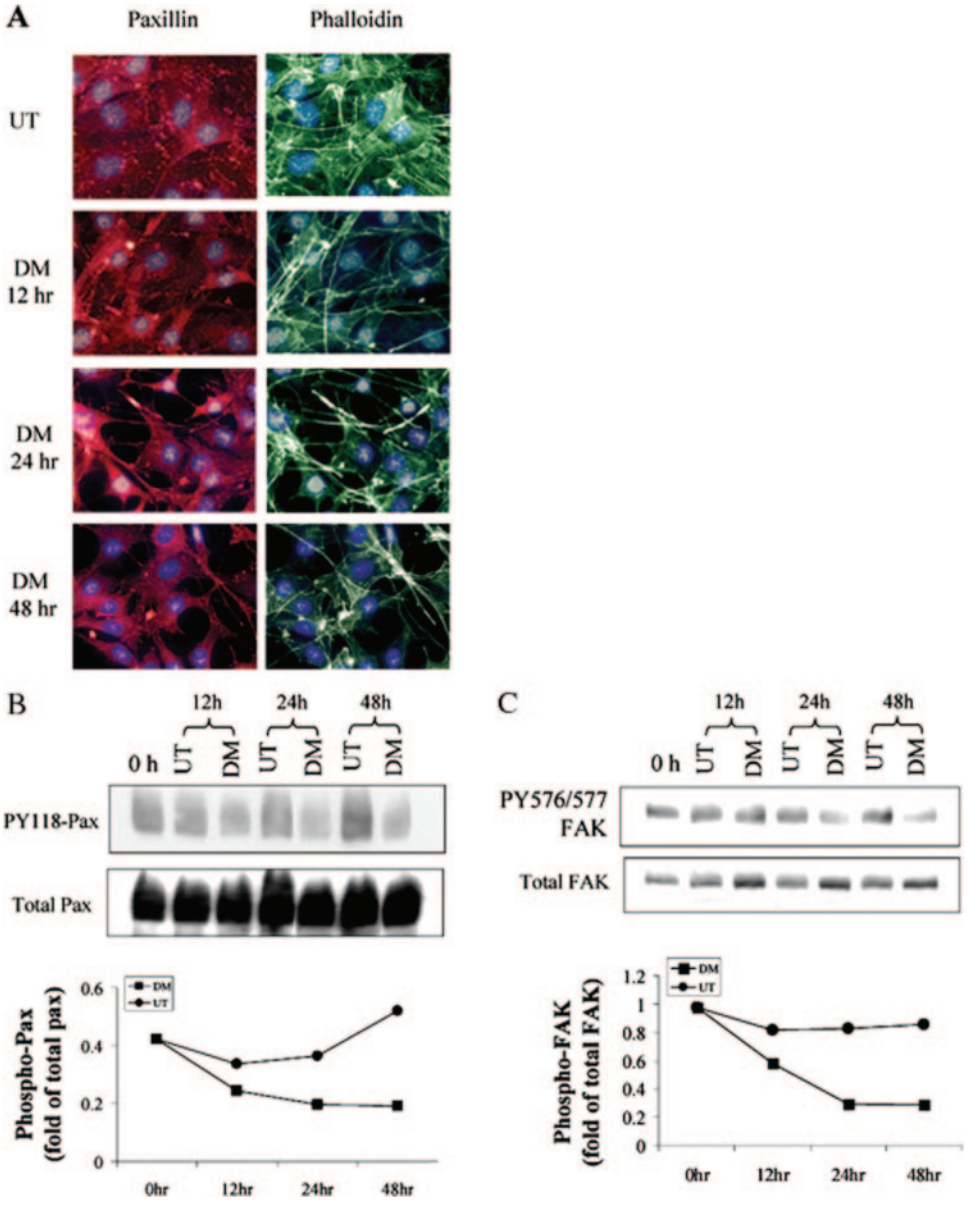

Fig. 4. DM treatment induces loss of cell adhesion. A, time course of adhesion loss in C3H10T1/2 cells, as shown by disruption of focal adhesion complex (paxillin) and actin stress fibers (phalloidin). Cells were either untreated (UT) or treated with DM for 12, 24, or 48 h, then immunostained using anti-paxillin antibody for paxillin focal adhesion, phallotoxin for actin stress fiber and DAPI for nuclei as described under *Materials and Methods;* pictures shown for each treatment reflect cells from the same field. B and C, time courses of tyrosine dephosphorylation of paxillin and FAK under DM treatment. Cells were treated as described in A, and phosphorylations of paxillin (Tyr118) and FAK (Tyr576/577) were analyzed by phosphospecific antibodies as described under *Materials and Methods*.

Src Is Acutely Regulated through Tyr416 Phosphorylation That Is Determined through a Src-Dependent Feedback Loop. Src kinase activity is greatly enhanced by phosphorylation adjacent to the catalytic site at Tyr416. EGF did not increase the phosphorylation of Src during the period of maximum Erk phosphorylation (initial 20 min) (Fig. 3B). Src phosphorylation, however, is tightly coupled to PTPase activity (Roskoski, 2005). EGF slowly increased this steady-state Tyr416 phosphorylation between 12 and 24 h, but this was not affected by TCDD (Fig. 3E). The kinase activity of immunoprecipitated Src on a synthetic peptide (poly–Glu-Tyr) paralleled the Tyr416 phosphorylation (Fig. 3D). The phosphorylation of the inhibitory Src regulatory site (Tyr527) was relatively insensitive to EGF, TCDD, and PP2 (data not shown).

It is noteworthy that PP2 alone caused a minor increase in Src Y416 phosphorylation during the same 20-min acute treatment. This increase showed a sustained dose-response when cells were treated for 24 h (Fig. 3C). This indicates a feedback loss of pTyr416 caused by an effect of Src kinase activity either on the mediating kinase (inhibition) or on PTPases acting on this site (activation). The similarity of PP2 effects with and without EGF (Fig. 3, compare B and C) shows that this feedback loop is largely independent of EGF addition. This stimulatory effect of PP2 establishes that Tyr416 phosphorylation does not arise from autophosphorylation by the adjacent catalytic site.

DM Induces Loss of Focal Adhesion Complexes and F Actin Stress Fibers. To determine how Src is participating in TCDD suppression, we examined the established participation of Src in cell adhesion mediated by the interaction of $\alpha\beta$ integrins with extracellular matrix proteins (Parsons et al., 2000). Cell rounding caused by a loss of surface and intercellular adhesion is seen in mouse embryo fibroblasts at an early stage in differentiation (Spiegelman and Farmer, 1982; Hausman et al., 1996). We first characterized the time course of the DM-induced morphological changes in relation to the increases in PPARγ1 described in Fig. 1. Immunostaining of paxillin foci identifies the number and location of $\alpha\beta$ integrin associated focal complexes. Fluorescein isothiocyanate-labeled phalloidin binding to F-actin visualizes the length and distribution of stress fibers that cross-link integrin-associated focal complexes. Figure 4A shows that DM decreased the number of paxillin foci that are visualized as white or red dots. These foci decreased appreciably after 12 h, although few were detectable after 24 and 48 h. In the untreated cells, F-actin stress fibers distribute from nucleus to plasma membrane and form extended structures under the plasma membrane that produce larger clustered focal adhesion complexes. Long projections extending between cells are also visible. These intracellular stress fibers decline appreciably by 12 h, although in some cells more than others (Fig. 4A). Because paxillin foci and F-actin stress fibers are visu-

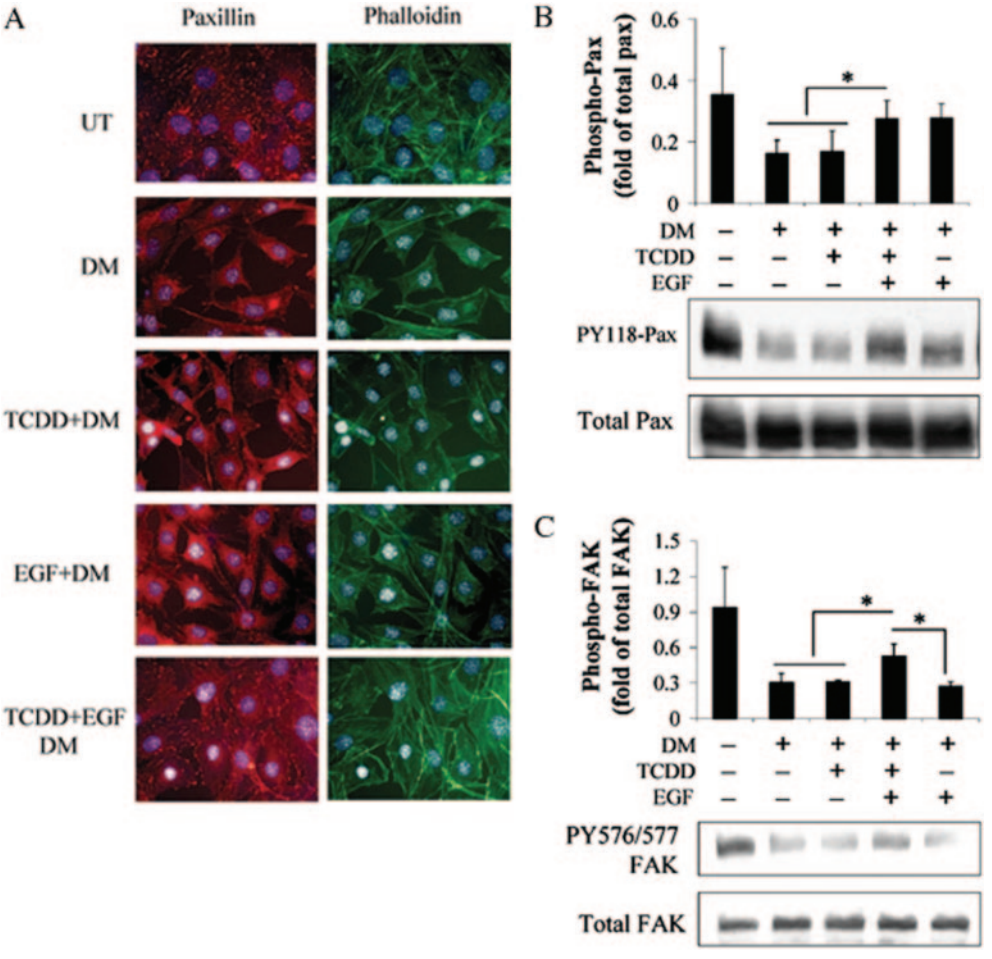

Fig. 5. TCDD and EGF cooperate to restore the DM-induced loss of adhesion. A, Effects of TCDD and EGF on focal adhesion complexes (paxillin) and actin stress fibers (phalloidin). Cells were either untreated (UT) or treated with TCDD (10 nM), EGF (10 nM), or TCDD+EGF in the presence of DM for 24 h, and then were immunostained as described under *Materials and Methods*. Pictures shown for each treatment reflect the same field of cells. B and C, effects of TCDD and EGF on the phosphorylation of paxillin (Tyr118) and FAK (Tyr576/577). Cells were treated as described in A; total proteins were harvested at 24 h, and the phosphorylations of paxillin (Tyr118) and FAK (Tyr576/577) were analyzed using phospho-specific antibodies as described under *Materials and Methods*. The levels of phosphorylation of paxillin and FAK versus control were expressed as mean ± S.E.M from triplicated samples. *, $p < 0.05$, Student's t test.

alized in the same cells, it is apparent that retention of F-actin fibers is associated with residual paxillin foci.

We also tested the phosphorylations of, respectively FAK and paxillin, which are each typically elevated in adhesion complexes (Turner, 2000a). In parallel with the morphology changes, paxillin phosphorylation at Tyr118 declined at 12 h, with maximum decreases after 24 h. The decrease in FAK phosphorylation at Tyr576/577 was slower and probably a consequence rather than a cause of the adhesion changes (Fig. 4, B and C). A gradual basal increase in these phosphorylations is observed and probably arises from the extensive basal expression of secreted extracellular matrix proteins, such as fibronectin and collagens that we and others have reported under these conditions (Spiegelman and Farmer, 1982; Hanlon et al., 2005a).

TCDD and EGF Cooperatively Prevent the DM-Induced Loss of Focal Adhesion Complexes and F Actin Stress Fibers. Figure 5A shows that TCDD and EGF alone were largely ineffective in preventing these losses of paxillin foci and actin stress fibers after 24 h of DM treatment. By contrast, the TCDD/EGF combination restored most of the morphology characteristics seen in the untreated cells (Fig. 5A). Additional changes have also occurred because the F-actin fibers project less between TCDD/EGF-treated cells than in the untreated cells. Major differences between untreated cells and TCDD/EGF-treated cells are indicated by our previous expression profiling of the cells under these two conditions (Hanlon et al., 2005a). The adipogenic stimulus produced substantial losses in the expression of mRNA corresponding to extracellular matrix and structural proteins, including F actin, that were either unaffected or enhanced by TCDD/EGF treatment (Hanlon et al., 2005a).

The DM-induced loss of phosphorylation of paxillin (Tyr118) was appreciably restored by EGF at 24 h (Fig. 5B). However, because EGF did not restore the focal complexes, this increased paxillin phosphorylation is evidently insufficient for the increase in adhesion. The combination of TCDD and EGF, which restored the adhesion complexes and stress fibers, did not further increase paxillin phosphorylation. This paxillin phosphorylation is therefore insufficient to restore adhesion without TCDD induction of some additional participant. EGF did not increase FAK Tyr576/577 phosphorylation (Fig. 5C), which is therefore unlikely to mediate the EGF stimulation of paxillin phosphorylation. The combination of TCDD and EGF significantly increased the FAK phosphorylation compared with the single treatments ($p < 0.05$, $n = 3$) (Fig. 5C). Although this parallels the restoration of adhesion, as noted in Fig. 4, the slow DM-induced loss of this FAK phosphorylation appeared after the adhesion change.

Src Kinase, but Not Erk, Is Required for the TCDD and EGF Cooperation in Restoring the DM-Induced Loss of Cell Adhesion changes. Src kinases participate in the signaling between $\alpha\beta$ integrin complexes by mediating tyrosine phosphorylation of intracellular partners including FAK and growth factor receptors (Schlaepfer et al., 1994). When appropriately activated by ECM binding, intracellular regions of β1 integrins interact with FAK, leading to autophosphorylation of Tyr397. This site then interacts with the SH2 domain of Src to initiate further phosphorylations, including Y-576/577 and paxillin Tyr118. We have used paxillin pTyr118 and FAK pTyr576/577 as indicators of Src kinase activity in the focal adhesion complexes.

Figure 6A shows that restoration of paxillin foci and F-actin stress fibers by the TCDD/EGF combination was blocked by 1 μM PP2 and is therefore dependent on Src kinase activity. The TCDD/EGF restoration of paxillin and FAK phosphorylations was also completely blocked by PP2 (1 μM) (Fig. 6B). This potent inhibition by PP2 corresponded closely to the inhibition of sustained Erk phosphorylation and the reversal of PPARγ1 suppression (Figs. 1D and 2F). The synergistic effects of TCDD and EGF that restore adhesion, sustain the Erk activation, and suppress PPARγ1 expression are therefore likely to be mechanistically linked.

These effects of the TCDD/EGF combination on cell morphology were insensitive to U0126 (Fig. 6A), thus providing further evidence that MEK/Erk does not participate in the initial TCDD induction process that controls adhesion. However, FAK phosphorylation was blocked by U0126 (Fig. 6B). As noted above, this effect of TCDD/EGF on FAK Tyr576/577 phosphorylation seems to be a later step that results from the

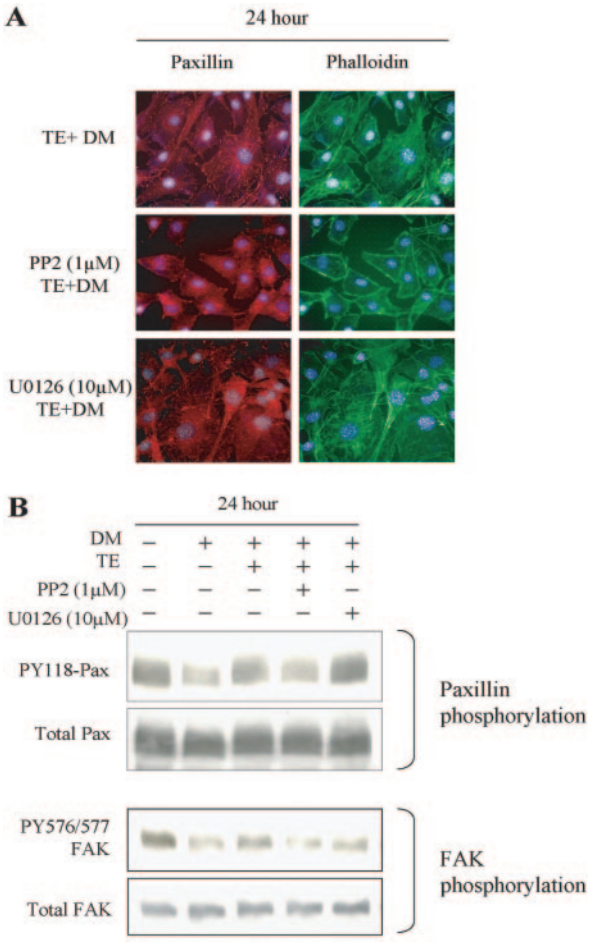

Fig. 6. Effects of Src inhibition (PP2) and MEK inhibition (U0126) on TCDD/EGF-induced restoration of cell adhesion. A, PP2, but not U0126, blocks the TCDD/EGF-induced restoration of adhesion complexes (paxillin) and stress fibers (phalloidin). Cells were treated as indicated in the figure and immuno-stained for paxillin and phalloidin as described under *Materials and Methods;* pictures shown for each treatment reflect cells from the same field. B, restoration of paxillin Tyr118 phosphorylation is sensitive to PP2, not U0126, whereas both inhibitors suppress FAK Tyr576/577 phosphorylation. After the indicated treatments for 24 h, total proteins were harvested, and the Tyr118 phosphorylation of paxillin and the Tyr576/577 phosphorylation of FAK were analyzed using the phosphospecific antibody as described under *Materials and Methods*. Results are representative of at least two experiments.

increased adhesion. The effect of U0126 demonstrates that FAK phosphorylation needs the MEK/Erk activity, which increases after the adhesion restoration. Recruitment of Erk to focal adhesion complexes has been reported (Fincham et al., 2000).

TCDD and EGF Cooperation Is Not Blocked by Inhibition of Rho Kinase. ROCK stimulates the conversion of integrin/FAK/F-actin focal complexes to highly extended adhesion structures that are responsible for cell-spreading (Amano et al., 1997). ROCK increases the phosphorylation of myosin light chains, which then form bridges between F actins. The resulting extended stress fibers, which are seen with the phalloidin staining in Figs. 4 to 6, greatly enhance the clustering of integrins (Amano et al., 1996; Matsui et al., 1996). Inhibition of ROCK provides the means to determine whether the more extended structures and restoration of the spread cell morphology are necessary for the sustained stimulation of Erk by TCDD/EGF and the suppression of PPARγ1.

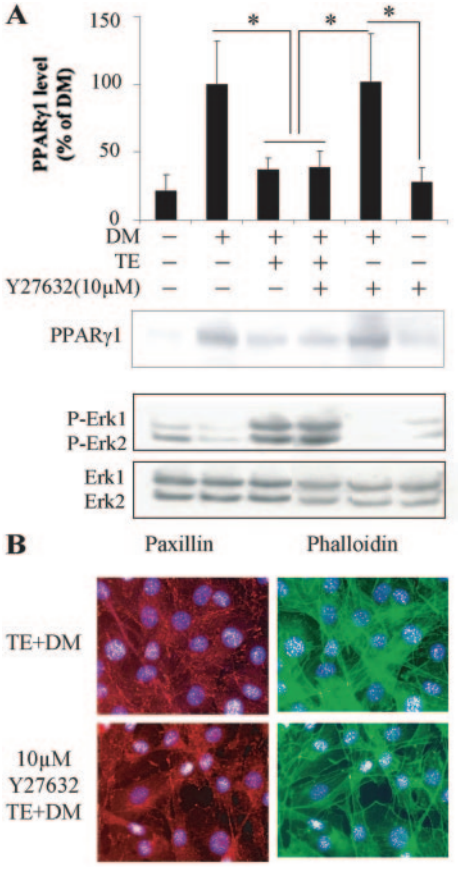

Fig. 7. Inhibition of Rho kinase blocks the restoration of cell adhesion by TCDD/EGF but does not affect either the suppression of PPARγ1 expression or the sustained Erk1/2 activation. A, the suppression of DM-induced PPARγ1 expression and the sustained Erk1/2 activation by TCDD/EGF are not affected by Rho kinase inhibitor Y27632 (10 μM). Cells were treated as indicated, total proteins were harvested at 24 h, and PPARγ1 expression was analyzed by Western blotting. The level of PPARγ1 versus DM induction is expressed as mean ± S.E.M from triplicate samples. *, $p < 0.05$, Student's t test. The phosphorylation of Erk1/2 (Thr202/Tyr204) was analyzed by phosphospecific antibody using Western blotting as described under *Materials and Methods*. B, inhibition of Rho kinase by Y27632 blocks TCDD/EGF effects on cell adhesion during DM treatment. Cells were treated with TCDD/EGF+DM in the presence or absence of Y27632 (10 μM) for 24 h. Paxillin focal adhesion complexes and phalloidin stress fiber were fluorescently stained as described under *Materials and Methods*. Pictures shown are from the same field of cells.

Figure 7A shows that the selective ROCK inhibitor Y27632 (10 μM) blocked neither the TCDD/EGF suppression of PPARγ1 nor the sustained Erk activation but nevertheless inhibited the restoration of cell adhesion by TCDD/EGF. This is visualized by the effect of TCDD/EGF on the distribution of paxillin and F actin stress fibers (Fig. 7B). This result indicates that TCDD/EGF maintenance of extended focal contacts and stress fibers are not needed for either the PPARγ1 suppression or the sustained Erk activation produced by TCDD/EGF. Therefore, ROCK and the extended clustering of focal integrin complexes are not involved in either of these processes. This parallel further supports the causal relationship between sustained Erk activation and the suppression of PPARγ1. By contrast, Y27632 (10 μM) prevented the increases in the phosphorylations of FAK (Tyr576/577) and paxillin (Tyr118) produced by TCDD/EGF (X. Liu and C. Jefcoate, unpublished data). These effects of Y27632 confirm that these steady-state phosphorylation levels are set by the extended adhesion structures.

Orthovanadate (OVA), a General PTPase Inhibitor, Restores EGF Stimulation of FAK (Tyr576/577) Phosphorylation and Enhances Paxillin (Tyr118) Phosphorylation. The steady-state levels of paxillin (Tyr118) and FAK (Tyr576/577) phosphorylation depend on the net activities of Src and opposing PTPases. Several PTPases have been implicated in the dephosphorylation of FAK and paxillin, including SHP2, PEST-PTPase, and PTP1B (Oh et al., 1999; Jin et al., 2000; Turner, 2000b). These steady-state measurements, however, may not provide a good indicator of onward kinase signaling because the PTPases also compete with the recruitment of SH2 domains. This includes proteins such as Grb2, which activates the MEK/Erk pathway (Fig. 8A, scheme). PTPase activity may actually target excess pTyr that exceed the availability of SH2 proteins. We therefore varied the concentrations of a general PTPase inhibitor OVA to test the impact of PTPase activity on Src-dependent phosphorylations of FAK and paxillin. We also examined effects on both adhesion characteristics and PPARγ1 expression. Figure 8B shows that 24 h after DM, several steady-state phosphorylations were modestly increased by 1 μM OVA and substantially increased by 5 μM OVA.

Paxillin and FAK phosphorylations were increased much more by 1 μM OVA in combination with EGF (Fig. 8C). This synergy between OVA and EGF correlated with restoration of adhesion, which was also blocked by 1 μM PP2 (X. Liu and C. Jefcoate, manuscript in preparation). Higher levels of OVA produce more than 5 times higher levels of phosphorylation as shown in Fig. 8B. These experiments show that Src phosphorylation of paxillin and FAK is much slower than dephosphorylation, which prevents an increase in steady state.

The modest inhibition of PTPases by OVA (1 μM) in combination with EGF suppressed PPARγ1 in much the same way as the combination of TCDD with EGF (Fig. 8D). This suppression of PPARγ1 depended on both Src and Erk, as evidenced by reversal effects of, respectively, PP2 (1 μM) and U0126 (10 μM) (Fig. 8E). OVA (1 μM) had little effect alone and only slightly increased the effectiveness of TCDD. It is noteworthy that EGF and 1 μM OVA not only restore adhesion but also sustain Erk phosphorylation after DM treatment (X. Liu and C. Jefcoate, manuscript in preparation). Thus, this partial inhibition of PTPase activity by OVA syn-

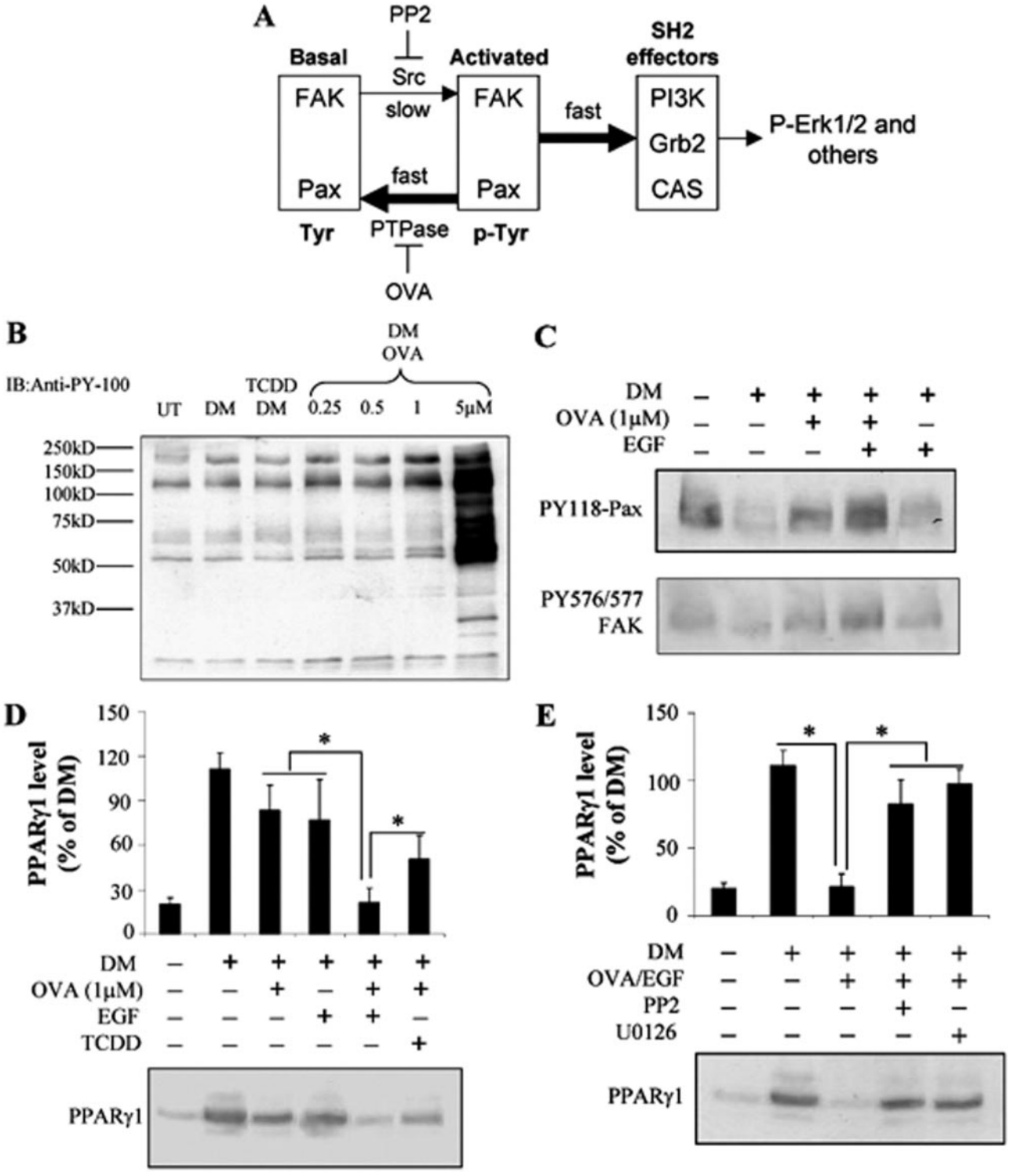

Fig. 8. Inhibition of PTPase by low levels of OVA cooperates with EGF to restore FAK and paxillin phosphorylation and to suppress PPARγ1 expression after DM treatment. A, proposed model for the role of PTPase played in the activation of FAK and paxillin by Src kinases. The Src generation of the activated pTyr forms of FAK and paxillin is limited by more active PTPases. This results in a low steady-state phosphorylation; the activity of SH2 effectors such as Grb2 depends on competition with the PTPases for pTyr sites. B, low levels of OVA produce modest effects on the overall phosphorylation status of cells in the presence of DM. Cells were treated with increasing concentrations of OVA (0.25, 0.5, 1, 5 μM) in the presence of DM for 24 h. The overall phosphorylations were analyzed by anti-pTyr100 antibody as described under *Materials and Methods*. C, low level of OVA (1 μM) cooperates with EGF (10 nM) to restore the phosphorylation of paxillin and FAK in the presence of DM. Cells were treated as indicated for 24 h; the phosphorylation of paxillin (Tyr118), FAK (Tyr576/577) was analyzed by Western blotting as described under *Materials and Methods*. D, low levels of OVA (1 μM) cooperates with EGF (10 nM) to suppress the DM-induced PPARγ1 expression, which is not seen in the cells treated with OVA (1 μM) and TCDD. Cells were treated as indicated for 24 h. PPARγ1 expression was analyzed by Western blotting as described under *Materials and Methods*. E, the suppression of PPARγ1 by OVA/EGF is sensitive to the inhibition of Src and Erk. Src inhibitor PP2 (1 μM) or Erk inhibitor U0126 (10 μM) was added with OVA/EGF for 24 h, and the suppressed PPARγ1 expression was restored by both inhibitors. In D and E, the level of PPARγ1 versus that of DM induction was expressed as mean ± S.E.M. from triplicate samples. *, $p < 0.05$, Student's t test.

ergizes with EGF in essentially the same way as processes induced by TCDD.

TCDD/EGF Suppression of PPARγ1 Is Sensitive to β-Methylcyclodextrin Treatment. TCDD with DM did not affect the levels of protein tyrosine phosphorylation (Fig. 8B). Because inhibition of PTPases enhances the effectiveness of Src, we looked for ways in which TCDD induction could produce an equivalent effect but not through inhibiting PTPase activity (see p-Tyr levels in Fig. 8B). Integrins initially form adhesion complexes in noncaveolin lipid rafts (Leitinger and Hogg, 2002; Upla et al., 2004), which also provide a major location for Src (Hur et al., 2004). Src complexes with phosphorylated caveolin, a protein that also complexes with cholesterol in caveolae rafts (Williams and Lisanti, 2004). We hypothesized that the activity of Src kinases in lipid rafts may be selectively affected by a TCDD-induced protein. We therefore tested the effect of progressively depleting cholesterol from the plasma membranes with the sequestering agent MCD (0.1–4 mM) (Huo et al., 2003). MCD produces only a modest effect on DM stimulation of PPARγ1 synthesis, even at a high concentration (4 mM), which depletes most plasma membrane cholesterol (Fig. 9, B and C). However, MCD, at 0.1 to 1 mM, significantly reverses TCDD/EGF suppression of PPARγ1 (Fig. 9B). By contrast, 4 mM MCD enhanced this suppression. The stimulatory effect of TCDD on Erk phosphorylation was progressively attenuated as by MCD (Fig. 9C). This linkage between MCD and TCDD effects provides a first indication that the suppression of PPARγ1 and the sustained stimulation of Erk kinase are each linked in some way to plasma membrane cholesterol rafts.

Discussion

During adipogenesis in C3H10T1/2 cells, PPARγ1 increases before the appearances of C/EBPα and PPARγ2 (Cho et al., 2005). These nuclear factors collectively mediate insulin-dependent lipogenesis (Rosen and Spiegelman, 2000). We have shown previously that TCDD suppression of PPARγ1 and of these later processes each depends on synergy with EGF activation of the MEK/Erk pathway (Hanlon et al., 2003, 2005a). A cluster of approximately 40 adipogenic gene responses each exhibit cooperative inhibition by TCDD and EGF, many of which have clear links to various aspects of cell metabolism (Table 1). This synergy functions in a relatively narrow time window immediately before the increased transcription of PPARγ1. Some of these transitional gene changes, typified by those shown in Table 1, may contribute to the suppression PPARγ1 (Fig. 10A). Here we show that during this time window, TCDD and EGF cooperatively enhance adhesion-associated signaling, which is linked to a sustained large increase in Erk phosphorylation. We have previously found that the level of Erk phosphorylation in early adipogenesis, when TCDD is present, is proportional to the later suppression of PPARγ1 and lipogenesis (Hanlon et al., 2003). This signaling is likely to also target the expression changes produced by cooperation between TCDD and EGF shown in Table 1, some of which may target the PPARγ1 promoter.

We have shown that 10T1/2 cells, like human preadipocytes, undergo adipogenesis without the clonal expansion that is usually necessary for 3T3-L1 cells (Tang et al., 2003; Cho and Jefcoate, 2004). Erk activity is required for clonal

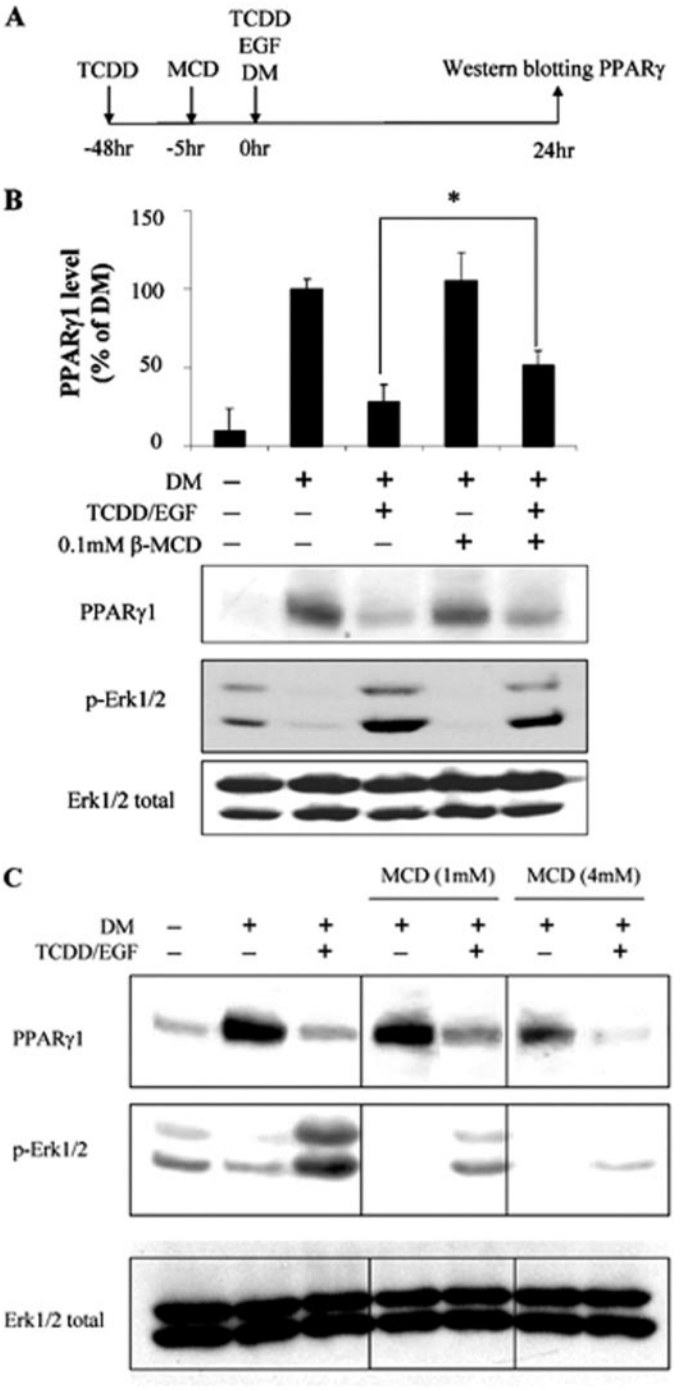

Fig. 9. Mild MCD treatment partially reverses the TCDD/EGF-induced suppression of PPARγ1 and the sustained Erk activation. A, experimental scheme. B, low level of MCD (0.1 mM) partially restores the TCDD/EGF-induced suppression of PPARγ1 with partial blocking of the sustained Erk1/2 activation. Cells were treated as indicated in the experimental scheme, in the presence or absence of TCDD/EGF (TE). Total proteins were harvested at 24 h; PPARγ1 expression and phosphorylation of Erk1/2 (Thr202/Tyr204) were analyzed by Western blotting as described under *Materials and Methods*. The relative levels of PPARγ1 were quantified by densitometry and expressed as mean ± S.E.M from triplicate experiments. *, $p < 0.05$, Student's t test. C, higher doses of MCD further block the TCDD/EGF-sustained Erk activation but gradually diminish the restoration of PPARγ1 expression. Cells were treated as described in B, with 1 and 4 mM MCD. After 24 h, total proteins were harvested to analyze PPARγ1 expression and phosphorylation of Erk1/2 (Thr202/Tyr204).

expansion with the consequence that MEK inhibitors prevent adipogenesis in 3T3-L1 cells. However, clonal expansion is not necessary for PPARγ stimulation and adipogenesis in 10T1/2 cells (Hanlon et al., 2003). We demonstrate this further here by showing that nonmitogenic adipogenic stimulation of 10T1/2 cells (DM/URS) fully retains the cell contraction and loss of adhesion before a particularly vigorous PPARγ1 increase (Figs. 1 and 4).

Cell adhesion is restored synergistically by the combination of TCDD and EGF, which also maximally suppresses PPARγ1 (Figs. 1 and 5). This restoration of adhesion by TCDD requires Src kinase activity, again exactly paralleling a requirement for Src activity in the suppression of PPARγ1 (Figs. 1 and 6). Phosphorylation of Erk is sustained at 10% of the peak transient level of acute stimulation for more than 12 to 24 h, but only with TCDD treatment. This increase in phosphorylation of Erk is maintained throughout the time window associated with suppression of PPARγ1 transcription. This window largely precedes the increase in PPARγ1, which is not directly sensitive to MEK inhibition. We previously hypothesized that this period corresponds to TCDD/EGF cooperation in the synthesis of a suppressor of PPARγ1 transcription (Hanlon et al., 2003). We can now add that an important contributor to this suppression process is the sustained elevation of Erk phosphorylation. This shared dependence on Src activity of enhanced cell adhesion, sustained Erk activation, and PPARγ1 suppression suggests that the three processes are mechanistically linked.

In contrast to PPARγ1 suppression, the MEK/Erk activity has no role in the TCDD restoration of adhesion (Fig. 6). This suggests that the TCDD-induced step initiates the adhesion response before the requirement for Erk. This separation of TCDD and Erk steps also provides the strongest evidence that Erk does not participate in PPARγ1 suppression by directly modifying AhR or ARNT. However, this activation of the heterodimer occurs in other processes (Tan et al., 2004), and we find that EGF stimulation of TCDD responses during adipogenesis depends on the particular target gene. Most responses, including an xenobiotic response element reporter, are little affected by further addition of EGF, but some genes exhibit appreciable synergy (Table 1) (Hanlon et al., 2005b). The preponderance of our data points to separate contributions by TCDD induction and EGF/Src signaling to adhesion, which initiates the signaling that sustains Erk activation (Fig. 10A, scheme).

In this model, we propose that TCDD induces a protein that enhances EGF activation of Src activity within adhesion sites. Enhanced effects of EGF on the Src-mediated restoration of cell adhesion and the suppression of PPARγ1 are similarly seen when TCDD is replaced by low levels of the PTPase inhibitor OVA (1 μM) (Fig. 8). This treatment inhibits its PTPase activities nonspecifically by approximately 50% (data not shown) and increases the phosphorylation of Src substrates such as FAK and paxillin in adhesion sites. OVA directly enhances Src effectiveness by diminishing the reverse dephosphorylation, notably at adhesion proteins like paxillin (Fig. 8).

Despite these close parallels between the TCDD/EGF syn-

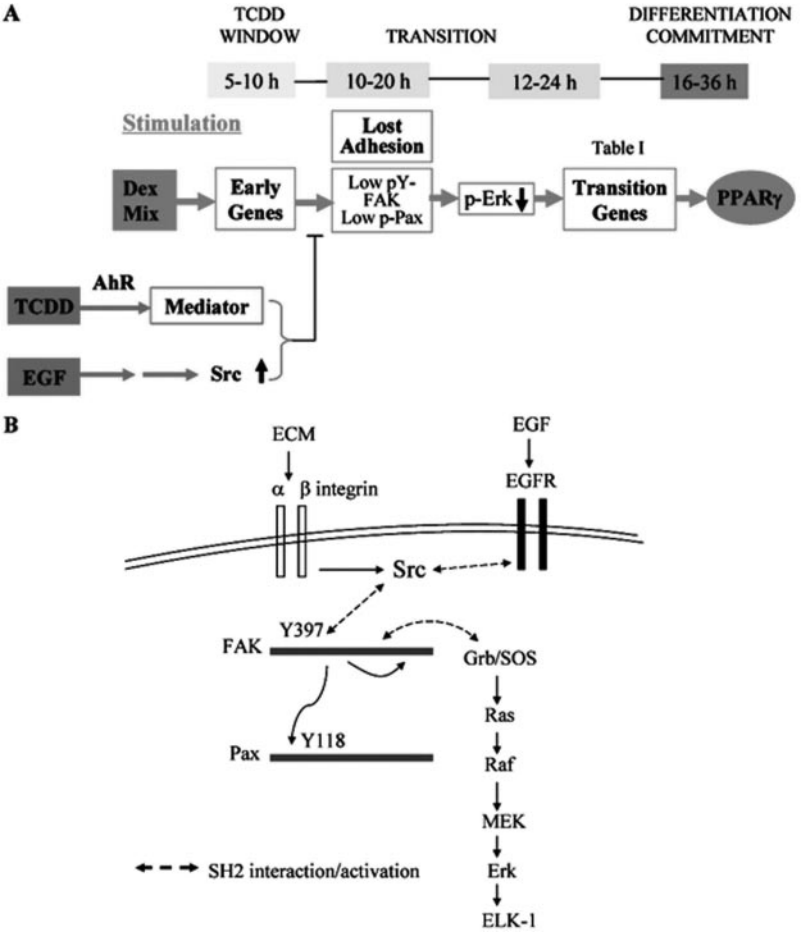

Fig. 10. Model for cooperation between TCDD and EGF in adhesion, sustained Erk activation, and suppression of PPARγ1. A, after DM stimulation, a "transition" period occurring after 10 to 24 h precedes the stimulation of PPARγ expression. In this period, DM induces a loss of cell adhesion and expression of transition genes (Table 1). TCDD and EGF cooperatively suppress the events occurred in this transition period, leading to suppression of DM-induced PPARγ1 expression. TCDD is likely to induce a mediator, which enhances the effectiveness of Src kinase stimulation by EGF. This cooperation between the unknown mediator and Src kinase leads to the restoration of focal adhesion and the sustained Erk1/2 activation. This sustained Erk activation initiates synthesis of a suppressor of PPARγ expression, which may be a member of the group of transition genes. Erk activation does not contribute directly during PPARγ1 synthesis. B, possible cooperation between integrin signaling and EGF/EGFR signaling to sustain Erk activation. Interaction of ECM components leads to the aggregation of α and β integrin, which activates FAK, leading to Src-mediated tyrosine phosphorylation of FAK. A further Src phosphorylation step leads to sustained Erk activation through SH2 recruitment of Grb/SOS to FAK pY 576/925. EGF/EGFR enhances FAK phosphorylation through Src/CAS cross-talk.

ergy in cell morphology and the effects on PPARγ1 suppression and Erk activation, our data show that they are not causally related. The cross-linking of F actin fibers with myosin light chains that is controlled by ROCK directs extensive clustering of integrin adhesion complexes, which are attached to F actin via paxillin (Amano et al., 1997). Inhibition of Rho kinase (ROCK) by Y27632 prevents TCDD effects of cell morphology but retains the activation of Erk and suppression of PPARγ1 expression (Fig. 7).

The high sensitivity of TCDD effects on PPARγ1 and Erk to cholesterol perturbation by β-MCD suggests an important role for cholesterol rafts. The ECM/integrin adhesion processes are initiated within noncaveolin cholesterol rafts and then shift to cytoplasmic sites as the extensive clustering occurs (Leitinger and Hogg, 2002; Upla et al., 2004; Williams and Lisanti, 2004). Caveolin−/− mice exhibit an intracellular accumulation of the markers of noncaveolin rafts along with a loss of adipocytes in the fat pads (Razani et al., 2002). TCDD may enhance the distribution of Src to these rafts. It is notable that glypican 1, which is highly induced by TCDD in these cells (Table 1) and locates to these lipid rafts, also activates Src (Bianco et al., 2003; Chu et al., 2004). The concentration of β-MCD that reverses TCDD effects on PPARγ1 and Erk (0.1 mM) does not significantly deplete total cholesterol from plasma membrane but is likely to affect cholesterol distribution. Higher concentrations of MCD (4 mM) deplete cholesterol inhibited PPARγ1 expression, as previously reported in 3T3-L1 cells (Huo et al., 2003).

Adhesion in preadipocytes primarily involves fibronectin interaction with α5β1 integrins (Fukai et al., 1993; Kubo et al., 2000). The importance of adhesion has been emphasized by the finding that α5 integrin is expressed early and then expression switches to the α6 integrin in mature adipocytes, where laminin provides the preferred ECM (Liu et al., 2005a). An initial FAK phosphorylation (pTyr397) then recruits Src, which then further phosphorylates FAK and adaptor proteins such as paxillin, which binds F actin stress fibers (Schlaepfer et al., 1994; Giancotti and Ruoslahti, 1999; Turner, 2000a) (Fig. 10B, scheme). This activation of integrins leads to Src phosphorylation of FAK (Tyr576, Tyr925) and paxillin (Tyr118), consistent with our findings. FAK activated by Src also mediates cross-talk with the EGF receptor via CAS phosphorylation (Moro et al., 2002). This cross-talk is clearly a candidate for Src mediation of Erk activation.

Src, FAK, and paxillin play key roles in adhesion, but the correlation between their phosphorylations and cell adhesion is poor in these TCDD-mediated responses. Although FAK and paxillin phosphorylations decline substantially after adipogenic stimulation (Fig. 4), paxillin phosphorylation is restored by EGF alone without full adhesion (Figs. 5), and FAK phosphorylation is sensitive to MEK inhibition by U0126, which does not affect adhesion restoration (Figs. 5, 6). Likewise, the increase in Src Tyr416 phosphorylation and kinase activity appear only after adhesion increases. However, the effects of OVA inhibition on steady-state phosphorylation establish the far greater activity of the several PTPases, which function in adhesion sites (SHP2, PTPα, PTP-1B, etc.) (Oh et al., 1999; Jin et al., 2000; Turner, 2000b). Nevertheless, increased Src activity can increase downstream processes without an increased steady-state activation, if the recruitment of SH2-dependent protein mediators such as Grb2 to FAK pY sites is faster than the competing PTPase inactivation (Fig. 8). The effectiveness of these PTPases is enhanced by a negative control of Src activity during adipogenesis (Fig. 3). Inhibition of kinase activity with PP2 alone substantially increases phosphorylation of the activation site (Tyr416) adjacent to the catalytic site, largely independent of the addition of EGF. This establishes a feedback loop in which pTyr416 and Src activity are attenuated by certain PTPases that are regulated by Src kinase itself.

Although we have seen changes in Src, PP2 also inhibits other Src family kinases that participate in adipogenesis. For example, interactions between the Src-family kinase fyn located in lipid rafts with flotillin and F-actin play an important role in insulin action in mature adipocytes (Liu et al., 2005b). At this stage, we cannot exclude contributions from other Src-family members to PP2-sensitive processes that mediate the TCDD/EGF synergy.

This work establishes a persistent link between TCDD suppression of PPARγ1 and the sustained Src-mediated activation of Erk kinase. This is seen under a wide range of conditions, including treatment with the selective inhibitors PP2 (for Src), U0126 (for MEK/Erk), and Y27632 (for ROCK) and cholesterol re-distribution (MCD). However, this work also establishes that the observed TCDD-induced and Src-mediated actin polymerization and extended paxillin clustering is not required for PPARγ1 suppression. These morphology changes therefore occur subsequently or in parallel to the suppression pathway. Our working hypothesis is that PPARγ1 suppression is mediated by a TCDD-induced early mediator protein that enhances the effectiveness of EGF to Src within the initial integrin/ECM complexes that form in cholesterol rafts. How Erk produces suppression remains a key question. We have excluded a direct phosphorylation of a PPARγ1 transcriptional activator because MEK/Erk inhibition is ineffective during PPARγ1 elevation (Hanlon et al., 2003). C/EBPβ activation that participates in PPARγ transcription was not affected by TCDD (Phillips et al., 1995; Chen et al., 1997). Thus, increased expression of a suppressor, including proteins listed in Table 1, is likely to mediate the suppression of PPARγ1 by TCDD. Future experiments will address the links between the TCDD and EGF stimulated changes described here including raft formation and the molecular control of PPARγ1 transcription.

References

Alexander DL, Ganem LG, Fernandez-Salguero P, Gonzalez F, and Jefcoate CR (1998) Aryl-hydrocarbon receptor is an inhibitory regulator of lipid synthesis and of commitment to adipogenesis. *J Cell Sci* **111:**3311–3322.

Amano M, Chihara K, Kimura K, Fukata Y, Nakamura N, Matsuura Y, and Kaibuchi K (1997) Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. *Science (Wash DC)* **275:**1308–1311.

Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y, and Kaibuchi K (1996) Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J Biol Chem* **271:**20246–20249.

Andrieux L, Langouet S, Fautrel A, Ezan F, Krauser JA, Savouret JF, Guengerich FP, Baffet G, and Guillouzo A (2004) Aryl hydrocarbon receptor activation and cytochrome P450 1A induction by the mitogen-activated protein kinase inhibitor U0126 in hepatocytes. *Mol Pharmacol* **65:**934–943.

Bianco C, Strizzi L, Rehman A, Normanno N, Wechselberger C, Sun Y, Khan N, Hirota M, Adkins H, Williams K, et al. (2003) A Nodal- and ALK4-independent signaling pathway activated by Cripto-1 through Glypican-1 and c-Src. *Cancer Res* **63:**1192–1197.

Camps M, Nichols A, and Arkinstall S (2000) Dual specificity phosphatases: a gene family for control of MAP kinase function. *Faseb J* **14:**6–16.

Castro-Munozledo F, Marsch-Moreno M, Beltran-Langarica A, and Kuri-Harcuch W (1987) Commitment of adipocyte differentiation in 3T3 cells is inhibited by retinoic acid, and the expression of lipogenic enzymes is modulated through cytoskeleton stabilization. *Differentiation* **36:**211–219.

Chen CL, Brodie AE, and Hu CY (1997) CCAAT/enhancer-binding protein beta is not

affected by tetrachlorodibenzo-*p*-dioxin (TCDD) inhibition of 3T3–L1 preadipocyte differentiation. *Obes Res* **5:**146–152.
Cho YC and Jefcoate CR (2004) PPARgamma1 synthesis and adipogenesis in C3H10T1/2 cells depends on S-phase progression, but does not require mitotic clonal expansion. *J Cell Biochem* **91:**336–353.
Cho YC, Zheng W, Yamamoto M, Liu X, Hanlon PR, and Jefcoate CR (2005) Differentiation of pluripotent C3H10T1/2 cells rapidly elevates CYP1B1 through a novel process that overcomes a loss of Ah Receptor. *Arch Biochem Biophys* **439:**139–153.
Chu CL, Buczek-Thomas JA, and Nugent MA (2004) Heparan sulphate proteoglycans modulate fibroblast growth factor-2 binding through a lipid raft-mediated mechanism. *Biochem J* **379(Pt 2):**331–341.
Cimafranca MA, Hanlon PR, and Jefcoate CR (2004) TCDD administration after the pro-adipogenic differentiation stimulus inhibits PPARgamma through a MEK-dependent process but less effectively suppresses adipogenesis. *Toxicol Appl Pharmacol* **196:**156–168.
Cowherd RM, Lyle RE, and McGehee RE Jr (1999) Molecular regulation of adipocyte differentiation. *Semin Cell Dev Biol* **10:**3–10.
Defilippi P, Olivo C, Venturino M, Dolce L, Silengo L, and Tarone G (1999) Actin cytoskeleton organization in response to integrin-mediated adhesion. *Microsc Res Tech* **47:**67–78.
Fincham VJ, James M, Frame MC, and Winder SJ (2000) Active ERK/MAP kinase is targeted to newly forming cell-matrix adhesions by integrin engagement and v-Src. *EMBO (Eur Mol Biol Organ) J* **19:**2911–2923.
Fukai F, Iso T, Sekiguchi K, Miyatake N, Tsugita A, and Katayama T (1993) An amino-terminal fibronectin fragment stimulates the differentiation of ST-13 preadipocytes. *Biochemistry* **32:**5746–5751.
Giancotti FG, and Ruoslahti E (1999) Integrin signaling. *Science (Wash DC)* **285:**1028–1032.
Hanlon PR, Cimafranca MA, Liu X, Cho YC, and Jefcoate CR (2005a) Microarray analysis of early adipogenesis in C3H10T1/2 cells: cooperative inhibitory effects of growth factors and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Toxicol Appl Pharmacol* **207:**39–58.
Hanlon PR, Ganem LG, Cho YC, Yamamoto M, and Jefcoate CR (2003) AhR- and ERK-dependent pathways function synergistically to mediate 2,3,7,8-tetrachlorodibenzo-*p*-dioxin suppression of peroxisome proliferator-activated receptor-gamma1 expression and subsequent adipocyte differentiation. *Toxicol Appl Pharmacol* **189:**11–27.
Hanlon PR, Zheng W, Ko AY, and Jefcoate CR (2005b) Identification of novel TCDD-regulated genes by microarray analysis. *Toxicol Appl Pharmacol* **202:**215–228.
Hausman GJ, Wright JT, and Richardson RL (1996) The influence of extracellular matrix substrata on preadipocyte development in serum-free cultures of stromal-vascular cells. *J Anim Sci* **74:**2117–2128.
Hoelper P, Faust D, Oesch F, and Dietrich C (2005) Evaluation of the role of c-Src and ERK in TCDD-dependent release from contact-inhibition in WB-F344 cells. *Arch Toxicol* **79:**201–207.
Huo H, Guo X, Hong S, Jiang M, Liu X, and Liao K (2003) Lipid rafts/caveolae are essential for insulin-like growth factor-1 receptor signaling during 3T3–L1 preadipocyte differentiation induction. *J Biol Chem* **278:**11561–11569.
Hur EM, Park YS, Lee BD, Jang IH, Kim HS, Kim TD, Suh PG, Ryu SH, and Kim KT (2004) Sensitization of epidermal growth factor-induced signaling by bradykinin is mediated by c-Src. Implications for a role of lipid microdomains. *J Biol Chem* **279:**5852–5860.
Jin S, Zhai B, Qiu Z, Wu J, Lane MD, and Liao K (2000) c-Crk, a substrate of the insulin-like growth factor-1 receptor tyrosine kinase, functions as an early signal mediator in the adipocyte differentiation process. *J Biol Chem* **275:**34344–34352.
Kamiya S, Kato R, Wakabayashi M, Tohyama T, Enami I, Ueki M, Yajima H, Ishii T, Nakamura H, Katayama T, et al. (2002) Fibronectin peptides derived from two distinct regions stimulate adipocyte differentiation by preventing fibronectin matrix assembly. *Biochemistry* **41:**3270–3277.
Kubo Y, Kaidzu S, Nakajima I, Takenouchi K, and Nakamura F (2000) Organization of extracellular matrix components during differentiation of adipocytes in long-term culture. *In Vitro Cell Dev Biol Anim* **36:**38–44.
Lazar MA (2005) PPAR gamma, 10 years later. *Biochimie* **87:**9–13.
Leitinger B and Hogg N (2002) The involvement of lipid rafts in the regulation of integrin function. *J Cell Sci* **115**(Pt 5):963–972.
Liu J, DeYoung SM, Zhang M, Cheng A, and Saltiel AR (2005a) Changes in integrin expression during adipocyte differentiation. *Cell Metab* **2:**165–177.
Liu J, Deyoung SM, Zhang M, Dold LH, and Saltiel AR (2005b) The stomatin/prohibitin/flotillin/HflK/C domain of flotillin-1 contains distinct sequences that direct plasma membrane localization and protein interactions in 3T3–L1 adipocytes. *J Biol Chem* **280:**16125–16134.
Liu PC and Matsumura F (1995) Differential effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on the "adipose- type" and "brain-type" glucose transporters in mice. *Mol Pharmacol* **47:**65–73.
Matsui T, Amano M, Yamamoto T, Chihara K, Nakafuku M, Ito M, Nakano T, Okawa K, Iwamatsu A, and Kaibuchi K (1996) Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. *EMBO (Eur Mol Biol Organ) J* **15:**2208–2216.
Moro L, Dolce L, Cabodi S, Bergatto E, Erba EB, Smeriglio M, Turco E, Retta SF, Giuffrida MG, Venturino M, et al. (2002) Integrin-induced epidermal growth factor (EGF) receptor activation requires c-Src and p130Cas and leads to phosphorylation of specific EGF receptor tyrosines. *J Biol Chem* **277:**9405–9414.
Nakajima I, Yamaguchi T, Ozutsumi K, and Aso H (1998) Adipose tissue extracellular matrix: newly organized by adipocytes during differentiation. *Differentiation* **63:**193–200.
Ntambi JM and Young-Cheul K (2000) Adipocyte differentiation and gene expression. *J Nutr* **130**:3122S–3126S.
Oh ES, Gu H, Saxton TM, Timms JF, Hausdorff S, Frevert EU, Kahn BB, Pawson T, Neel BG, and Thomas SM (1999) Regulation of early events in integrin signaling by protein tyrosine phosphatase SHP-2. *Mol Cell Biol* **19:**3205–3215.
Parsons JT, Martin KH, Slack JK, Taylor JM, and Weed SA (2000) Focal adhesion kinase: a regulator of focal adhesion dynamics and cell movement. *Oncogene* **19:**5606–5613.
Phillips M, Enan E, Liu PC, and Matsumura F (1995) Inhibition of 3T3–L1 adipose differentiation by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J Cell Sci* **108:**395–402.
Razani B, Combs TP, Wang XB, Frank PG, Park DS, Russell RG, Li M, Tang B, Jelicks LA, Scherer PE, et al. (2002) Caveolin-1-deficient mice are lean, resistant to diet-induced obesity, and show hypertriglyceridemia with adipocyte abnormalities. *J Biol Chem* **277:**8635–8647.
Rosen ED and Spiegelman BM (2000) Molecular regulation of adipogenesis. *Annu Rev Cell Dev Biol* **16:**145–171.
Roskoski R Jr (2005) Src kinase regulation by phosphorylation and dephosphorylation. *Biochem Biophys Res Commun* **331:**1–14.
Safe S (2001) Molecular biology of the Ah receptor and its carcinogenesis. *Toxicol Lett* **120:**1–7.
Schlaepfer DD, Hanks SK, Hunter T, and van der Geer P (1994) Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature (Lond)* 372(6508):786–791.
Spiegelman BM and Farmer SR (1982) Decreases in tubulin and actin gene expression prior to morphological differentiation of 3T3 adipocytes. *Cell* **29:**53–60.
Tan Z, Huang M, Puga A, and Xia Y (2004) A critical role for MAP kinases in the control of Ah receptor complex activity. *Toxicol Sci* **82:**80–87.
Tang QQ, Otto TC, and Lane MD (2003) Mitotic clonal expansion: a synchronous process required for adipogenesis. *Proc Natl Acad Sci USA* **100:**44–49.
Tice DA, Biscardi JS, Nickles AL, and Parsons SJ (1999) Mechanism of biological synergy between cellular Src and epidermal growth factor receptor. *Proc Natl Acad Sci USA* **96:**1415–1420.
Turner CE (2000a) Paxillin and focal adhesion signalling. *Nat Cell Biol* **2:**E231–E236.
Turner CE (2000b) Paxillin interactions. *J Cell Sci* **113:**4139–4140.
Upla P, Marjomaki V, Kankaanpaa P, Ivaska J, Hyypia T, Van Der Goot FG, and Heino J (2004) Clustering induces a lateral redistribution of alpha 2 beta 1 integrin from membrane rafts to caveolae and subsequent protein kinase C-dependent internalization. *Mol Biol Cell* **15:**625–636.
Vogel CF and Matsumura F (2003) Interaction of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) with induced adipocyte differentiation in mouse embryonic fibroblasts (MEFs) involves tyrosine kinase c-Src. *Biochem Pharmacol* **66:**1231–1244.
Vogel CF, Zhao Y, Wong P, Young NF, and Matsumura F (2003) The use of c-src knockout mice for the identification of the main toxic signaling pathway of TCDD to induce wasting syndrome. *J Biochem Mol Toxicol* **17:**305–315.
Williams TM and Lisanti MP (2004) The Caveolin genes: from cell biology to medicine. *Ann Med* **36:**584–595.
Zhao L, Gregoire F, and Sul HS (2000) Transient induction of ENC-1, a Kelch-related actin-binding protein, is required for adipocyte differentiation. *J Biol Chem* **275:**16845–16850.

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