Research article

Coordinate regulation of RARgamma2, TBP, and TAF$_{II}$135 by targeted proteolysis during retinoic acid-induced differentiation of F9 embryonal carcinoma cells

Lucia Perletti, Eliezer Kopf, Lucie Carré and Irwin Davidson*

Address: Institut de Génétique et de Biologie Moléculaire et Cellulaire. CNRS/INSERM/ULP. B.P. 163-67404 Illkirch Cédex. C.U. de Strasbourg France

E-mail: Lucia Perletti - lucy@titus.u-strasbg.fr; Eliezer Kopf - eliezerkopf@hotmail.com; Lucie Carré - carre@titus.u-strasbg.fr; Irwin Davidson* - irwin@titus.u-strasbg.fr
*Corresponding author

Abstract

Background: Treatment of mouse F9 embryonal carcinoma cells with all-trans retinoic acid (T-RA) induces differentiation into primitive endodermal type cells. Differentiation requires the action of the receptors for all trans, and 9cis-retinoic acid (RAR and RXR, respectively) and is accompanied by growth inhibition, changes in cell morphology, increased apoptosis, proteolytic degradation of the RARγ2 receptor, and induction of target genes.

Results: We show that the RNA polymerase II transcription factor TFIID subunits TBP and TAF$_{II}$135 are selectively depleted in extracts from differentiated F9 cells. In contrast, TBP and TAF$_{II}$135 are readily detected in extracts from differentiated F9 cells treated with proteasome inhibitors showing that their disappearance is due to targeted proteolysis. This regulatory pathway is not limited to F9 cells as it is also seen when C2C12 myoblasts differentiate into myotubes. Targeting of TBP and TAF$_{II}$135 for proteolysis in F9 cells takes place coordinately with that previously reported for the RARγ2 receptor and is delayed or does not take place in RAR mutant F9 cells where differentiation is known to be impaired or abolished. Moreover, ectopic expression of TAF$_{II}$135 delays proteolysis of the RARγ2 receptor and impairs primitive endoderm differentiation at an early stage as evidenced by cell morphology, induction of marker genes and apoptotic response. In addition, enhanced TAF$_{II}$135 expression induces a novel differentiation pathway characterised by the appearance of cells with an atypical elongated morphology which are cAMP resistant.

Conclusions: These observations indicate that appropriately timed proteolysis of TBP and TAF$_{II}$135 is required for normal F9 cell differentiation. Hence, in addition to transactivators, targeted proteolysis of basal transcription factors also plays an important role in gene regulation in response to physiological stimuli.

Background

RNA polymerase II (pol II) transcription factor TFIID comprises the TATA-binding protein (TBP) and a set of TBP-associated factors (TAF$_{II}$s) [123]. At least 12 TAF$_{II}$s have been identified in TFIID and cloning of their cDNAs has shown an evolutionary conservation of TAF$_{II}$s from

yeast to mammals [4567]. TAF$_{II}$s are not only components of the TFIID complex, but a subset of TAF$_{II}$s are also found in the SAGA, PCAF, TFTC/STAGA complexes which lack TBP [89101112].

TAF$_{II}$ function in living cells has been studied in yeast where the use of temperature sensitive (TS) mutants has shown that many TAF$_{II}$s are required for transcription of the majority of yeast genes [1314151617]. In contrast, TS lesions in TAF$_{II}$145, TAF$_{II}$150, and TAF$_{II}$90 have a less dramatic effect affecting the expression of only a specific subset of genes mainly involved in the cell cycle [1819] (for reviews see [3, 20].

In mammalian cells, a TS mutation in TAF$_{II}$250 shows that one of the functions of this protein is cell cycle regulation [21222324]. Genetic experiments indicate that TAF$_{II}$30 is required for the viability of mouse F9 embryonal carcinoma cells as well as for their differentiation into parietal endoderm [25]. In the absence of TAF$_{II}$30, undifferentiated F9 cells die through apoptosis, but TAF$_{II}$30 is not required for survival of retinoic acid differentiated F9 cells.

Several studies have also focused on TAF$_{II}$135. TAF$_{II}$135 comprises 1083 amino acids and contains multiple functional domains. At least four glutamine-rich domains have been described. Sp1 and CREB interact with distinct glutamine-rich domains of TAF$_{II}$135 and TAF$_{II}$135 acts as a coactivator *in vitro* for these activators. In transfected cells, subdomains of TAF$_{II}$135 can act as dominant negative repressors of CREB activity [262728]. It has further been suggested that some neurodegenerative diseases may result from sequestration of TAF$_{II}$135 by expanded polyglutamine domains and consequent interference with CREB activity [29]. TAF$_{II}$135 also contains two conserved regions, CR-I and CR-II, which are shared with the *Drosophila* homologue dTAF$_{II}$110 and mammalian TAF$_{II}$105 [27, 30] The CR-II region is also shared with the yeast homologue yTAF$_{II}$48 [31, 32] and contains a histone fold domain required for heterodimerisation with hTAF$_{II}$20/yTAF$_{II}$68 [33, 34]. The CR-II domain plays an essential role in the ability of TAF$_{II}$135 to potentiate ligand-dependent transactivation by the the receptor for all-trans retinoic acid (RAR) in transfected mammalian cells [5, 33]. Aside from these studies, little is known concerning the role of TAF$_{II}$135 in more physiological situations.

An increasing body of evidence indicates that targeted 26S proteasome-mediated proteolysis of transcription factors is an integral part of the transactivation process. There is a very tight relationship between the potency of activation domains and their stability [35363738]. Activation domains and sequences required for degradation

overlap and mutations in the VP16 activation domain which impair its function result in enhanced protein stability [35]. Similarly, ligand-dependent targeted proteolysis of several nuclear receptors has been observed [39404142]. In the estrogen receptor, the RARα, and the RXRα, deletion of the α-helix H12 of the ligand binding domain which is essential for ligand-dependent activation stabilises these proteins showing that proteolysis and transactivation are intimately linked [424344]. In the case of nuclear receptors, their targeted proteolysis in the presence of ligand may be a mechanism for attenuating the physiological response to the ligand. It has also been suggested that targeted proteolysis is a means of regulating other physiological responses, such as signaling through STAT factors [45] and heat shock [46].

Although targeted proteolysis of transcriptional activators has been investigated, it is not known whether components of the basal transcription apparatus are subject to this type of regulation in response to physiological stimuli. We show here that TBP and TAF$_{II}$135 are selectively depleted in extracts from T-RA differentiated F9 cells and from differentiated C2C12 cells. This depletion is due to the selective targeting of TBP and TAF$_{II}$135 for proteolytic degradation since depletion is blocked when cells are treated with proteasome inhibitors. During F9 cell differentiation, degradation of TBP and TAF$_{II}$135 is concomitant with that of the RARγ2, a critical activator in primitive endoderm differentiation. These results reveal a novel pathway controlling the intracellular levels of these two TFIID components and show that in F9 cells RA not only induces targeted proteolysis of the RARγ2, but also of the basal transcription factors which mediate transcriptional activation. We further show that stable ectopic expression of TAF$_{II}$135 in F9 cells delays the targeted degradation of endogenous TAF$_{II}$135, TBP, and the RARγ2 in response to T-RA. The cells have an enhanced growth rate and their differentiation into primitive endoderm is impaired at an early stage, but they readily differentiate into parietal endoderm. Treatment of these cells with T-RA also induces the appearance of a population of cells with an atypical elongated morphology, distinct from that of the primitive endodermal cells, which have not been previously documented with wild type F9 cells, and which are resistant to differentiation with bt2cAMP.

Results

TBP and TAF$_{II}$135 are selectively depleted in extracts from T-RA differentiated F9 cells

F9 cells are a well characterised model for cellular differentiation. In the presence of T-RA, F9 cells differentiate into primitive or visceral endoderm, and into parietal endoderm in the presence of T-RA and bt$_2$cAMP [4748]. Differentiation is accompanied by growth inhibition,

characteristic changes in cell morphology, targeted degradation of RARγ2, induction of marker genes, and increased apoptosis.

To determine whether components of basal transcription factor TFIID were subject to regulation during F9 cell differentiation, immunoblotting with monoclonal antibodies against TBP and a number of TAF$_{II}$s, was used to monitor the levels of the respective proteins in differentiated cell extracts (see Materials and Methods).

No significant changes in the levels of TAF$_{II}$100, TAF$_{II}$55, TAF$_{II}$20, TAF$_{II}$30, and TAF$_{II}$18, or the largest subunit of RNA polymerase II were observed between 3 and 10 days of T-RA treatment (Fig. 1A, lanes 1-6 and data not shown). In striking contrast, TAF$_{II}$135 and TBP were strongly and selectively depleted in extracts made from cells differentiated with T-RA for more than 3 days (Fig. 1A lanes 1-2) and remained at low levels up until at least day 10 (lanes 2-5). No such changes were observed in vehicle treated control cultures (Fig. 1A, lanes 7-12). In these experiments, TAF$_{II}$135 and TBP were detected using two different monoclonal antibodies directed against distinct epitopes (see Materials and methods). The relevant extracts from T-RA treated cells were also analysed by staining with Coomassie Brilliant Blue showing that there was no overall change in the protein profile (Fig. 1B). Therefore, there is a selective depletion of these two TFIID components in extracts from differentiating cells.

To evaluate the levels of TBP and TAF$_{II}$135 in extracts from primitive endodermal cells we performed titration experiments using serial dilutions of undifferentiated F9 cell extract. Upon prolonged exposure, TAF$_{II}$135 could still be detected in a 20-fold dilution of the F9 cell extract, whereas it was virtually undetectable in extracts from day 7 differentiated cells (Fig. 1C, lanes 1-5 compared with lane 6). Similarly, TBP was readily detected in the 20-fold dilution of F9 cell extract, while the levels detected in the differentiated cell extracts were significantly lower (compare lanes 5 and 6).

The above experiments were performed with total cell extracts made in the presence of 0.5 M KCl. We also asked whether the depletion of TBP and TAF$_{II}$135 would be observed in nuclear extracts from differentiated F9 cells where the nuclei were extracted with 1.0 M KCl to ensure efficient protein extraction. As observed in total cell extracts, TAF$_{II}$135 and TBP were selectively depleted in nuclear extracts from differentiated cells (Fig. 1D).

TBP and TAF$_{II}$135 were strongly and selectively depleted in extracts from visceral endoderm differentiated F9 cells analogous to what was observed in primitive endodermal cells (data not shown). When F9 cells were treat-

ed with T-RA and bt$_2$cAMP to induce parietal endoderm differentiation, a selective depletion of TBP and TAF$_{II}$135 also took place between day 3 and day 5 (Fig. 1E lanes 1, 2 and 6), however, TBP, but not TAF$_{II}$135, reappeared in parietal endodermal cell extracts around day 9 (lanes 4-5). Together the above results indicate that there is a specific regulation of TBP and TAF$_{II}$135 during F9 cell differentiation.

Depletion of TBP and TAF$_{II}$135 is not observed in F9 cells which are refractory to T-RA.
To determine whether the depletion of TBP and TAF$_{II}$135 was intimately linked to the differentiation process, we examined the regulation of these proteins in mutant F9 cells with altered differentiation properties. F9 cells in which the retinoid X receptor α gene has been disrupted by homologous recombination (RXRα$^{-/-}$ cells) are impaired in their response to T-RA [49]. When these cells are treated with T-RA, the depletion of TBP and TAF$_{II}$135 observed in the cell extracts is delayed by almost 4 days and takes place only between days 7 and 9 rather than between days 3 and 5 in wild-type cells (Fig. 2A), while no such depletion is seen in extracts from vehicle treated cells (Fig. 2B). In contrast, no significant change in TBP and TAF$_{II}$135 is seen in extracts from T-RA treated RXRα$^{-/-}$RARγ$^{-/-}$ cells, in which the RARγ gene has also been disrupted [50, 51] and which are known to be refractory to T-RA induced differentiation (Fig. 2C). Thus, the selective depletion of TBP and TAF$_{II}$135 requires that the F9 cells respond, at least partially, to T-RA, but does not require full differentiation to take place.

Depletion of TBP and TAF$_{II}$135 is due to targeted proteolytic degradation
The depletion of TBP and TAF$_{II}$135 in extracts from day 3-10 T-RA treated cells could result from transcriptional or post-transcriptional events. Semi-quantitative reverse-transcription PCR (RT-PCR) experiments using exon-specific oligonucleotide primers showed that there were no significant changes in the TAF$_{II}$135 or TBP mRNA levels during differentiation (data not shown). This suggests that disappearance of TBP and TAF$_{II}$135 in extracts from differentiated cells does not occur at the transcriptional level, but rather results from a post-transcriptional event.

The peptide aldehyde proteasome inhibitors MG132 and ALLN have previously been used to investigate the post-transcriptional regulation of proteins during physiological processes [52, 53]. To determine whether the depletion of TAF$_{II}$135 and TBP observed in the extracts from late T-RA treated F9 cells was due to their selective proteolysis, we treated T-RA differentiated cells with these inhibitors on days 3, 4, 5, 6, and 7. After an overnight ex-
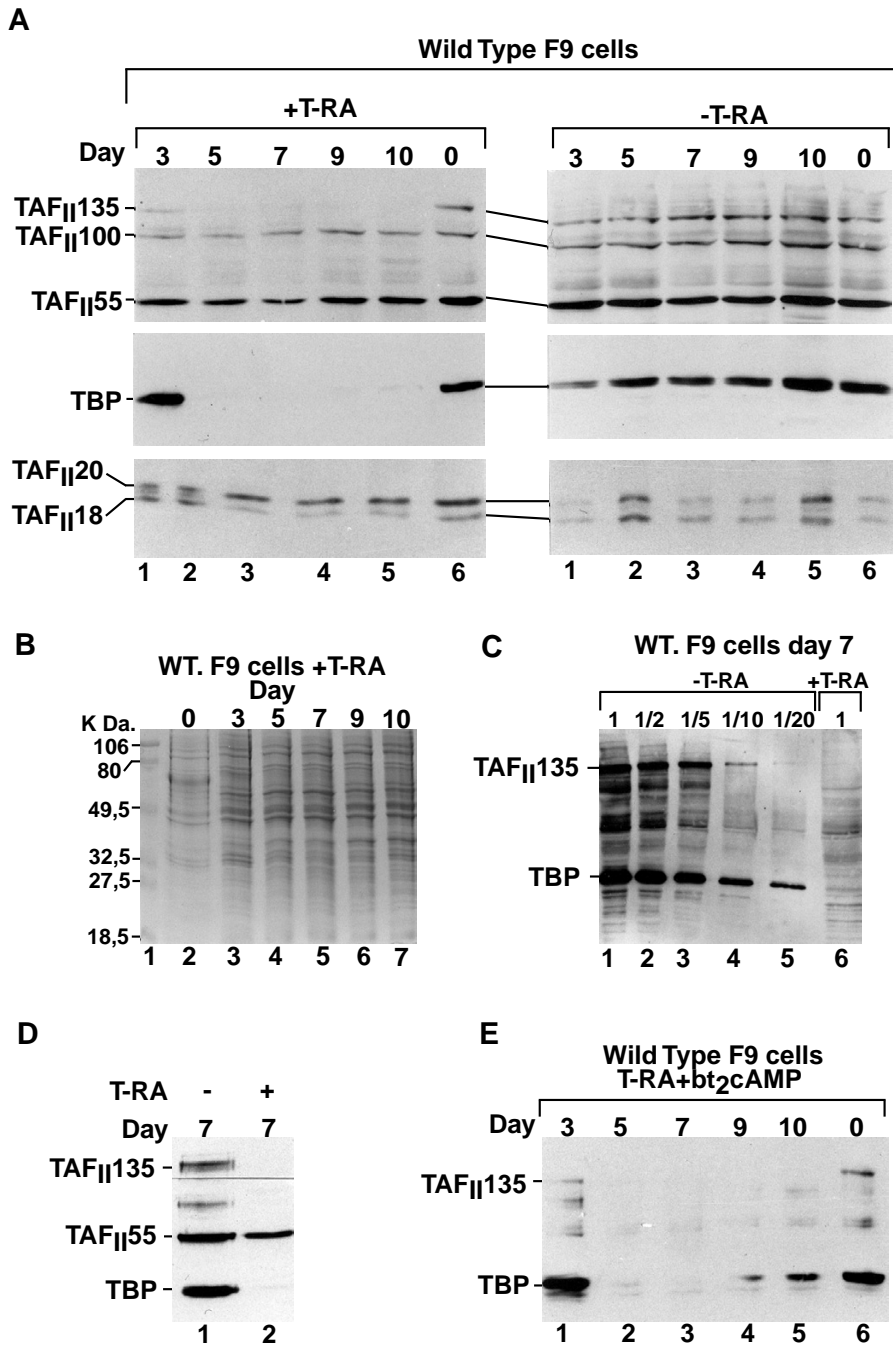
Figure 1
A. Depletion of TBP and TAF$_{II}$135 in extracts from differentiated F9 cells. Replica plates of cells were treated with T-RA (or vehicle, right panel) at day 0 and extracts were subsequently prepared form one 10 cm plate at the day indicated above each lane. 10 μg of each cell extract were then subjected to SDS-PAGE transferred to nitrocellulose membranes and the presence of TBP, and TAF$_{II}$s was detected using the described antibodies. The locations of the detected proteins are indicated to the left. **B**. The extracts from cells differentiated for 0-9 days used in panel A were subjected to SDS-PAGE and stained with Coomasie Brilliant Blue. **C.** Western blot with anti-TBP and TAF$_{II}$135 antibodies performed on serial dilutions of an extract from undifferentiated F9 cells were compared with an undiluted extract from 7 day differentiated cells. Lanes 1-5 contain 20, 10, 4, 2, and 1 μg of undifferentiated cell extract and lane 6, 20 μg of differentiated cell extract. **D.** Immunoblotting shows that TAF$_{II}$135 and TBP are selectively depleted in F9 cell nuclear extracts. **E.** Extracts were prepared from F9 cells differentiated by the addition of T-RA and bt$_2$cAMP for the number of days indicated above each lane.
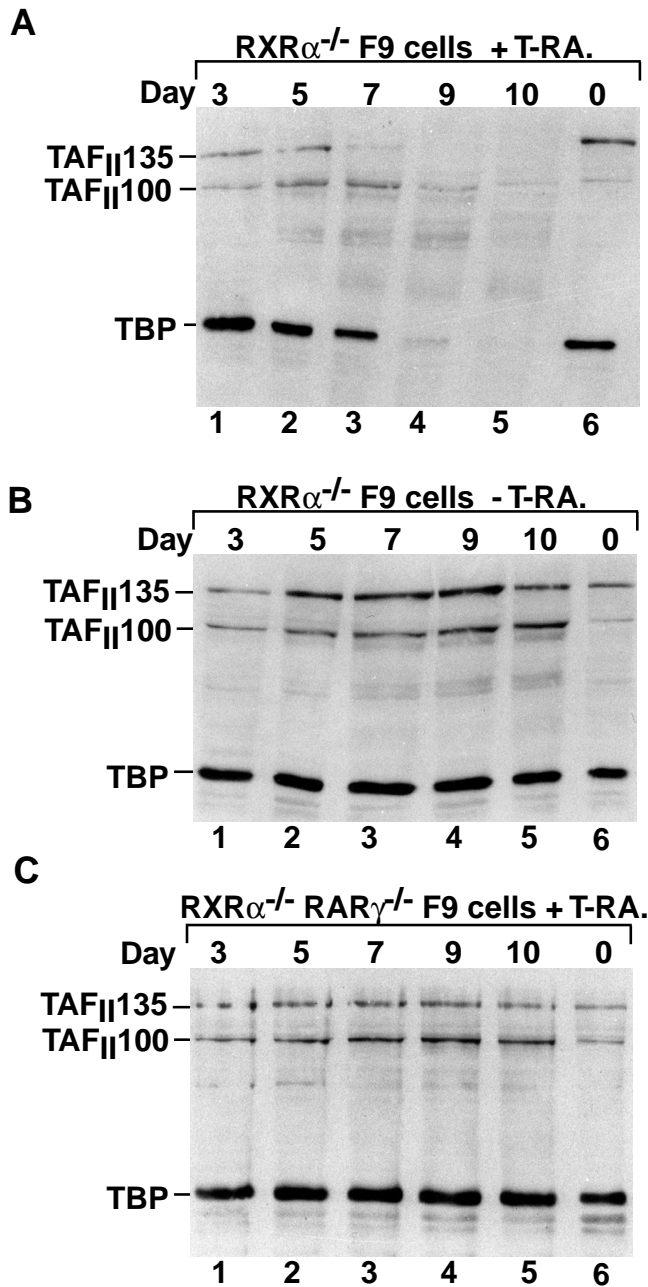
Figure 2
Depletion of TBP and $TAF_{II}135$ is integral to the differentiation process. The positions of TBP and $TAF_{II}135$ detected by immunoblotting are indicated. **A.** Extracts were prepared from mutant $RXR\alpha^{-/-}$ cells differentiated with T-RA for the number of days indicated above each lane. **B.** Control panel showing extracts from undifferentiated $RXR\alpha^{-/-}$ cells. **C.** Immunoblotting of extracts from T-RA treated $RXR\alpha^{-/-}$ $RAR\gamma^{-/-}$ cells.

ALLN, TBP and $TAF_{II}135$ disappeared in the differentiated cell extracts (middle panel Figs. 3A and 3B lanes 6-10). In contrast, in the presence of the inhibitors both proteins were readily detected even at late times where they are normally not observed (lanes 1-5). The levels were nevertheless lower than those detected in undifferentiated cell extracts. The level of $TAF_{II}55$, which remains constant during differentiation, is only mildly increased by the addition of MG132 or ALLN (Figs. 3A and 3B lower panel, lanes 1-5 and 6-10). The addition of MG132 or ALLN to non-differentiated cells also resulted in only a small increase in TBP and $TAF_{II}135$ levels (Figs. 3A and 3B, top panel compare lanes 1-5 and 6-10). The above results indicate that when proteolysis is inhibited the depletion of TBP and $TAF_{II}135$ is no longer observed showing that it is due to T-RA-induced proteolytic degradation.

We also prepared extracts from differentiated cells where MG132 was added only at the time of extract preparation. Extracts made in this way are a measure of the intracellular level of these proteins since synthesis and degradation are both stopped at the same time. Comparison of the TBP and $TAF_{II}135$ levels in extracts from day 7 differentiated cells prepared in the presence of MG132 showed that they were indeed significantly reduced compared to undifferentiated cells or day 3 differentiated cells (approximately 3 fold, Fig. 3C, lanes 1, 4 and 5, similar results were obtained in the presence of ALLN data not shown). Nevertheless, these proteins do not disappear completely as they do in the absence of MG132 (lanes 2-3). Therefore, the complete disappearance of these proteins seen in the absence of protease inhibitors indicates that there is further degradation in the extracts which exaggerates the differences between differentiated and undifferentiated cells. Although, less TBP could be immunopurified from the MG132 blocked differentiated cell extracts using the anti-TBP antidody 2C1, when equal amounts of immunopurified TBP from differentiated and undifferentiated cells was loaded, equivalent amonts of $TAF_{II}135$ were detected in the TFIIDs (data not shown). Thus, while the overall level of TFIID is diminished through the reduction in TBP and $TAF_{II}135$, the relative stoichiometry of the remaining TFIID is not significantly altered. The apparently larger decrease in $TAF_{II}135$ seen in Fig. 3C results from the lower efficiency of the antibody (see also Fig. 1C.)

Taken altogether, the above results show that the intracellular level of TBP and $TAF_{II}135$ and consequently TFIID is significantly reduced in differentiated cells through T-RA-induced proteolytic degradation.
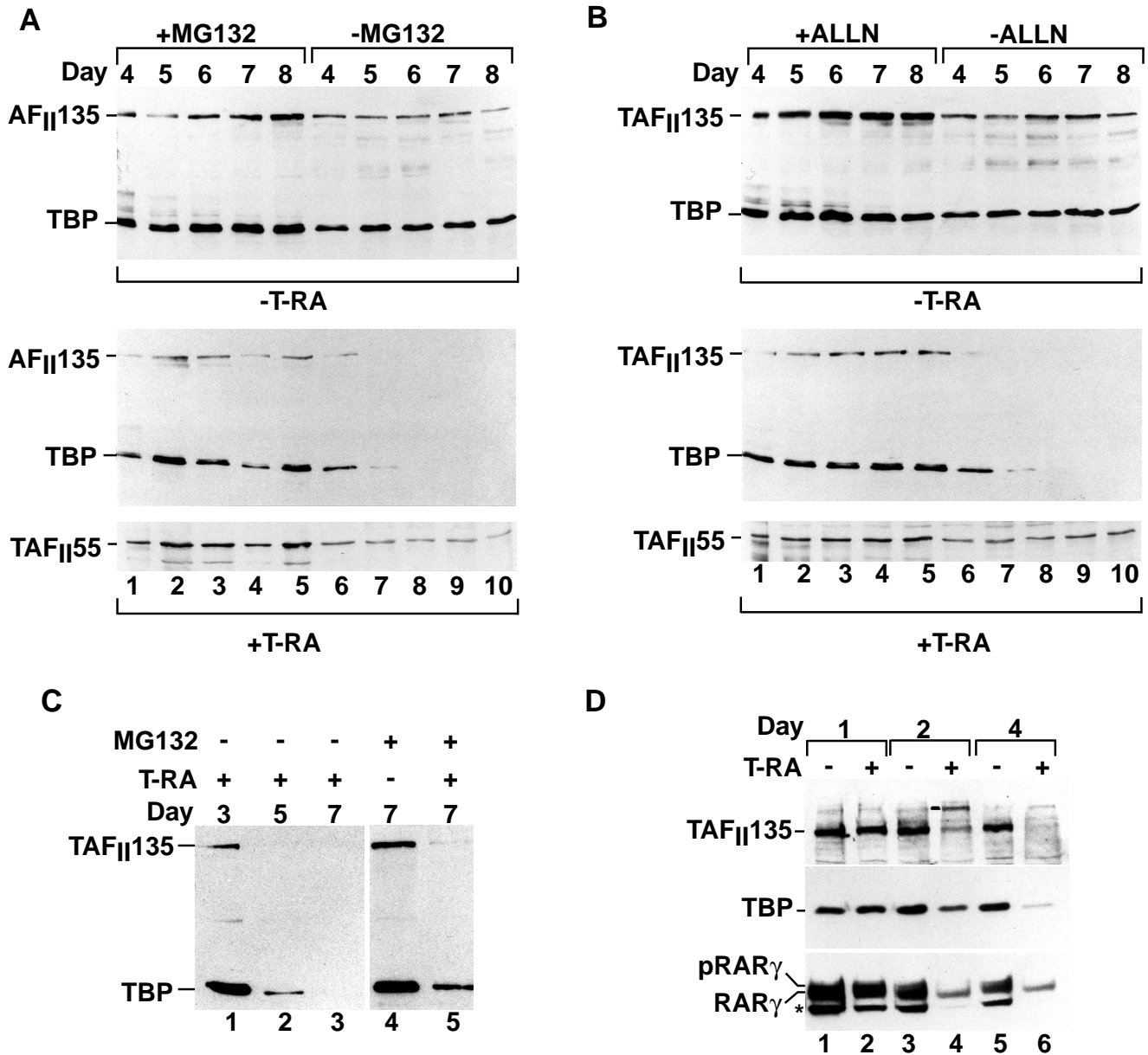
posure, extracts were prepared (day 4, 5, 6, 7 and 8 extracts in Figs. 3A and 3B). In the absence of MG132 or

Figure 3

Depletion of TBP and TAF$_{II}$135 is abrogated in the presence of protease inhibitors MG132 and ALLN. **A**. Replica plates of cells were treated with T-RA or vehicle and after 3, 4, 5, 6, and 7 days MG132 was added overnight (12 hours) and extracts then prepared designated day 4 to day 8 as indicated above each lane. TAF$_{II}$135, TBP and TAF$_{II}$55 were detected by immunoblotting. **B**. An experiment analogous to that shown in panel A was performed using the protease inhibitor ALLN. **C**. Measurement of the intracellular level of TAF$_{II}$135. In lanes 1-3 cell extracts were made in the absence of MG132. In lanes 4-5 cells were resuspended in extraction buffer containing 50 μM MG132. **D**. Coordinate degradation of TBP, TAF$_{II}$135 and the RARγ2. Cell extracts were made on the days indicated above each lane from T-RA treated or untreated cells and the presence of TBP, TAF$_{II}$135, and the RARγ2 was detected by immunobloting. The native RARγ and the phosphorylated species (pRARγ) are indicated along with a breakdown product indicated by [*]. A slower migrating species of TAF$_{II}$135 seen after 48 hours is indicated by -.

Coordinate degradation of TBP, TAF$_{II}$135, and the RARgamma2 receptor during F9 cell differentiation

It has previously been shown that the RARγ2 is subject to

degradation beginning 48 hours after T-RA-induced differentiation of F9 cells [42]. To determine whether there is a coordinate degradation of RARγ2, TBP and

$TAF_{II}135$, we examined extracts made at these early times. After 24 hours of T-RA treament the levels of $RAR\gamma2$, TBP and $TAF_{II}135$ were unchanged (Fig. 3D, lanes 1-2). However after 48 hours, there was a T-RA-dependent reduction in the amount of $RAR\gamma2$ and TBP and a significant shift in the electrophoretic mobility of $TAF_{II}135$ (lanes 3-4). By 4 days the levels of all three proteins were strongly reduced (lanes 5-6). These results indicate that the onset of degradation of TBP and $TAF_{II}135$ is concomitant with that of the $RAR\gamma2$ suggesting that these events are interdependent.

Constitutive ectopic expression of $TAF_{II}135$ impairs F9 cell primitive endoderm differentiation

The above results show that the level of $TAF_{II}135$ and TBP is strictly controlled during the differentiation of F9 cells by a post-transcriptional mechanism involving targeted proteolysis. To test whether artificially increasing the level of $TAF_{II}135$ would affect the proper differentiation of F9 cells, we established cell lines which constitutively express flag-tagged versions of human $TAF_{II}135$ [$fTAF_{II}135(372-1083)$ and $fTAF_{II}135(805-1083)$ see Materials and methods]. $TAF_{II}135(372-1083)$ contains all the known functional domains, but lacks an unconserved proline-alanine-rich region whereas, $TAF_{II}135(805-1083)$ comprises only the CR-II region.

For $fTAF_{II}135(372-1083)$ 12 cell lines were examined and we chose two, lines A and B, since they expressed the highest levels of the $fTAF_{II}135$ protein. Immunoblotting with an anti-flag monoclonal antibody showed a significantly higher expression level in line A compared to line B (Fig. 4A, lanes 1-2). This result is confirmed by immunoblotting with monoclonal antibody 20TA which detects both the endogenous and ectopically expressed $TAF_{II}135$ proteins (Figs. 4B and 4C, lane 1). In line A, the level of the ectopically expressed protein is higher than that of the endogenous protein, while in line B both the exogenous and the endogenous proteins are expressed at similar levels. For $fTAF_{II}135(805-1083)$ which contains only the conserved C-terminal domain, 8 cell lines were examined of which 3 expressed the $fTAF_{II}135$ protein (Fig. 6A). Both of the cell lines expressing $fTAF_{II}135(372-1083)$ showed an approximately 2-fold accelerated growth rate compared to wild type cells while no significant increase was seen in the $fTAF_{II}135(805-1083)$ expressing cells (Fig. 4E, and data not shown, note that the apparent slowing of growth between day 8 and 10 for lines A and B is due to their reaching confluence).

We first verified whether treatment of these cells with T-RA would lead to the depletion of the ectopically expressed $TAF_{II}135$. In line A, both the ectopically expressed $TAF_{II}135$ and the endogenous $TAF_{II}135$ were depleted with the same kinetics (Fig. 4B, lanes 1-5). TBP

was also concomitantly depleted in these extracts, and all three proteins were stabilised when cells were treated with MG132 (lanes 6-8). Importantly however, the disappearance of all three proteins was clearly delayed by 48 hours compared to wild-type cells (compare Figs. 4B and Fig. 1A or Fig. 3C). In line B, there was also coordinate disappearance of TBP and the exogenous and endogenous $TAF_{II}135$ (Fig. 4C), although no obvious delay such as that seen in line A is observed. In contrast, no depletion of $fTAF_{II}135(805-1083)$ was seen following T-RA treatment, while the endogenous TBP and $TAF_{II}135$ were depleted normally between days 3 and 5 (Fig. 6B, and data not shown).

As the targeted proteolysis of TBP and $TAF_{II}135$ is linked to the differentiation process, the delayed depletion in line A suggests that T-RA induced differentiation may be perturbed. Their differentiation was therefore compared to that of the wild-type cells.

In wild type cells, the degradation of $RAR\gamma2$ begins at day 2 and the protein is strongly depleted from extracts made at day 3 (Fig. 4D, lane 3). At later times, low levels of only the unphosphorylated form of $RAR\gamma2$ can be seen in the extracts (lanes 4-6). In contrast, in line A, elevated levels of $RAR\gamma2$ are observed even at day 5 (Fig. 4D, lanes 3-4). In line B, an intermediate situation is observed since high levels of $RAR\gamma2$ are seen at day 3 which decrease by day 5 (lanes 3-4). In all cases, low levels of only the unphosphorylated $RAR\gamma2$ are detected by days 7-9 (lanes 5-6). These results indicate that proteolytic degradation of $RAR\gamma2$ is delayed in lines A and B suggesting that an early event in the differentiation process has been perturbed.

Comparison of the growth rate of line A with that of wild-type cells in the presence of T-RA, shows that line A is less sensitive to the anti-proliferative effect of T-RA than wild-type, however growth is eventually slowed compared to undifferentiated cells (Fig. 4E). Line B also grows faster than wild-type over the first 4-6 days, but subsequently its growth slows and is considerably retarded compared to undifferentiated cells (Fig. 4E). Together these observations show that line A is poorly sensitive to T-RA-induced growth arrest and retains an essentially undifferentiated morphology, while growth of line B slows after 4-6 days of T-RA treatment and morphological differentiation is retarded.

We determined whether $fTAF_{II}135(372-1083)$ expression modified T-RA induced apoptosis. The proportion of early apoptotic cells in lines A, B, and wild-type was determined by flow cytometry in the presence of propidium iodide and annexin V-FITC [25, 54, 55]. In wild-type cells, the proportion of early apoptotic cells increased
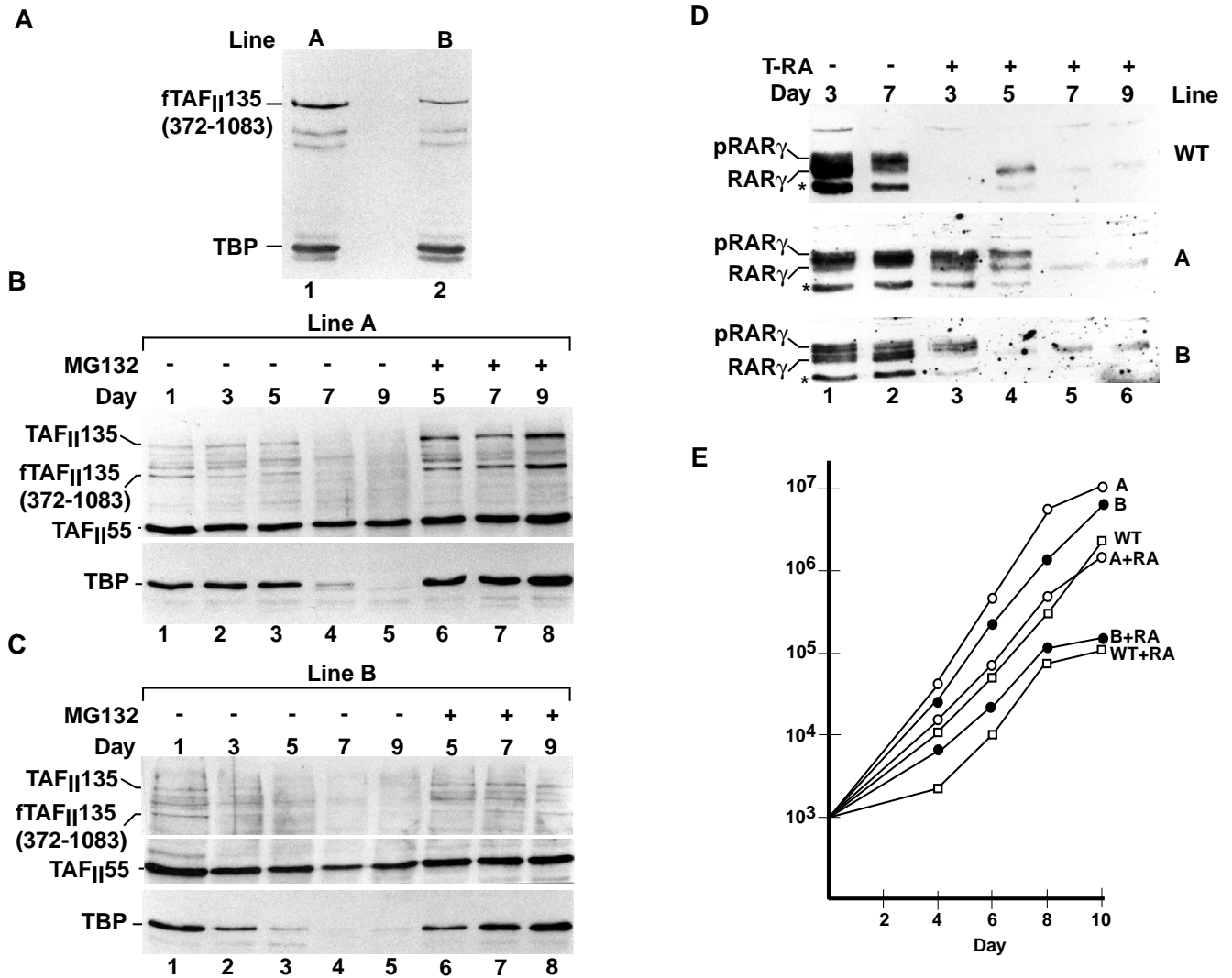
Figure 4
Characterisation of F9 cell lines A and B expressing ectopic f TAF$_{II}$135(372-1083). **A.** The presence of f TAF$_{II}$135(372-1083) in extracts from each cell line was determined using the commercial anti-flag monoclonal antibody. **B**. Immunoblot analysis of extracts from line A differentiated for the number of days indicated above each. Lanes 6-8 show extracts from cells treated for 12 hours with MG132. **C.** Immunoblot analysis of differentiated cell extracts from line B. **D.** Immunoblot analysis of extracts from wild-type cells and lines A and B with antibodies against RARγ2. The native and phosphorylated species and the breakdown product are indicated as in Fig. 4D. **E**. Cell growth was measured for 10 days in the presence or absence of T-RA. The cell number is indicated on the X axis and the number of days of growth is indicated on the Y axis. Analogous results were obtained in three independent experiments and the graph shows the results of a representative experiment.

from 13% in the absence of T-RA to 26% after 5 days of T-RA treatment (Fig. 5). In line A, spontaneous apoptosis was lower than wild type (7% compared to 13%) and only a mild increase (9%) was seen by day 5 (Fig. 5). In line B, spontaneous apoptosis was also lower than wild type (6% compared to 13%), while in the presence of T-RA the proportion rose to 16% (Fig. 5). Thus, line A is resistant to T-RA induced apoptosis, while line B shows reduced sensitivity to T-RA induced apoptosis.

Differentiation of lines A and B was further assessed from changes in cellular morphology. 6-8 days after the addition of T-RA, wild-type F9 cells adopt a typical flattened primitive endodermal morphology (see upper panel of Fig. 7). In contrast, line A cells retain an essentially undifferentiated morphology even after 8 days of T-RA treatment (centre panel Fig. 7). The differentiation of line A is severely impaired, as the cells never adopt a differentiated morphology even after 10-12 days of RA-treatment (data not shown). Similarly, line B cells appear
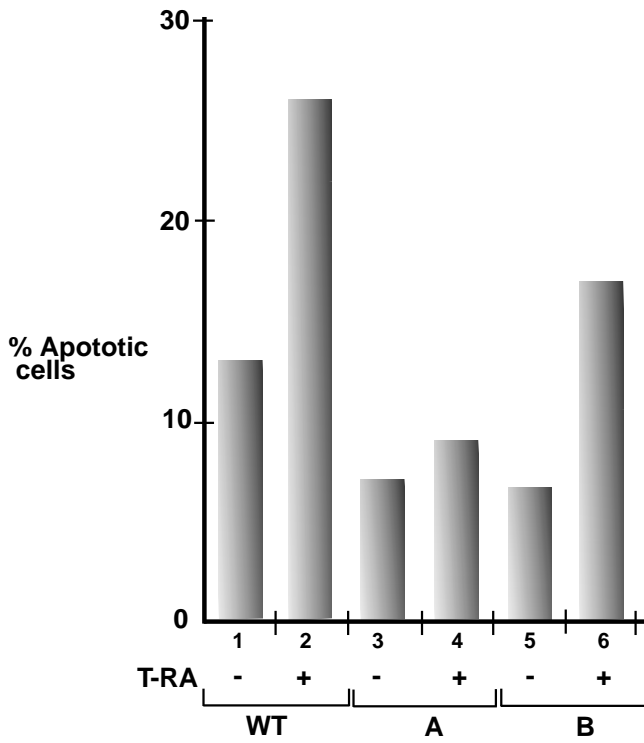
Figure 5
Detection of early apoptotic cells by flow cytometry. The percentage of early apoptotic cells in undifferentiated cultures and in day 5 T-RA-differentiated cultures is indicated for wild type cells and for lines A and B.

essentially undifferentiated after 6 days of T-RA treatment (lower panel, Fig. 7). After 8 days, a fraction of the cells begin to adopt a primitive endodermal type morphology, although the cells are much less flattened than wild-type cells, and the majority of the cells retain an undifferentiated morphology (Fig. 7). Only after 10 days of T-RA treatment did the majority of the cells adopt a differentiated morphology (data not shown).

F9 cell differentiation is characterised by the induction of different marker genes. We used reverse transcription coupled-PCR (RT-PCR) to compare the induction of some of these genes in lines A and B with that observed in wild-type cells. The RARβ2 gene is a marker which is rapidly induced in wild-type cells normally, after 12-24 hours [495051, 56]. In agreement with this, the RARβ2 mRNA is fully induced by day 3 in wild-type cells. Expression persists until day 5 and decreases only by day 7 (Fig. 8A, lanes 2-5, note that the small increase in lane 5 in this experiment is not reproducibly observed). In contrast, in lines A and B, RARβ2 expression is weak at day 3, peaks only at day 5, and drops off by day 7 (lanes 7-10 and 12-15). Thus, induction of RARβ2 is severely retard-
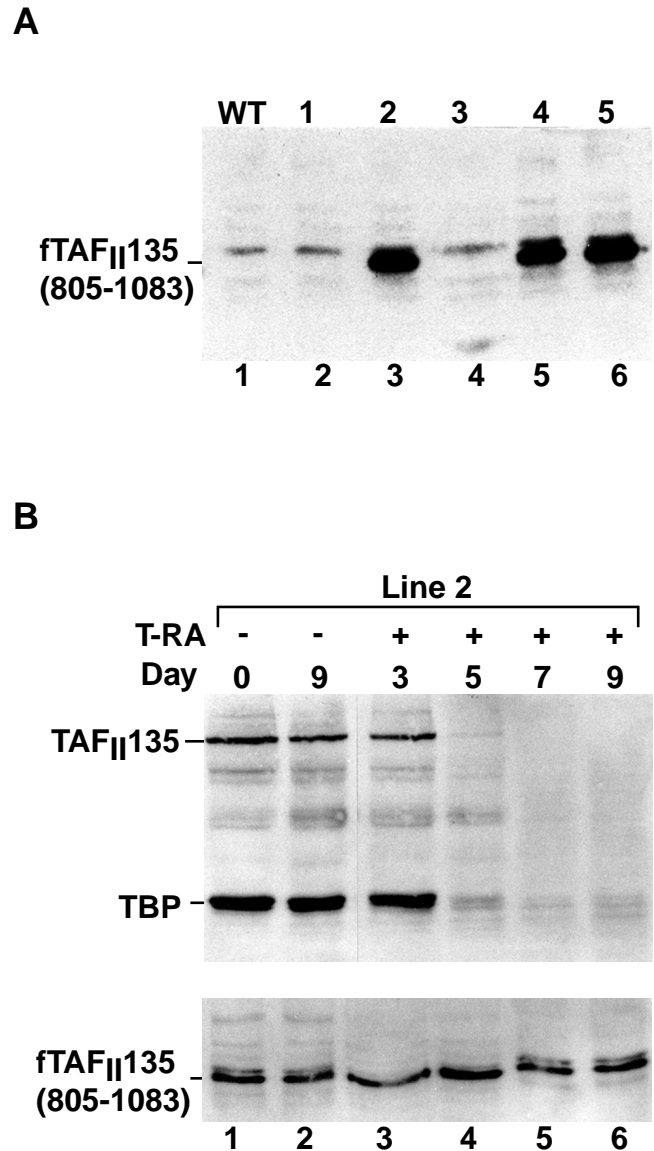

Figure 6
Isolation and analysis of cells expressing fTAF$_{II}$135(805-1083). **A**. Extracts from wild-type cells and 5 different clones were analysed by immunoblotting using an anti-flag antibody. **B** Extracts were made from clone 2 cells differentiated with T-RA for the number of days indicated above each lane. The fTAF$_{II}$135(805-1083) deletion mutant was detected in the cell extracts using the anti-flag antibody.

ed and transient in lines A and B. In the same RNA samples, constant expression levels of HPRT were observed in differentiated and undifferentiated cells. In contrast to the RARβ2 gene, induction of the collagen IVα1 and laminin B1 mRNAs was almost identical in the wild-type and mutant cells (Fig. 8B and data not shown) showing that the delay in induction is specific to RARβ2.
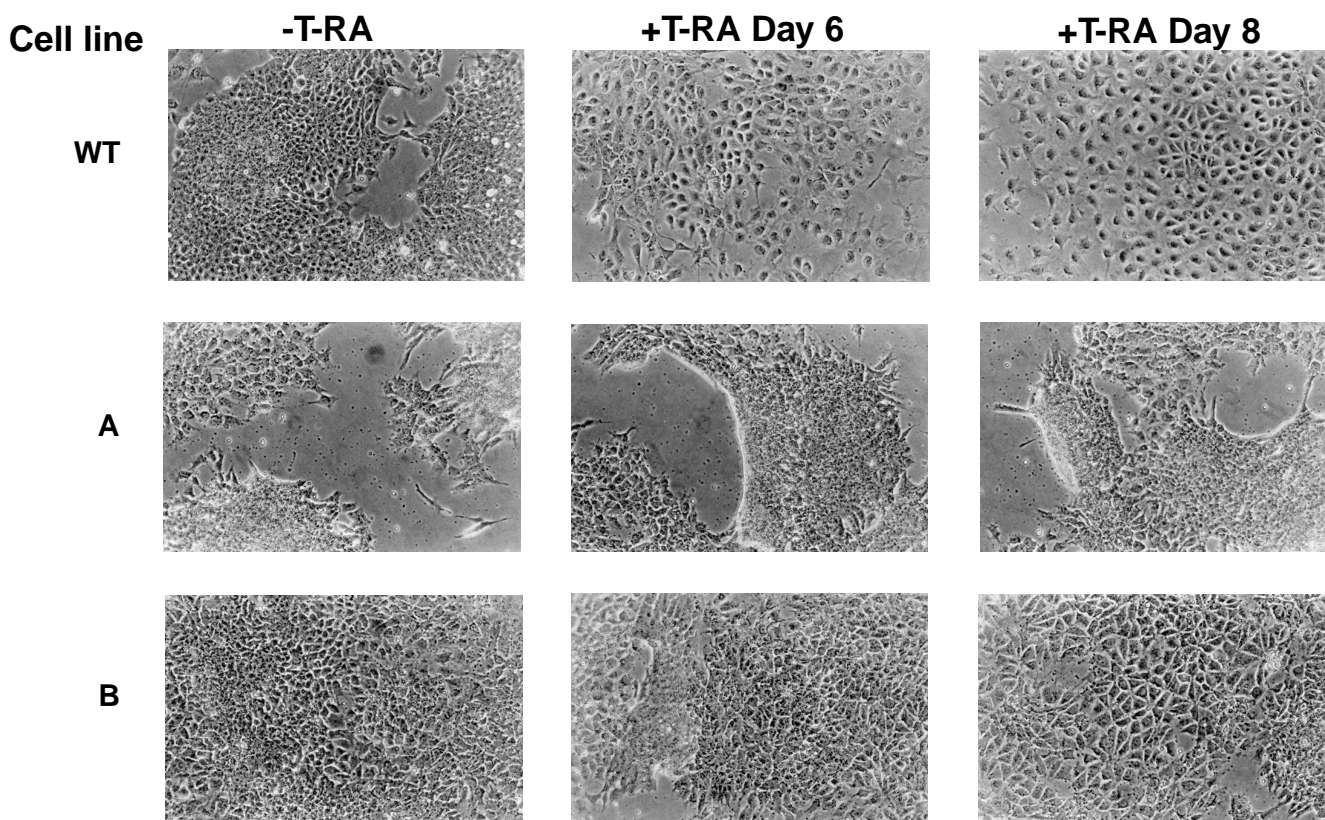
Cell line	-T-RA	+T-RA Day 6	+T-RA Day 8
WT			
A			
B			

Figure 7
Morphology of lines A and B in the presence of T-RA. **A**. Each panel shows representative phase-contrast photography at 125-fold magnification of wild-type cells or lines A and B after the indicated number of days treatment with T-RA.

After 6 days of T-RA treatment, bt_2cAMP was added to induce parietal endoderm differentiation. Addition of bt_2cAMP induced the rapid appearance of rounded cells with typical parietal endoderm morphology for both the wild type cells and lines A and B (Fig. 9). Thus, while primitive endoderm differentiation of lines A and B is impaired, they readily differentiate into parietal endoderm.

In lines A and B, we noticed after 6-8 days of T-RA treatment the appearance of a novel cell type characterised by elongated tightly packed cells (Fig. 10A). The morphology of these cells was completely different from those of undifferentiated or primitive endoderm cells. Although the majority of the cell population differentiates when bt_2cAMP is added to these cultures, the elongated cells persisted even after several days of bt_2cAMP treatment (Fig. 10B) showing that these cells are refractory to the differentiating effect of bt_2cAMP. Cells with this atypical morphology were not observed when wild-type cells or $fTAF_{II}135(805-1083)$ expressing cells are differentiated either with T-RA or T-RA and bt_2cAMP. Therefore, ectopic expression of $fTAF_{II}135(372-1083)$ has induced a

novel differentiation pathway leading to the appearance of atypical elongated cells.

Taken altogether, the above results show that primitive endoderm differentiation of F9 cells is blocked in line A expressing the highest level of $TAF_{II}135(372-1083)$ and is significantly retarded in line B cells which express lower levels of $TAF_{II}135(372-1083)$. In contrast, all three $fTAF_{II}135(805-1083)$ expressing cell lines and several control lines picked at the same time all showed a normal T-RA response with respect to growth inhibition, differentiated cell morphology, and targeted depletion of $TAF_{II}135$ and TBP (Fig. 6B and data not shown).

$TAF_{II}135$ and TBP are depleted in extracts from differentiated C2C12 cells
To determine whether the down regulation of TBP and $TAF_{II}135$ is specific to F9 cell differentiation we asked whether this phenomenon could be observed in an unrelated cell type. C2C12 are mouse skeletal muscle myoblasts which differentiate into multinucleated myotubes [57]. This differentiation process does not require the addition of retinoic acid, but takes place spontaneously

A

B
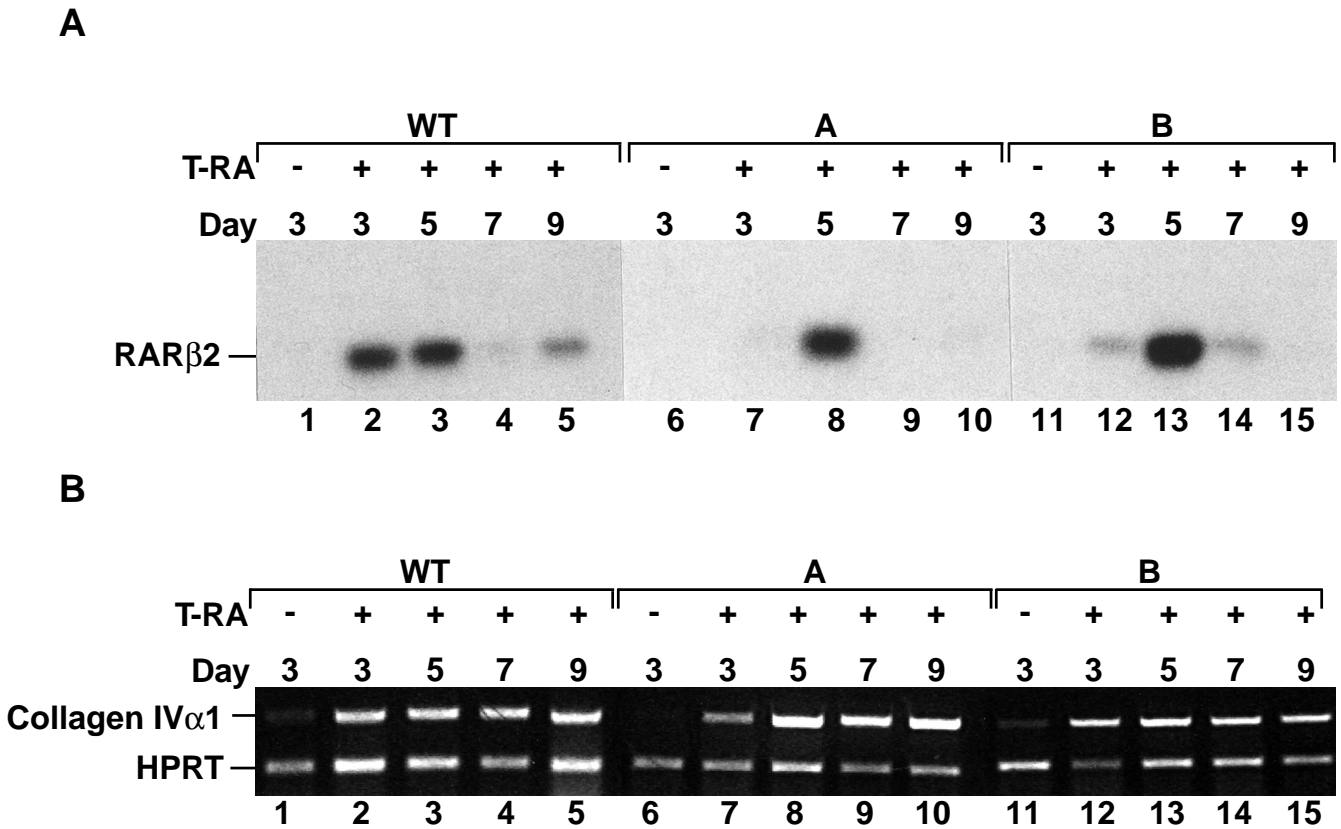
Figure 8
RT-PCR analysis of gene expression during differentiation. A. Delayed induction of the RARβ2 gene. Aliquots of the PCR reactions made with RNA from the day shown above each lane were electrophoresed, blotted, and hybridised with an internal oligonucleotide probe for the RARβ2 gene. **B.** Aliquots of the PCR reactions performed with the same RNA samples as in panel A were analysed by electrophoresis on an agarose gel and staining with ethidium bromide.

when cultures are grown to high density and undergo growth arrest.

In extracts from differentiating C2C12 cells, a progressive decrease in $TAF_{II}135$ and TBP can be seen beginning on day 6 such that these proteins are strongly depleted from the extracts by day 11 (Fig. 11A, lanes 1-5 compare with lane 6). In contrast, the levels of $TAF_{II}55$ remain constant (Fig. 11A). The observed depletion of TBP and $TAF_{II}135$ was not observed when the cells on day 10 were treated with MG132 for 12 hours prior to extract preparation on day 11 (Fig. 11B, lanes 1-3). These results indicate that TBP and $TAF_{II}135$ are also targetted for proteolytic degradation during C2C12 cell differentiation.

Discussion
Post-transcriptional regulation of TAF$_{II}$135 and TBP in differentiated F9 cells
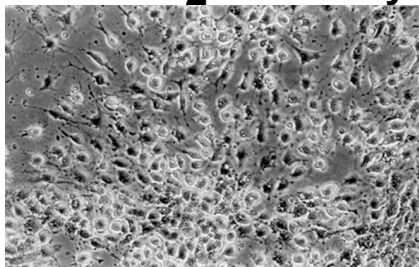We show here that targeted proteolysis of TBP and $TAF_{II}135$ is an integral part of the T-RA response in F9

cells. This process is specific for TBP and $TAF_{II}135$ as it is not observed with the other TFIID components analysed, and is induced concomitantly with that of the RARγ2. In F9 cells with constitutive ectopic expression of $TAF_{II}135$, targeted degradation is delayed, primitive endodermal differentiation is impaired and a novel differentiation pathway is induced. Hence, targeted degradation of $TAF_{II}135$ and TBP is a prerequisite for proper F9 cell differentiation.
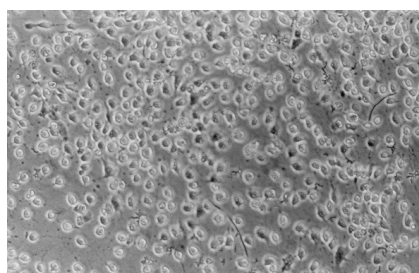
$TAF_{II}135$ and TBP progressively disappear from whole cell or nuclear extracts made from T-RA differentiated F9 cells. This depletion does not result from altered transcription of these genes, since no significant changes in the corresponding mRNA levels were observed during differentiation. In lines A and B, the ectopic expression of f-$TAF_{II}135$ is driven by a synthetic promoter, yet coordinate depletion of the exogenous and endogenous $TAF_{II}135$ is observed further confirming that this is indeed a post-transcriptional event.

Cell line +T-RA +bt$_2$cAMP Day 8

A

Cell line **+T-RA Day 10**

B

Cell line **+T-RA +bt$_2$cAMP Day 10**

Figure 10
Abberant differentiation of lines A and B in the presence of T-RA. **A.** Phase contrast photograph of line B cells differentiated with T-RA showing the presence of elongated fibroblast-like cells. **B**. Phase contrast photograph of line A cells differentiated with T-RA and bt$_2$cAMP. The rounded parietal endoderm cells can be seen alongside cells with an elongated morphology.
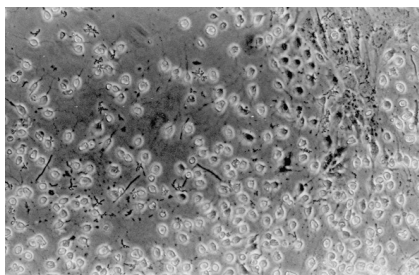
Figure 9
Parietal differentiation of lines A and B. Each panel shows representative phase-contrast photography at 125-fold magnification of wild-type cells or lines A and B after 6 days of treatment with T-RA and two days with T-RA and bt$_2$cAMP.
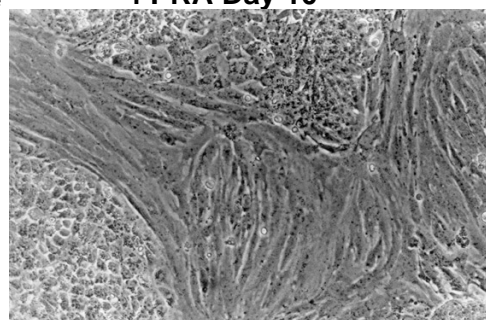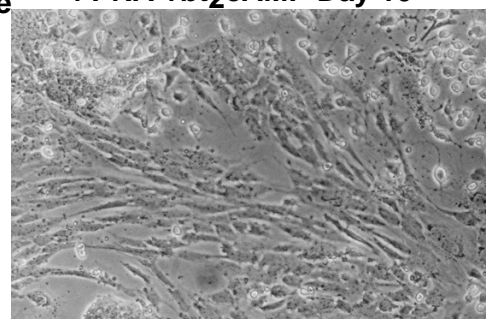
In contrast, TBP and TAF$_{II}$135 are readily detected in extracts made from differentiated cells treated with MG132, ALLN, and the proteasome-specific inhibitor lactacystin (our unpublished data) showing that their disappearance is due to selective proteolysis. In the presence of the inhibitors, the detected levels are significantly lower than those seen in equivalent extracts made from undifferentiated cells. Addition of the protease inhibitors only during the extraction procedure is another way to measure of the intracellular level of TBP and TAF$_{II}$135. In these extracts also, the levels of TBP and TAF$_{II}$135 are significantly reduced in differentiated cells compared with undifferentiated cells. Therefore, the intracellular levels of TBP and TAF$_{II}$135 and consequently TFIID are reduced in differentiated cells through selective proteolysis.

Targeted degradation of TAF$_{II}$135 does not require amino acids 1-372 since deletion mutant (372-1083) is targeted along with the endogenous protein in lines A and B. In contrast, mutant (805-1083) is not degraded during differentiation indicating that the sequences required for targeting are located between amino acids 372-805. Many of proteins which are targeted for proteolytic degradation contain signal sequences such as PEST or cyclin destruction boxes which mediate their proteolysis, however no recognised destabilising motifs are present in TBP and TAF$_{II}$135 and indeed these proteins are stable in non-differentiated F9 cells and in many other cell types. It will therefore be interesting to identify the sequences are required for degradation of TBP and TAF$_{II}$135 in differentiating F9 cells. In this respect we note that we have not been able to recover F9 cell lines significantly overexpressing TBP suggesting that TBP overexpression is toxic.
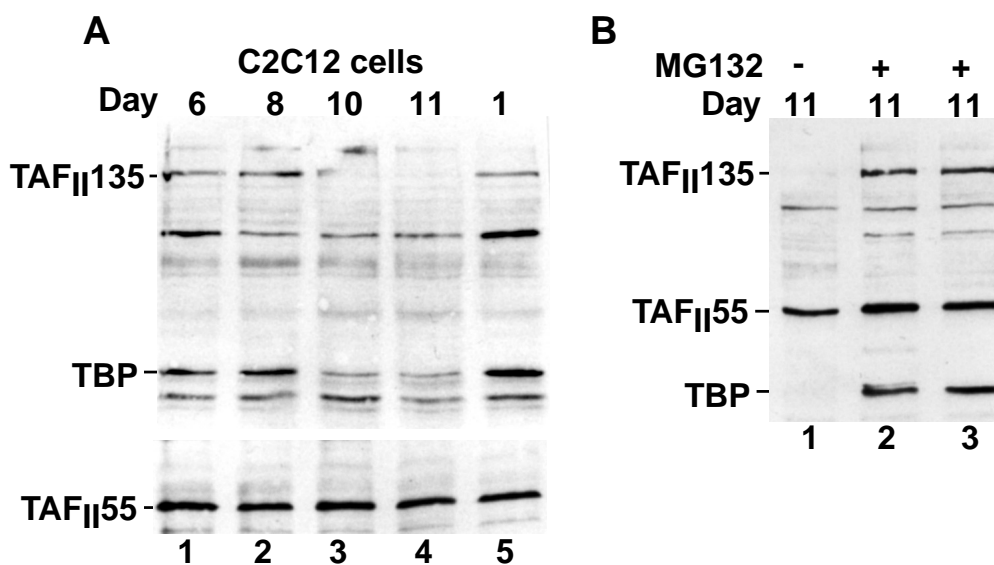
Figure 11
Depletion of TBP and TAF$_{II}$135 in extracts from differentiated C2C12 cells. **A**. Immunoblot analysis of extracts from C2C12 cells grown at high density in the absence of serum for the number of days indicated above the panel. **B**. Immunoblot analysis of extracts from C2C12 cells in the presence or absence of MG132 added at day 10 and extracts prepared on day 11. Lanes 2 and 3 show duplicate extracts from two dufferent paltes treated with MG132.

In F9 cells, RARγ2 plays a key role in primitive endoderm differentiation [58,59]. 48 hours after addition of T-RA degradation of this protein begins such that by 3-4 days most of the protein is depleted [42]. The onset of RARγ2 degradation coincides with that of TBP and TAF$_{II}$135 suggesting that these events are causaly related. This idea is supported by the fact that in line A degradation of TBP, TAF$_{II}$135, and RARγ2 are all delayed to similar extents. A lesser delay in degradation of RARγ2 in line B was also observed. If the corresponding delay in TBP and TAF$_{II}$135 degradation in line B was less than two days, it would be missed since we prepared extracts at two day intervals. All of these observations are consistent with the idea that upon exposure to T-RA there is an early transcriptional response in which a RARγ2/RXR heterodimer activates genes required for differentiation. As a consequence, the RARγ2, and the TAF$_{II}$135 and TBP components of the basal transcription machinery are down-regulated through proteolytic degradation. It has been suggested that degradation of the RARγ2 or the ER is a means of regulating the cellular response to these ligands both in terms of magnitude and time [42, 44]. The coordinate degradation of RARγ2, TBP, and TAF$_{II}$135 in response to T-RA indicates that the cellular T-RA response is not only regulated through degradation of the activator, but also through degradation of components of the basal transcription machinery.

The differentiation of C2C12 myoblasts into myotubes does not require T-RA, but is induced by growth inhibition and serum starvation. Despite the important differences between these cells and F9 cells, targeted degradation of TBP and TAF$_{II}$135 is also observed when C2C12 cells differentiate. Hence, targeted degradation can be induced by stimuli other than T-RA. Our report is the first showing that basal transcription factors can be regulated in this way in response to different physiological stimuli.

As described in the Introduction, recent studies indicate that the activation process is linked to targeted proteolysis of transcriptional activators. In the case of the RARγ2, degradation requires a constitutive phosphorylation of the AF-1 domain, heterodimerisation with RXR, and the presence of ligand indicating that the receptor has to be transcriptionally active to be degraded [42]. Under these conditions the receptor is ubiquitinated and degraded. While we have not been able to directly detect ubiquitination of TBP or TAF$_{II}$135 in extracts where ubiquitination of RARγ2 can be detected (our unpublished data), at early times we do observe a significant shift in the electrophoretic mobility of TAF$_{II}$135 which may correspond to a polyubiquitinated intermediate While the molecular events involved in degradation of transcription factors are in general poorly understood, it is noteworthy that both NRs and TBP have been reported to interact with the proteasome component SUG1 [60,61] which may act

to target these proteins for degradation. In addition, other components of the proteasome/ubiquitin degradation pathway have also been shown to interact with either nuclear receptors or components of the preinitiation complex (for review see [38]). Further experiments will be required to determine whether TBP and $TAF_{II}135$ are modified directly to allow targeting, or whether they are targeted indirectly through the formation of transcriptionally active complexes with the RARγ2 which, as it is itself degraded, acts to recruit the degradation machinery.

A decreased level of TBP in differentiated F9 cells has previously been noted [62,63]. These studies did not address the mechanism underlying its down-regulation, but they revealed a down-regulation of the pol III TAF BRF and the pol I TAF_I48 and TAF_I95 proteins and diminished transcription by the corresponding polymerases in T-RA differentiated F9 cells. In the light of the present study, it is possible that the BRF, TAF_I48, and TAF_I95 proteins are also post-transcriptionally down-regulated by targeted proteolysis in the same way as TBP and $TAF_{II}135$. This pathway may therefore be used to more generally regulate the activity of all three RNA polymerases during differentiation.

$TAF_{II}135$ plays a key role in F9 cell growth and differentiation

We show here that T-RA-induced primitive endoderm differentiation is impaired in cell lines constitutively expressing ectopic $TAF_{II}135(372-1083)$ as judged from, retarded targeted degradation of RARγ2, cell morphology, growth inhibition, apoptotic response, and induction of the RARβ2 marker gene. The effects are most pronounced in line A cells which express the highest level of ectopic $TAF_{II}135$ and do not show a differentiated cell morphology even upon prolonged exposure to T-RA. An intermediate, but significant, effect is seen using all criteria tested in line B cells, where $fTAF_{II}135(372-1083)$ expression is lower. In contrast, cells expressing $fTAF_{II}135(805-1083)$ differentiate normally.

The RARβ2 gene is normally fully induced by 12-24 hours [56] and elevated expression persists up until day 5. In lines A and B, induction was seen only after 5 days and this induction was transient compared to wild-type cells. As the RARβ2 gene is important for T-RA-induced growth arrest [64], its delayed induction in lines A and B may in part explain the phenotype of these cells. Nevertheless, the collagen IVα1 and laminin B1 genes (as well as several other known marker genes, our unpublished data), are induced normally in lines A and B by day 3-5 even when no obvious morphological differentiation is observed. This differential induction of the RARβ2 versus other marker genes has not been previously noted in

the various RAR and RXR knock out cell lines analysed. The phenotype generated by ectopic $TAF_{II}135$ expression is therefore distinct from that seen in these knock out cell lines. Most of the currently identified marker genes are not appropriate for the analysis of the phenotype of these lines. A detailed understanding of how ectopically expressed $TAF_{II}135$ blocks primitive endoderm differentiation will require the identification of novel marker genes whose expression is significantly altered in lines A and B and which may be directly regulated by $TAF_{II}135$.

Despite the fact that primitive endoderm differentiation of these lines is perturbed, they differentiate normally into parietal endoderm. This shows that, in contrast to what has previously been assumed, differentiation into parietal endoderm does not require the cells to first be fully differentiated into primitive endoderm since line A cells go from an essentially undifferentiated morphology to a parietal morphology. However, even line A is not totally refractory to T-RA, since several marker genes are induced and they have become bt_2cAMP responsive. Again, the phenotype of these cells is not the same as that of the $RXRα^{-/-}RARγ^{-/-}$ cells where none of these events take place.

Our results rather indicate that ectopic $TAF_{II}135$ expression acts at an early stage to impair primitive endoderm differentiation, but does not prevent the T-RA-induced changes which render the cells bt_2cAMP responsive. The initial elevated $TAF_{II}135$ expression in lines A and B impairs both the T-RA transcriptional response and its associated degradation of RARγ2, TBP, and $TAF_{II}135$ prolonging the period of elevated expression of these proteins resulting in a subsequent block of primitive endoderm differentiation. It has previously been shown that phosphorylation of the RARγ2 AF-1 is indispensable for primitive endoderm differentiation [59]. When F9 cells expressing the RARγ2 in which the phosphorylation site has been mutated are treated with T-RA, no degradation of this protein is observed. There is therefore an intimate relationship between differentiation and RARγ2 degradation suggesting that proper RARγ2 degradation is essential for differentiation [42]. This idea is supported by our present results since we show that $TAF_{II}135$ overexpression impairs both RARγ2 degradation and primitive endoderm differentiation. It is therefore probable that the increased stability of the RARγ2 in lines A and B is the major cause of their impaired primitive endoderm differentiation. It is interesting to note that the $TAF_{II}135(805-1083)$ deletion mutant is not degraded in response to T-RA and its expression does not interfere with differentiation. This suggests that $TAF_{II}135$ is only targeted for degradation if it contributes functionally to the T-RA response.

The severity of the phenotype observed in lines A and B correlates with the expression level of the exogenous f TAF$_{II}$135(372-1083) protein, a version of TAF$_{II}$135 in which the N-terminal proline-alanine rich region has been deleted. The deleted region is not conserved in hTAF$_{II}$105 and dTAF$_{II}$110, is not involved in interactions with other TAF$_{II}$s, SP1, or CREB [26,27, 33], and has no known function. In agreement with this, the fTAF$_{II}$135(372-1083) [and the fTAF$_{II}$135(805-1083)] protein can be coprecipitated with the endogenous TFI-ID of lines A and B (our unpublished data). It is likely therefore that the phenotype of lines A and B results from the TAF$_{II}$135 overexpression *per se*, although we cannot exclude the possibility that the effects are not due only to the elevated expression levels, but that they may additionally reflect a dominant negative property of this mutant. Investigation of this has been hampered by the fact that we have not been able to isolate F9 cell lines stabley expressing full length TAF$_{II}$135.

Treatment of lines A and B with T-RA induces a fraction of the cells to adopt an atypical elongated cell morphology, which to our knowledge has not been previously seen upon differentiation of F9 cells. Unlike the other cells in the population, these cells do not differentiate into parietal endoderm showing that they are refractory to bt$_2$cAMP. These observations suggest that ectopic TAF$_{II}$135 expression promotes T-RA induction of genes which are normally not activated in F9 cells leading to a novel differentiation pathway. It is also possible that TAF$_{II}$135 expression modulates the activity of transcription factors in addition to the RARs leading to the appearance of this novel cell type.

Conclusions
We report for the first time that components of the basal transcription machinery are subject to regulation by targeted proteolysis in response to a physiological stimulus. Our results point to a critical role of TAF$_{II}$135 in F9 cell physiology. It is downregulated during F9 cell differentiation and expression of TAF$_{II}$135 at elevated levels promotes cell growth, impairs the normal T-RA response, and induces a novel differentiation pathway.

Materials and methods
F9 and C2C12 cell culture and differentiation
Wild-type and mutant F9 cells were grown as described [25, 49]. Differentiation was induced by addition of 1.0 μM all-trans retinoic acid (T-RA) with or without 250 μM di-butaryl bt$_2$cAMP. In most experiments an appropriate number of 10 cm plates were seeded with 10^5 cells and after 24 hours (day 0) T-RA was added and protein or RNA extracts were made from one plate of cells on each of the indicated days. Stably transformed cell lines were isolated by electroporating plasmid pXJ-fTAF$_{II}$135(372-1083)

along with the puromycin resistance gene under the control of the SV40 promoter. Puromycin resistant cell clones were selected in the presence of 1μg/ml of puromycin, amplified, and the presence of exogenous fTAF$_{II}$135(372-1083) was detected by immunoblotting and immunofluoresence (data not shown). Aliquots of each clone were frozen after the minimum number of passages and three independent differentiation experiments giving analogous results were started from the frozen stocks. For cell growth rates, 3 cm plates were seeded with 10^3 cells and after 4-10 days cells were counted with a particle counter (Coulter Z2). C2C12 cells were maintained undifferentiated by growth at low density. C2C12 cells were differentiated by plating at high density and growth in 0.1% serum for the number of days indicated on the figures. Morphological differentiation was clearly seen by light microscopy.

Extract preparation and immunoblotting
Protein extracts from cells were made essentially as previously described [4, 65]. Briefly, cells were washed and harvested with ice cold phosphate buffered saline. Cells were then resuspended in buffer A (50 mM Tris-HCl pH 7.5, 20% glycerol, 1 mM Dithiothreitol, 0.2% Nonidet P-40) with 0. 5 M KCl and subjected to two rounds of freeze-thaw followed by a 30 min incubation on ice. The cell debris was then removed by centrifugation and the soluble protein fraction was frozen in liquid nitrogen and stored at -80°C. Nuclear extracts were prepared by first lysing the cells for 10 minutes in 1 ml of isotonic buffer (50 mM KCl, 50 mM Tris-HCl, pH 7.9, 0.2% NP40, 0.5 mM EDTA and 1.5 mM MgCl2). The nuclei were then recovered by centrifugation at 2000 rpm for 5 minutes and the supernatant containing the cytoplasmic proteins was discarded. Nuclear protein was then extracted from the purified nuclei with buffer A containing 1.0 M KCl. Protein concentrations were determined by the method of Bradford using a Biorad protein assay kit. Equal amounts of protein, normally 10 μg unless otherwise stated, were then subjected to SDS PAGE and immunoblotting. TBP was detected using monoclonal antibodies 3G3 and 2C1 [666768]. TAF$_{II}$135 was detected using monoclonal antibody 20TA [5] which recognizes an epitope in the conserved C-terminal domain and monoclonal antibody 32TA raised against peptide P1 in the N-terminal region as described in [5]. TAF$_{II}$100, TAF$_{II}$55, TAF$_{II}$30, and TAF$_{II}$20, were detected using the previously described monoclonal antibodies ([4, 69, 70] and refs therein). RARγ2 was detected using the previously described antibody [42]. Anti-flag monoclonal antibody was puchased from Sigma. Blots were revealed using peroxidase conjugated goat anti-mouse IgG antibody and chemiluminescence with an ECL kit (Amersham). Protease inhibitors MG132 (Cbz-leu-leu-leucinal) and ALLN (acetyl-leu-leu-norleucinal) were purchased from

CalBiochem and ICN Biochemicals respectively. Each was resuspended in DMSO and added to cells or extracts at a final concentration of 50 μM.

RT-PCR and flow cytometry

Total cellular RNA was isolated using Trizol Reagent (Life Technologies) from F9 cells grown in parallel with those used for making protein extracts. 1 μg of total RNA was then analysed by RT-PCR performed as described [50] Control reactions (not shown) contained all components except reverse transcriptase. Details of the exon-specific primers for HPRT, TBP, and TAF$_{II}$135, are available upon request. The primers used for RARβ2, laminin B1 and collagen IVa1 were as previously described [49]. Southern blotting and hybridisation for RARβ2 were performed by standard procedures. Flow cytometry was performed as described by Metzger et al., (1999) using a final concentration of 25 μg/ml of propidium iodide and FITC-labelled annexin V (CALTAG Laboratories). Samples were analysed on a FACScan (Becton Dickinson) equipped with a single aron laser. A minimum of 10^5 cells per sample were analysed with the CELLQuest software.

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