

Cell-type-selective induction of *c-jun* by TAF4b directs ovarian-specific transcription networks

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Cell-type-selective expression of the TFIID subunit TAF_{II}105 (re-named TAF4b) in the ovary is essential for proper follicle development. Although a multitude of signaling pathways required for folliculogenesis have been identified, downstream transcriptional integrators of these signals remain largely unknown. Here, we show that TAF4b controls the granulosa-cell-specific expression of the proto-oncogene *c-jun*, and together they regulate transcription of ovary-selective promoters. Instead of using cell-type-specific activators, our findings suggest that the coactivator TAF4b regulates the expression of tissue-specific genes, at least in part, through the cell-type-specific induction of *c-jun*, a ubiquitous activator. Importantly, the loss of TAF4b in ovarian granulosa cells disrupts cellular morphologies and interactions during follicle growth that likely contribute to the infertility observed in TAF4b-null female mice. These data highlight a mechanism for potentiating tissue-selective functions of the basal transcription machinery and reveal intricate networks of gene expression that orchestrate ovarian-specific functions and cell morphology.

ovary | TFIID | short interfering RNA | chromatin immunoprecipitation | granulosa cells

Specific targeting of RNA polymerase (Pol) II to heteromorphous promoters dispersed throughout the genome and packaged into chromatin requires the function of a multitude of individual proteins and multiprotein complexes that collectively direct the modulation of RNA synthesis. At the core of this machinery are the general transcription factors that were first posited to be global in nature and not directly implicated in control of cell-type-specific gene expression. Rather, cell-type-specific expression patterns were largely attributed to DNA-binding activator and repressor proteins that were thought to be expressed in a highly cell-type-specific fashion (1). The general transcription factor IID (TFIID) is a large multiprotein transcription factor that lies at the core of the basal transcription machinery. TFIID is composed of the TATA-box-binding protein (TBP) and a common subset of TBP-associated factors (TAFs) that together perform promoter-selective and coactivator functions during transcription initiation (2). Together, genetic and biochemical studies suggest a conserved and formidable role of TFIID in the execution of global programs of gene expression.

The identification of specialized, tissue-restricted components of TFIID revealed that the basal machinery has evolved additional critical roles in the execution of cell-type-specific patterns of gene expression, especially during germ-line development (3). In *Drosophila*, multiple testis-specific TAFs have been shown to be precisely expressed in the male germ line and absolutely required for spermatogenesis (4). In mammals, the TBP homologue TRF 2 (also called TLF) has been shown to be required for spermiogenesis during male gametogenesis (5). To examine the cell-type-specific role of TFIID in mammals, we have characterized the function of the TAF4b subunit of TFIID, which was the first identified tissue-specific TAF (6). In mice, the elevated expression level of *Taf4b* mRNA in the ovary and testis indicates that TAF4b may play a selective role in the regulation of gonadal-specific programs of gene

expression (7). The presence of cell-type-specific components of the general transcription machinery in metazoan organisms suggests that, in addition to performing global functions in gene expression, selective components of the basal machinery may be intimately involved in promoting cell-type-specific patterns of gene expression.

Postnatal ovarian follicles are composed of intimately associated cell types including somatic granulosa cells (GCs) and germ cells that collectively contribute to the production of a functional oocyte. Folliculogenesis is a cyclic process in which cohorts of meiotically arrested oocytes resume growth while their accompanying GCs proliferate and then further differentiate in response to pituitary gonadotropins (8). Intriguingly, in the wild-type ovary, *Taf4b* mRNA is expressed highly in the layers of GCs that support the growing oocyte through ovulation. To elucidate such a selective role in the gonads, we have previously targeted disruption of *Taf4b* in mice, revealing essential functions of TAF4b in ovarian follicle development in addition to the maintenance of spermatogenesis in males (7, 9). Female TAF4b-null mice are infertile owing to severe abnormalities in folliculogenesis. Because defective follicle development in TAF4b-null females is accompanied by detrimental changes in tissue-specific gene expression, the promoters of these genes may be direct targets of TAF4b transcriptional regulation in GCs. By using mammalian folliculogenesis as a model system of coordinated cell-type specification, we have begun to characterize how complex differential programs of gene expression are achieved in mammals and how such programs function during reproduction and ovarian follicle development.

Results

Establishment of TAF4b-Overexpressing GCs. To dissect the molecular mechanisms underlying female infertility in the *Taf4b*^{-/-} mice, a rat spontaneously immortalized GC (SIGC) line was used as a model system to identify TAF4b-dependent promoters and to probe additional networks of gene expression programs that are regulated by TAF4b (10). Because transcriptional activation by numerous activators can be potentiated by overexpression of individual TFIID subunits in mammalian cells (11), we established several stable SIGC lines that constitutively express the Flag-tagged TAF4b (f-TAF4b) or express the control Flag-tag alone (flag). From these stable cell lines, we further tested and selected one of each, a control flag and f-TAF4b cell line, for further analysis. Expression of Flag-TAF4b and endogenous TAF4b was monitored by Western blot analysis using anti-Flag and anti-TAF4b antibodies, respectively. SIGCs containing Flag-TAF4b express approximately three to five times more TAF4b than the control flag cell line, which had equivalent levels of the GAPDH control (Fig. 1A). Overexpression of TAF4b did not affect the levels of the 220-kDa subunit

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Abbreviations: AP-1, activator protein 1; ChIP, chromatin IP; GC, granulosa cell; FSH, follicle-stimulating hormone; IP, immunoprecipitation; PMSG, pregnant mare serum gonadotropin; Pol, polymerase; RPA, RNase protection assay; SIGC, spontaneously immortalized GC; siRNA, short interfering RNA; TBP, TATA-box protein; TAF, TBP-associated factor.

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Table 1. Microarray analysis of genes up-regulated in f-TAF4b SIGCs

Gene title	Average fold induction
Osteopontin	38.25
Vimentin	22.63
Insulin-like growth-factor-binding protein 3	13.22
Fibronectin	11.08
Matrix metalloproteinase-3	9.25
c-Jun	7.88
α -Tubulin	7.31
Vascular endothelial growth factor-B	6.38
Latent transforming growth-factor- β binding protein 1	4.35
Caldesmon-1	4.27
Disabled-2	3.79
Interleukin 1 α	3.28
Brain-derived neurotrophic factor	3.14
Amphiregulin	2.78
RhoB	2.76
Kruppel-like factor 6	2.54
Fos-related antigen 1	2.52
Follistatin	2.52
Transforming growth factor- α	2.38

suggest that an adaptation of TFIID by incorporation of TAF4b may be sufficient, at least in part, to reprogram the expression of a subset of cell-type-specific genes independent of cell-type-specific activators.

TAF4b Stimulates the Expression of c-jun. In an attempt to identify additional TAF4b-target genes expressed primarily in GCs, DNA microarray analysis was used to monitor changes in the gene expression profiles of SIGC stable cell lines. Approximately 82 genes were consistently and specifically up-regulated at least 2-fold in f-TAF4b stable cells compared with the control flag cells (Table 1 and data not shown). Interestingly, the data indicated that *c-jun* (7.9-fold) and *fra-1* (2.5-fold) are two of the potential TAF4b-responsive genes in GCs. Consistent with this result, a number of known c-Jun/activator protein 1 (AP-1) target genes were also highly up-regulated in f-TAF4b stable cells, some of which are growth factors and cytoskeletal proteins (Table 1). Real-time RT-PCR was performed to confirm a subset of the microarray results and to independently quantitate increases in mRNA levels (see Fig. 8, which is published as supporting information on the PNAS web site). This result suggests that TAF4b is involved in the transcription of a subset of GC-specific as well as non-cell-type-selective genes.

TAF4b Stimulation of c-jun Expression Is Cell-Type-Specific. The transcription factor c-Jun is an immediate early gene product that can be rapidly induced by a myriad of extracellular stimuli independent of cell type. Therefore, we next asked whether Flag-TAF4b-dependent induction of *c-jun* expression was cell-type-specific. In SIGCs expressing elevated levels of Flag-TAF4b, we detect 5-fold more *c-jun* mRNA than in the control flag cell line, consistent with our previous DNA microarray analysis (Fig. 3A Left). Surprisingly, however, in NIH/3T3 cells, both f-TAF4b-expressing and flag-control stable cell lines exhibited equivalent mRNA levels of *c-jun*, indicating that TAF4b-dependent *c-jun* induction may be cell-type-specific (Fig. 3A Right). To confirm that constitutive overexpression of a TFIID subunit does not indirectly induce c-Jun levels in the SIGCs, we established a Flag-tagged TBP (f-TBP) cell line and measured the levels of c-Jun protein. SIGCs expressing either the control Flag vector or Flag-TBP possessed

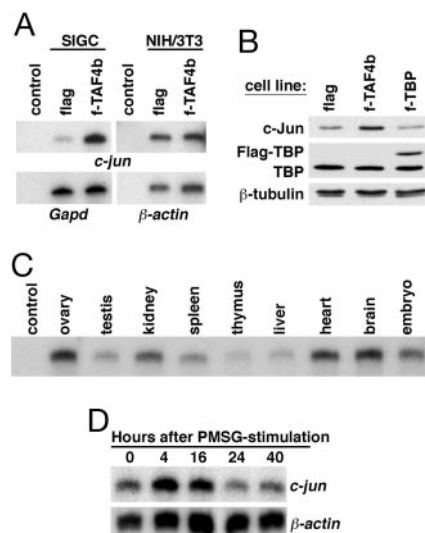


Fig. 3. Activation of gene expression in stable f-TAF4b cells and mouse tissues. (A) RPAs of *c-jun* expression were performed on RNA isolated from the SIGC and NIH/3T3 stable cell lines. *Gapd* and β -actin mRNAs were used as internal controls. Control, yeast RNA. (B) Immunoblotting of total cell extracts from stable control flag, f-TAF4b, and f-TBP SIGCs was performed to determine levels of c-Jun, TBP, and Flag-TBP. Anti- β -tubulin was used to confirm equal loading. (C) RPA of *c-jun* expression in adult mouse tissues and embryos. Control, yeast RNA. (D) Time course demonstrating the rapid induction of *c-jun* expression after PMSG-stimulation in adult mouse ovaries in comparison to β -actin as determined by RPA. Autoradiographic images are shown in A, C, and D.

equivalently low levels of c-Jun, whereas f-TAF4b cells had higher levels, as expected (Fig. 3B). Thus, although NIH/3T3 cells can be stimulated to activate the promoters of ovarian-specific reporter constructs (e.g., *Inhba*) by overexpression of TAF4b, a key ubiquitous activator like c-Jun is induced only in a cell-type-specific manner.

Elevated c-jun mRNA and Rapid Pregnant Mare Serum Gonadotropin (PMSG)-Responsiveness in the Mouse Ovary. We were surprised to find that overexpression of TAF4b resulted in elevated levels of *c-jun* in the SIGCs, because we did not observe detectable changes in the levels of *c-jun* in our initial microarray analysis of TAF4b-null ovaries. In wild-type mice, *c-jun* mRNA levels are most elevated in the ovary, heart, and brain compared with several other mouse tissues as determined by RPA (Fig. 3C). The control 18S rRNA had similar expression levels (data not shown), previously reported for these same tissue samples (7).

To correlate *c-jun* expression levels with folliculogenesis, we stimulated wild-type mice with PMSG, which mimics the *in vivo* activity of the follicle-stimulating hormone (FSH) by activating follicle growth, and measured *c-jun* levels by RPA. As compared with a β -actin control mRNA that was relatively unchanged, the *c-jun* mRNA levels accumulated at 4 and 16 h after PMSG injection to levels between 2- and 4-fold above uninduced levels (Fig. 3D). Our data suggest that, whereas *c-jun* mRNA is expressed at some basal level in many different cell types, it is highly expressed in the ovary, where it specifically responds to the action of gonadotropins. Accordingly, we postulate that c-Jun performs a critical function in the execution of the transcriptional response to gonadotropin signaling and GC proliferation in concert with TAF4b.

TAF4b Knockdown in SIGCs Decreases c-jun Expression. Short interfering (si)RNA-mediated knockdown of TAF4b activity in parental SIGCs was used to determine its effect on *c-jun* expression and GC function. An $\approx 90\%$ or greater reduction in TAF4b protein levels

1). TFIID and c-Jun binding was specific to the promoter region, because no DNA fragments were precipitated from the 3' non-coding region of the gene (Fig. 5B, 3' control). Importantly, we also detected the binding of TAF4b to the core promoter regions of the *Ccnd2*, *Fn1*, *Fst*, *Inhba*, and *Mmp-3* genes but not to the 5' distal AP-1 enhancer region of the *Vim* promoter. In contrast, c-Jun was bound at a 5' distal AP-1 site within the *Vim* promoter and at the core promoter regions of the *Ccnd2*, *Fn1*, and *Mmp-3* genes. TAF4b was readily detected only at the core promoters of *c-jun* and *Ccnd2* and, to a lesser extent, at the *Inhba* and *Fn1* promoters, unlike TAF4b, which was detected at each core promoter tested (Fig. 5B).

Because the ability of Flag-TAF4b to activate expression of *c-jun* was cell-type-specific, we reasoned that Flag-TAF4b may not be recruited to the *c-jun* promoter in NIH/3T3 cells. Indeed, we failed to detect the association of Flag-TAF4b above control levels in NIH/3T3 cells by ChIP assays (Fig. 5C, lane 3). However, as expected, c-Jun was bound to its own promoter in the NIH/3T3 cells (Fig. 5C, lane 2). These results suggest that TAF4b regulates the levels of *c-jun* expression in a cell-type-specific manner by directly associating with the core promoter.

Discussion

TAF4b-Dependent Regulation of Ovarian Gene Expression. Our initial analysis of putative TAF4b-target genes was performed with intact mouse ovaries (7). Identification of TAF4b-dependent genes whose expression is not cell-type-specific but essential for GC function may have been masked by their abundant expression in additional ovarian cells that do not express or require TAF4b for transcription initiation. Herein, we have uncovered additional TAF4b-dependent genes by analyzing the function of TAF4b in a pure population of GCs grown in culture. Importantly, we have identified key ovarian programs of gene expression that are directly depend on the levels of TAF4b. Specifically, we have determined that TAF4b binds to and activates the native promoters of the *Inh*-subunit, *Ccnd2*, and *Fst* genes when constitutively overexpressed in cultured GCs. These results suggest that the loss of TAF4b transcriptional activity in the knockout mice contributes to the down-regulated levels of their expression in TAF4b-null females, which ultimately leads to an inadequate supply of healthy follicles expressing these transcripts and, thus, the observed female fertility defect (7).

In addition to inducing GC-specific gene expression, we show that TAF4b also induced the expression of AP-1 family members and directly regulates the *c-jun* promoter in a cell-type-selective context. Consequently, only a subset of promoters that contain putative AP-1-binding sites or genes known to be responsive to c-Jun stimulation were up-regulated in these cells. Our results strongly support the notion that TAF4b can function as a specific transcriptional coactivator for only a limited subset of genes, some of which are also c-Jun/AP-1 targets, and whose coordinated expression is essential for proper folliculogenesis.

TAF4b Coactivation in the Absence of Ovary-Specific Activators. Surprisingly, we found that TAF4b was able to stimulate selective ovarian promoters in a dose-dependent manner without a complement of GC-specific activators as induction was also observed in NIH/3T3 cells that express elevated levels of TAF4b (Fig. 2C). Our results indicate that the restrictive expression of TAF4b in a cell-type-specific fashion may be sufficient to alter TFIID's activity or specificity for a subset of core promoter elements that is independent of cell-type-specific activators. Consistent with this notion, overexpression of TAF4b in *Taf4*-null embryonic fibroblasts is also capable of inducing transcription, especially that of osteopontin expression (20), which was highly induced in GCs that overexpress TAF4b (Table 1). Because TAF4 does not compensate for the loss of TAF4b in the knockout mice, it is likely that TAF4b may use ubiquitous transcription factors that differ from those used by TAF4.

Remarkably, *Taf4*-null embryonic fibroblasts that possess TAF4b also have increased expression of *Mmp-3* and *Bdnf*, two transcripts that were also induced in SIGCs that overexpress TAF4b (Table 1), indicating that TAF4b may regulate their expression in a non-cell-type-specific manner (20). However, *c-jun* was not induced in *Taf4*-null embryonic fibroblasts that express TAF4b, indicating that *c-jun*'s regulation by TAF4b is strictly cell-type-specific. Thus, in GCs, TAF4b-containing TFIID complexes may be constitutively bound to specific core promoter elements within the *c-jun* promoter to maintain basal levels of *c-jun* expression and to be poised for immediate induction. In this regard, *c-jun* expression in GCs can be simultaneously responsive to a greater spectrum of distinct stimuli by using dedicated TAF4b-TFIID promoter-recognition complexes.

TAF4b Is Essential for Ovarian Folliculogenesis. In response to gonadotropin-induced transcriptional signaling networks, GCs rapidly proliferate and continue to differentiate into more specialized GC subtypes as ovarian follicular development ensues. Importantly, hormonally primed TAF4b-null ovaries fail to activate the expression of a subset of FSH-responsive genes to the same levels as wild-type controls, suggesting an important role for TAF4b in a FSH signaling pathway (7). Therefore, we predict that FSH (an inducer of cAMP production) potentiates the transcriptional activity of TAF4b-containing TFIID complexes at target promoters such as *c-jun*, which can be partially bypassed by directly overexpressing TAF4b in GCs (see Fig. 9, which is published as supporting information on the PNAS web site). Indeed, forskolin/cAMP stimulation (used to mimic the effects of FSH) of a human GC line results in the transient phosphorylation of TAF4b, with the potential to affect its function (21).

In contrast, the loss of TAF4b activity results in the aberrant transcription of critical GC-specific genes that likely impact GC morphology and function. Specifically, intercellular communications and their requisite cellular adhesions are essential linkages for GC-cell and GC-oocyte interactions during normal folliculogenesis (22). Consistent with this necessity, we specifically demonstrate that TAF4b-null ovaries possess vastly disorganized GCs that appear to be deficient in intimate cell-cell attachments, which may contribute to the abnormal development of follicles in the TAF4b-null ovaries (Fig. 4D). Furthermore, knockdown of TAF4b in cultured GCs, which still express normal levels of TAF4, develop abnormal cell morphologies and subsequently display significantly disrupted cell-cell attachments. Similarly, *Taf4*-null embryonic fibroblasts also display irregular and elongated morphologies despite the presence of TAF4b in these cells, suggesting that the paralogous TAF4b and TAF4 proteins share a common nonredundant function in maintaining normal cellular morphologies in distinct cell types (20). Thus, loss of *Taf4b* and TAF4b-dependent target gene expression results in substantial defects in GC transcription, follicle development, and cell morphology in both cultured GCs and *in vivo*.

Taken together, our data suggest that TAF4b is, indeed, a key cell-type-specific transcription coactivator component essential for target gene expression such as that of *c-jun*, as we have determined by both TAF4b overexpression and knockdown in GCs. TAF4b represents a unique class of cell-type-specific transcription factors that, surprisingly, can control *c-jun* expression independent of its well documented regulation as a rapidly induced immediate early gene. The dynamic composition of AP-1 transcription factors during GC proliferation and differentiation likely reflects the necessity to direct specific programs of gene expression unique to each stage of follicle development in response to multiple signaling pathways. Therefore, the specific expression of TAF4b in GCs provides a potentially unique mechanism to initiate a cascade of gene-expression changes by modulating the expression and activity

of specific transcription activators and growth regulatory factors involved in cell proliferation, differentiation, and morphology.

Materials and Methods

Western Blot and IP. Total protein extracts were prepared from cells in log phase of growth that were lysed in 1× SDS-gel-loading buffer. Western blot analysis was performed with anti-Pol II (H-224; Santa Cruz Biotechnology), anti-TAF4, anti-TFIIB (BD Transduction Laboratories), anti-Flag M2 (Sigma), anti-GAPDH (Abcam), anti-TAF1, and anti-TAF4b antibodies. Partial purification of f-TAF4b nuclear extracts by phosphocelulose chromatography was performed as described in ref. 23. IP of TAF4b was performed with anti-TAF4b polyclonal antibodies or a control antibody (mouse preimmune serum), followed by Western blot analysis with anti-TAF4, anti-Flag M2, and anti-TBP (Bioscience Resource Project, Saco, ME) antibodies.

Indirect Immunofluorescence. SIGC stable cell lines were grown on chamber slides (Nalge Nunc International), fixed in 2% paraformaldehyde, and permeabilized with 0.5% Triton X-100. Cells were immunostained with anti-Flag M2 antibody, detected with anti-mouse Alexa Fluor 488 (Molecular Probes) containing Hoechst 33342 (Sigma), and mounted with Vectashield (Vector Laboratories). Cells were observed under a Zeiss Axioplan microscope and imaged with a Leica LEI-750TD digital video camera (Optronics, Goleta, CA).

Luciferase Assays. Stable cell lines were transiently transfected by using Effectene (Qiagen, Valencia, CA). The activities of promoter-luciferase reporters were normalized to the expression of HSV TK-*Renilla* control vector (Promega) for SIGC and SV40-*Renilla* control vector for NIH/3T3. A dual-luciferase reporter assay (Promega) was used to measure luciferase activities from three independent experiments that were performed in triplicate. The ratio of average luciferase values and SDs in the f-TAF4b cells as compared with control flag cells (set at 1) are shown.

RPA. Total RNA was isolated from cells and ovaries by using TRIzol (Invitrogen). RPA probes were synthesized with Maxiscript (Ambion). The RPAIII kit (Ambion) was used to perform RPA. RPA of mouse tissue RNAs is described in ref. 7. Mice were maintained in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. For hormone treatments, 7- to 8-week-old wild-type C57BL/6 female mice (The Jackson Laboratory) were i.p. primed with 5 IU of PMSG (Sigma). Ovariectomy was performed on one control-uninjected female at the start of the time course and subsequently on one PMSG-injected animal at indicated time points.

DNA Microarray Analysis. f-TAF4b and flag SIGC RNAs were used to probe microarray RG-U34A according to standard protocols and manufacturer's instructions (Affymetrix). Details of microarray analysis can be found in *Supporting Materials and Methods* and Tables 2 and 3, which are published as supporting information on the PNAS web site.

Quantitative RT-PCR Analysis. RNAs used for DNA microarray analysis and 48 h after siRNA treatments were digested with DNase I (Ambion) and reverse transcribed with Transcriptor (Roche) or iScript cDNA synthesis kit (Bio-Rad), respectively. Real-time PCR analysis was performed with 1× SYBR green PCR master mix (Bio-Rad) on a DNA Engine Opticon 2 (MJ Research). PCRs were performed three times in triplicate. *Gapd* was used for normalization. Fold-induction is defined as the ratio of relative average values for f-TAF4b cells versus flag control amplifications, or TAF4b siRNA versus nontargeting control siRNA. Primer sequences are available upon request.

TAF4b Knockdown in SIGCs. Parental SIGCs were transfected with rat TAF4b and nontargeting control siRNA SMART pools by using DharmaFECT4 according to manufacturer's suggestions (Dharmacon Research, Lafayette, CO). After 24 h, complexes were removed, and cells were harvested or cultured for an additional 24 h. Cells were lysed in either 1× SDS-gel-loading buffer or TRI reagent (Sigma). Live-cell images were acquired 48 h after siRNA treatment on a Zeiss Axiovert35 microscope equipped with a Zeiss ZVS-3C75DE video camera. Ovary sections from 4-week-old hormonally synchronized *Taf4b*^{+/+} and *Taf4b*^{-/-} mice were prepared as described in ref. 7.

ChIP. ChIP assays were performed essentially as described in ref. 24, but f-TAF4b cells were cross-linked by 1% formaldehyde for 12 min. Specific details of chromatin processing and IP can be found in *Supporting Materials and Methods*.

Plasmids, Cell Lines, and Antibodies. Details of plasmids, antibody production, and cell lines used in this study can be found in *Supporting Materials and Methods*.

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