Podoplanin Is a Novel Fos Target Gene in Skin Carcinogenesis

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Abstract

Expression and function of the oncogenic transcription factor activator protein (AP-1; mainly composed of Jun and Fos proteins) is required for neoplastic transformation of keratinocytes *in vitro* and tumor promotion as well as malignant progression *in vivo*. Here, we describe the identification of 372 differentially expressed genes comparing skin tumor samples of *K5-SOS-F* transgenic mice (*Fos*$^{f/f}$ *SOS*$^{+}$) with samples derived from animals with a specific deletion of *c-Fos* in keratinocytes (*Fos*$^{\Delta ep}$ *SOS*$^{+}$). Fos-dependent transcription of selected genes was confirmed by quantitative real-time PCR analysis using tumor samples and mouse back skin treated with the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA). One of the most differentially expressed genes encodes the small mucin–like glycoprotein Podoplanin (Pdpn), whose expression correlates with malignant progression in mouse tumor model systems and human cancer. We found Pdpn and Fos expression in chemically induced mouse skin tumors, and detailed analysis of the *Pdpn* gene promoter revealed impaired activity in *Fos*-deficient mouse embryonic fibroblasts, which could be restored by ectopic Fos expression. Direct Fos protein binding to the *Pdpn* promoter was shown by chromatin immunoprecipitation and a TPA-induced complex at a TPA-responsive element–like motif in the proximal promoter was identified by electrophoretic mobility shift assays. In summary, we could define a Fos-dependent genetic program in a well-established model of skin tumors. Systematic analysis of these novel target genes will guide us in elucidating the molecular mechanisms of AP-1–regulated pathways that are critically implicated in neoplastic transformation and/or malignant progression. [Cancer Res 2008; 68(17):6877–83]

Introduction

Cancer is a multistage disorder in which genetic and epigenetic changes result in characteristic alterations within the gene regulatory network and, thereby, influence the cellular decision of differentiation, proliferation, or survival (1). Regulation of gene transcription is a process that is primarily under the influence of nuclear-located transcription factors that exhibit tightly controlled DNA-binding as well as physical and functional interactions with transcriptional coregulators. Consequently, identifying transcription factors that activate or repress specific target genes is a prerequisite for understanding cell fate and function during neoplastic transformation.

Much of our current knowledge about the characteristics of transcription factors comes from the discovery and study of activator protein 1 (AP-1; ref. 2). AP-1 describes an activity that controls both basal and inducible transcription of target genes sharing AP-1 binding sites, also known as 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive element (TRE), within their genomic regulatory regions (3). AP-1 is mainly composed of Jun and Fos proteins that form homodimers (Jun-Jun) and heterodimers (Jun-Fos) and mediates gene transcription in response to a plethora of stimuli (4). Studies in genetically modified mice and cells derived thereof have highlighted a crucial role for AP-1 in a variety of cellular events implicated in normal development as well as in pathologic conditions, including cancer. As an example, expression of a dominant-negative Jun mutant (TAM67) in basal keratinocytes protects transgenic mice from UV- and chemically induced as well as human papillomavirus 16–driven skin tumor formation (5, 6). Additionally, mice harboring a mutant *Jun* allele that has the Jun-NH_2-kinase phospho-acceptor serines changed to alanines as well as mice with an epidermal-specific *Jun* knockout (*Jun*$^{\Delta ep}$) exhibit impaired skin tumor development in the *K5-SOS-F* transgenic tumor model (4). The important role of AP-1 for malignant transformation of keratinocytes is further supported by the fact that *Fos*-deficient mice fail to undergo malignant progression of skin tumors in a transgenic model of oncogenic Ras (7). Finally, the conditional expression of A-Fos, a dominant negative mutant that inhibits AP-1 DNA-binding, in epidermal keratinocytes of transgenic mice interferes with the development of characteristic benign or malignant squamous cell lesions during chemically induced skin carcinogenesis (8).

In this study, we used the tumor-prone *K5-SOS-F* transgenic mouse model to screen systematically for novel Fos-regulated genes performing global gene expression analysis with samples derived from skin tumors with a floxed *Fos* allele (*Fos*$^{f/f}$) or with an epidermis-specific *Fos*-deletion (*Fos*$^{\Delta ep}$). We could identify a comprehensive list of differentially expressed genes and confirmed Fos-dependent expression of selected genes in TPA-treated back skin. In line with published data that Fos induces epithelial-mesenchymal transition *in vitro* and is critical for malignant progression *in vivo* (7, 9), numerous differentially expressed genes were functionally associated with cellular movement and cell morphology. We found that the mucin-like glycoprotein Podoplanin (Pdpn), one of the highest differentially expressed genes, is a novel direct Fos target gene. Previously, Pdpn was found significantly up-regulated in advanced stages of the two-step skin carcinogenesis (10) as well as in human cancer, including squamous cell carcinomas of the skin (11, 12). Additionally, ectopic

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Pdpn expression accelerates cell motility and invasion *in vitro*, is sufficient to induce tumor growth in a xenograft model, and induces tumor cell invasion and metastasis *in vivo* (12, 13). In summary, this suggests a critical role for Pdpn in the Fos-dependent program of tumor cell invasion and malignant progression.

Materials and Methods

Animals. Mice carrying a floxed *Fos* allele ($Fos^{f/f}$; ref. 14) were bred to transgenic mice expressing a constitutively active form of hSOS (SOS-F) under the control of the keratinocyte-specific *Keratin 5* promoter (*K5-SOS-F*; ref. 15). To obtain $Fos^{\Delta ep}$ SOS^{+} mice, we crossed $Fos^{f/f}$ SOS^{+} with the *K5–cre2* line (16). Chemically induced skin carcinogenesis and TPA treatment of $Fos^{-/-}$ mice and *wt* controls were described elsewhere (17). Animals were housed in specific pathogen–free and in light, temperature, and humidity controlled conditions. Food and water were available *ad libitum*. The procedures for animal experiments were in accordance with the principles and guidelines of the ATBW (authority for animal welfare) and were approved by the Regierungspräsidium Karlsruhe, Germany (AZ. 129/02) and Austrian authorities.

Cell culture. Mouse embryonic fibroblasts (MEF) were established from *wt*, $Fos^{-/-}$, $Jun^{-/-}$, and $JunB^{-/-}$ embryos as described previously (18, 19). Serum-starved cells were treated with 100 ng/mL TPA dissolved in acetone for the indicated time points. Immortalized mouse keratinocytes (IMK) were established as described elsewhere (20).

Tissue preparation and immunohistochemistry analysis. For immunohistochemistry (IHC) staining, skin and tumor samples were fixed in 4% w/v paraformaldehyde [PFA in PBS (pH 7.2)], embedded in paraffin, and subsequently cut in 6-μm sections. IHC stainings were done with the Immunodetection kit (Vector Laboratories) according to manufacturer's instructions. Primary and secondary antibodies used in this manuscript are listed in Supplementary Table S2.

Chromatin immunoprecipitation analysis. For chromatin immunoprecipitation (ChIP) analysis, the ChIP assay kit (Upstate Biotechnology) was used following the manufacturer's instructions. The ChIP experiment was performed with IMKs treated for 1 or 2 h with 100 ng/mL of TPA or for 2 h with acetone as control. Cross-linked Fos protein-DNA complexes were immunoprecipitated with a Fos-specific antibody (Supplementary Table S2). PCR amplification of the immunoprecipitated samples was performed using primers located within the proximal *Pdpn* promoter (Supplementary Table S3). Immunoprecipitates in the absence of antibody and a portion of the sonicated chromatin before immunoprecipitation were used as controls. A PCR using a primer-pair specific for a *β-Tubulin* coding region (Supplementary Table S3) served as an additional control.

Transfection and reporter gene assay. The mouse proximal *Pdpn* promoter was amplified by PCR (see Supplementary Table S3 for primer sequences) and cloned in the pGEM-T easy plasmid (Promega). A 327-bp fragment was isolated by *Hind*III and *Nco*I digestion and cloned into the pGL3-basic vector (Promega) to generate the Pdpn-luci(−215/+113) reporter plasmid. The Pdpn-luci(−860/+113) reporter plasmid was generated by insertion of a 625-bp fragment (*Xho*I/*Hin*dII) of the pGEM-T easy clone into the Pdpn-luci(−215/+113) reporter plasmid. Both constructs were approved by DNA sequencing. MEFs were transiently transfected with reporter gene plasmids using FuGENE HD Transfection Reagent according to the manufacturer's instructions (Roche). A *Renilla* luciferase reporter gene plasmid was cotransfected as an internal control for transfection efficiency (Promega). Cells were harvested 48 h after transfection, and the measurement was performed using the Dual Luciferase Assay System (Promega). To calculate fold inductions, the relative light units of mock-transfected cells were set to 1. All values are means of at least three independent experiments and error bars represent SE. The TRE-luci reporter plasmid and the Fos expression plasmid were described previously (21).

Nuclear extracts, EMSA, and Western blot analysis. Isolation of nuclear extracts, electrophoretic mobility shift assay (EMSA), and Western Blot analysis were performed as described previously (22). Oligonucleotides for the TRE-Coll probe were described by Porte and colleagues (23), oligonucleotides of the Pdpn promoter are listed in Supplementary Table S3, and oligonucleotides for the Oct probe were kindly provided by T. Wirth (University of Ulm, Ulm, Germany). Antibodies for Western Blot are listed in Supplementary Table S2.

Microarrays, sample preparation and hybridization, quantitative real-time PCR analysis, and data processing. See Supplementary Materials and Methods.

Results and Discussion

Identification of Fos-regulated genes in the *K5-SOS-F* tumor model. We crossbred tumor-prone *K5-SOS-F* mice with genetically modified animals containing either floxed *c-Fos* alleles ($Fos^{f/f}$ SOS^{+}) or an epidermis specific *c-Fos* deletion ($Fos^{\Delta ep}$ SOS^{+}). As expected, $Fos^{f/f}$ SOS^{+} mice developed highly disorganized papillomatous lesions within 4 weeks after birth. In the absence of Fos, tumor volume was significantly reduced, supporting the crucial role of Fos expression and activity for neoplastic transformation of epidermal keratinocytes.[4] To identify tumor-associated genes whose expression critically depends on the presence of Fos function, we isolated total RNA from tumor samples of three independent $Fos^{f/f}$ SOS^{+} and $Fos^{\Delta ep}$ SOS^{+} mice and performed global gene expression analysis. We found 372 differentially expressed and annotated genes[5] of which 277 were up-regulated and 95 were down-regulated in $Fos^{f/f}$ SOS^{+} tumors compared with samples with *Fos* ablation (Supplementary Table S1). Numerous differentially expressed genes of the list were previously identified as tumor-associated genes in the two-step skin carcinogenesis model (24–26). Gene clustering according to their functional annotation showed that differentially expressed genes are implicated in distinct tumorigenic features, such as cellular movement and morphology, cell cycle control and proliferation, cell death, cell signaling, and interaction (Fig. 1).

We selected several up-regulated genes (*Fetub, Klk12, Mmp10, Pdpn*, and *Ptgs2*) and one down-regulated gene (*S100a3*) for quantitative real-time PCR (RQ-PCR) analysis and could confirm altered transcription in $Fos^{f/f}$ SOS^{+} compared with $Fos^{\Delta ep}$ SOS^{+} samples (Fig. 2*A*). Most of the analyzed genes also exhibited TPA-induced transcription in mouse back skin, which was impaired in *Fos*-deficient animals. Additionally, costimulation with dexamethasone, a well-known inhibitor of Fos-dependent transcription, repressed TPA-induced *Klk12* and *Pdpn* expression (Fig. 2*B*).

Efforts to highlight the role of AP-1 family members in neoplastic transformation often focus on enhanced gene transcription; however, recent experimental data also suggest a contribution of transcriptional repression in Fos-mediated cellular transformation (27). We found several candidate genes with significantly higher transcript levels in the absence of Fos protein, and as an example, we analyzed Fos-dependent regulation of *S100a3* transcription. In *wt* mice, *S100a3* transcript levels were dramatically reduced in TPA-treated compared with acetone-treated skin (33-fold; Fig. 2*B*). Although basal *S100a3* transcription was slightly reduced in $Fos^{-/-}$ compared with *wt* skin (2-fold), we found no further down-regulation after TPA treatment, suggesting that Fos function is essential for the repression of *S100a3* transcription.

Fos-dependent Pdpn expression in skin tumors. One gene that was highly reduced in $Fos^{\Delta ep}$ SOS^{+} compared with $Fos^{f/f}$ SOS^{+}

[4] J. Guinea-Viniegra and E.F. Wagner, unpublished data.
[5] http://www.ncbi.nlm.nih.gov/geo/

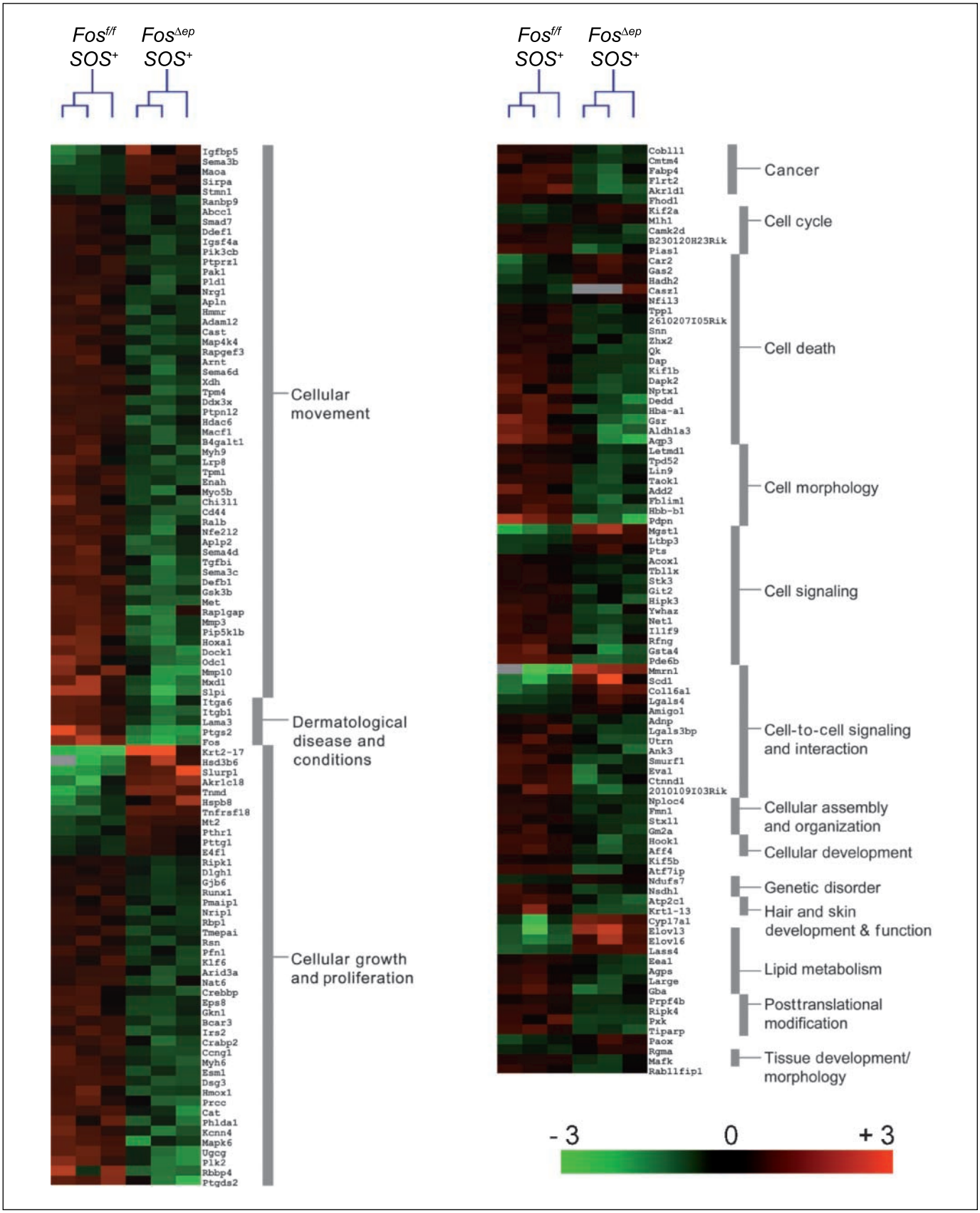

Figure 1. Heat-map for differentially expressed genes. Differentially expressed and annotated genes derived from the global gene expression analysis with cDNA from *Fos*$^{f/f}$ *SOS*$^{+}$ versus *Fos*$^{\Delta ep}$ *SOS*$^{+}$ tumors were clustered according to their functional annotation. Indicated values reflect the log ratio of gene expression between individual sample and average of all samples. Only genes with functional assignment according to Ingenuity Systems are shown.

tumors and whose TPA-induced transcription in mouse skin critically depends on Fos (Fig. 2) encodes the mucin-like glycoprotein Pdpn. To confirm Fos-dependent Pdpn expression in epidermal keratinocytes, we performed IHC on tissue sections derived from $Fos^{f/f}$ SOS^{+} and $Fos^{\Delta ep}$ SOS^{+} tumors. Whereas $Fos^{f/f}$ SOS^{+} tumors revealed strong staining for Pdpn protein in tumor cells adjacent to the stromal compartment, no or only minor staining was present in $Fos^{\Delta ep}$ SOS^{+} tumors (Fig. 3*A*). Additionally, we found Pdpn and Fos-positive tumor cells in the same area of consecutive sections derived from the two-step skin carcinogenesis model (Fig. 3*B*). Our data are in line with previous publications and support the hypothesis that Pdpn expression may be modulated by the tumor environment and is induced in epithelial cells by growth factors and cytokines secreted from cells within the tumor stroma (12).

***Pdpn* is a direct Fos target gene.** IHC analysis revealed enhanced Pdpn protein expression in epidermal keratinocytes as well as dermal fibroblasts of TPA-treated skin (Supplementary Fig. S1). To confirm TPA-induced *Pdpn* transcription in both cell types, we generated IMKs and used established MEFs (Fig. 3*C*). In contrast to IMKs and control MEFs, no up-regulation of *Pdpn* transcripts was detected in cells derived from *Fos*-deficient embryos. To investigate whether Fos directly binds to the *Pdpn* promoter, MEF and IMK cells were treated with TPA to induce endogenous Fos protein expression followed by ChIP with a Fos-specific antibody. PCR analysis using primers that amplify the proximal *Pdpn* promoter revealed direct Fos DNA binding 2 hours after TPA stimulation of IMK cells but not in control-treated cells or after 1 h of treatment (Fig. 4*A*; Supplementary Fig. S2). Enrichment of *Pdpn* promoter sequences in ChIP experiments with TPA-treated IMK cells could be confirmed by RQ-PCR analysis and was also detected in *wt* MEFs upon TPA stimulation but not in $Fos^{-/-}$ MEFs (data not shown).

Several TRE-like motifs were present in the proximal *Pdpn* promoter and downstream of the transcription start site. Therefore, we cloned two *Firefly*-luciferase reporter gene plasmids, Pdpn-luci(−215/+113) and Pdpn-luci(−860/+113), to analyze *Pdpn* promoter activity in *wt* and $Fos^{-/-}$ MEFs. Transient transfection experiments revealed basal activity of both promoter constructs in *wt* MEFs that was significantly enhanced by TPA treatment (Fig. 4*B*; data not shown). In line with Fos-dependent *Pdpn* transcription *in vitro* and *in vivo*, we found that both basal and induced promoter activity was impaired in the absence of Fos (Fig. 4*B*; data not shown). Because similar data were obtained with the Pdpn-luci(−860/+113) and the Pdpn-luci(−215/+113) reporter plasmid, the latter was used to confirm induced *Pdpn* transcription upon ectopic Fos expression. Cotransfection with Pdpn-luci(−215/+113) reporter and a Fos expression plasmid revealed a 3.6-fold induction in *wt* and a 2.6-fold induction in $Fos^{-/-}$ MEFs (Fig. 4*C*), demonstrating that ectopic Fos expression is sufficient to induce *Pdpn* transcription.

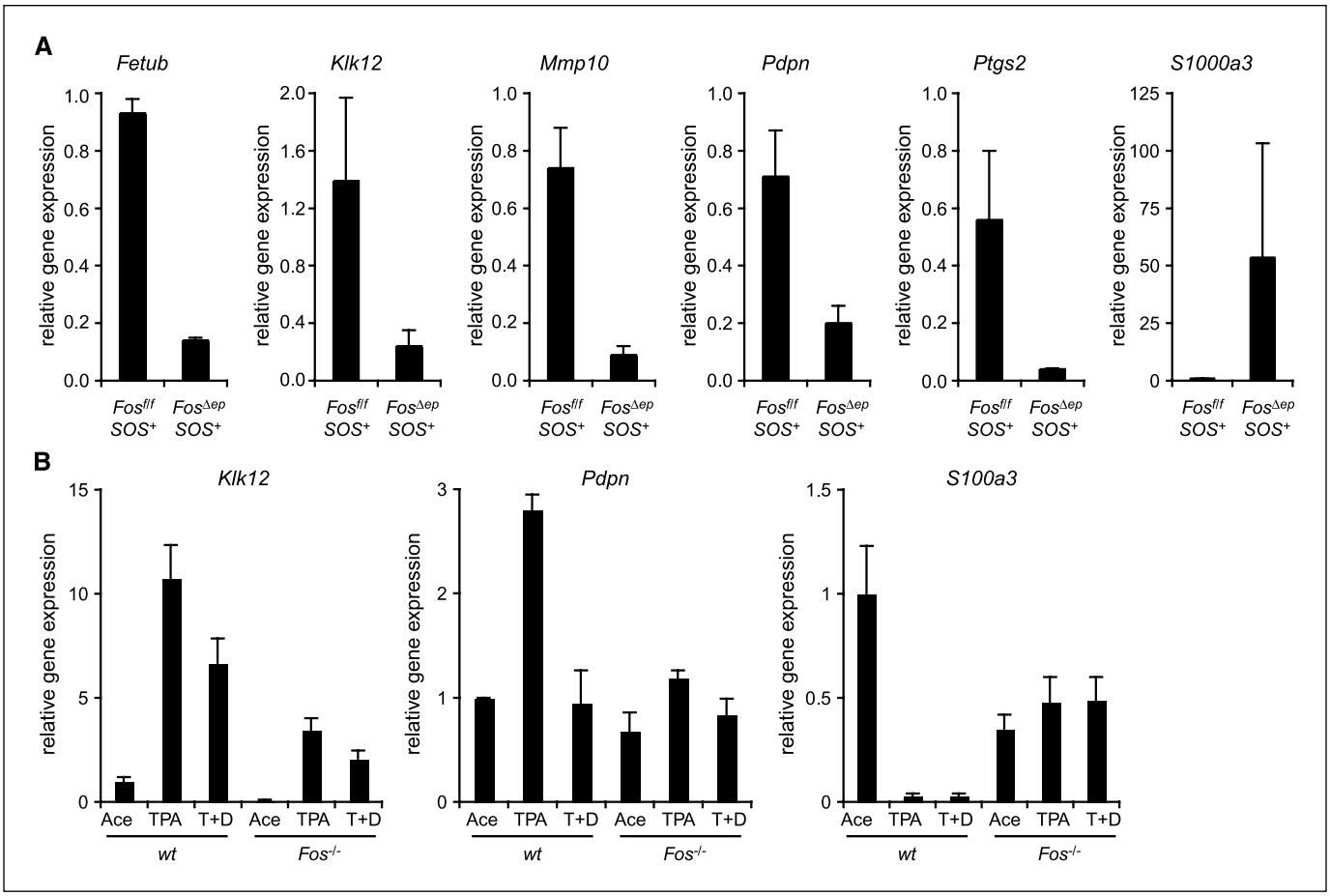

Figure 2. Fos-dependent transcription of candidate genes. RQ-PCR analysis was used (*A*) to confirm different gene transcription levels between $Fos^{f/f}$ SOS^{+} and $Fos^{\Delta ep}$ SOS^{+} tumors and (*B*) to quantify transcript levels of selected genes in back skin of *wt* and $Fos^{-/-}$ mice upon TPA treatment (*TPA*) without or with dexamethasone cotreatment (*T+D*).

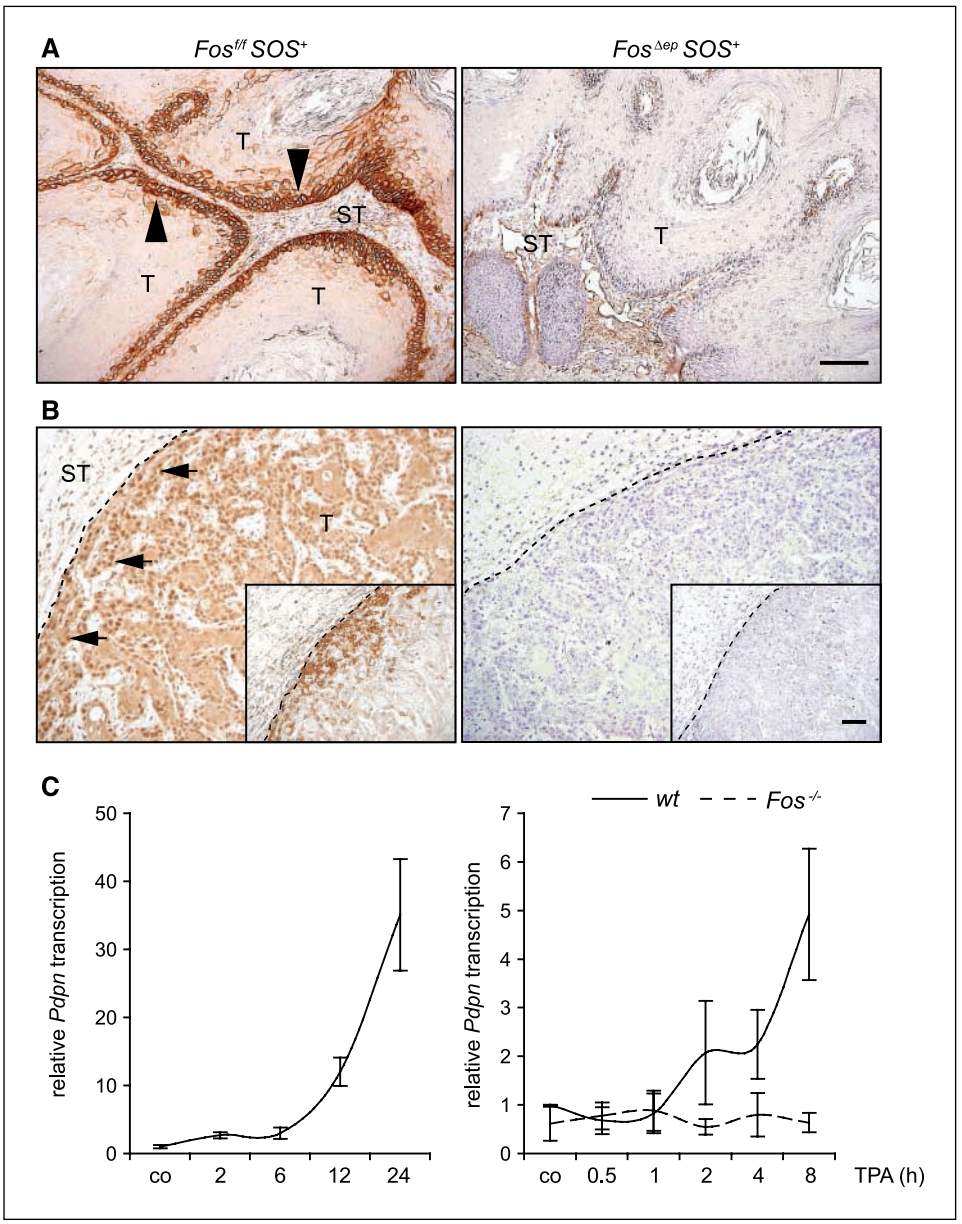

Figure 3. Fos-dependent Pdpn protein expression in mouse skin tumors. IHC analysis of tumor sections derived from (*A*) $Fos^{f/f}$ SOS^{+} and $Fos^{\Delta ep}$ SOS^{+} mice or (*B*) 7,12-dimethylbenz(*a*)anthracene/TPA-induced skin tumors of mouse back skin. Sections were counterstained with hematoxylin. *Scale bars,* 100 μm. *A,* specific staining for Pdpn protein (*brown signal*) was detected in tumor cells (*T, arrowheads*) adjacent to the stromal compartment (*ST*). *B,* strong nuclear staining of Fos protein (*brown signal, arrows*) was detected in tumor cells adjacent to the stromal compartment, which overlaps with Pdpn expression (*inset, left*). *Right,* antibody controls for Fos and Pdpn protein staining. *Dashed lines,* the border between tumor and stroma. *C,* TPA-induced *Pdpn* transcription was analyzed by RQ-PCR with cDNA derived from *wt* IMKs (*left*) or *wt* and $Fos^{-/-}$ MEFs (*right*).

To identify the TRE-like motif that is required for Fos binding to the *Pdpn* promoter, we performed EMSA experiments with distinct radioactive-labeled oligonucleotides covering the critical genomic region. An inducible bandshift was detected with nuclear extracts of TPA-treated MEFs and IMKs (Fig. 4*D*; Supplementary Fig. S3), and the respective oligonucleotide (PdpnIV) shares a TRE-like binding motif. Indeed, we found a complete loss of TPA-induced complex formation upon introduction of specific mutations (PdpnIV-mut1 and PdpnIV-mut2) as well as impaired binding by competition with nonlabeled oligonucleotides with a conserved TRE motif (Supplementary Fig. S3). In line with our ChIP assay, EMSA analysis showed enhanced complex formation at the PdpnIV oligonucleotide with nuclear extracts from HeLa cells that were transiently transfected with a Fos-expression plasmid compared with mock controls (Supplementary Fig. S3). Finally, we addressed the question whether Jun proteins are required in TPA-induced *Pdpn* transcription. RQ-PCR analysis with control- and TPA-treated MEFs revealed impaired *Pdpn* transcript levels in $Jun^{-/-}$ cells. Furthermore, *JunB*-deficient MEFs showed reduced basal and lack of TPA-induced *Pdpn* expression (Supplementary Fig. S2), suggesting a regulation by functional Jun-Fos heterodimers.

In summary, global gene expression analysis and the systematic analysis of a Fos-dependent genetic pattern using a well-established mouse model of skin carcinogenesis revealed a comprehensive list of well-known Fos/AP-1 target genes but also novel candidates. Further analysis of these genes concerning their promoter topology and biological function will certainly contribute to a better understanding of AP-1 mediated gene regulation and molecular principles of Fos-driven cellular transformation. Accordingly, we identified *Pdpn* as a novel direct Fos target gene in both fibroblasts and keratinocytes. Pdpn and Fos proteins are critically implicated in epithelial cell migration and invasion *in vitro* and closely correlated with malignant progression *in vivo*. Moreover, investigating cell lines with inducible Fos expression and a Fos-transgenic mouse model that develops bone tumors (28),

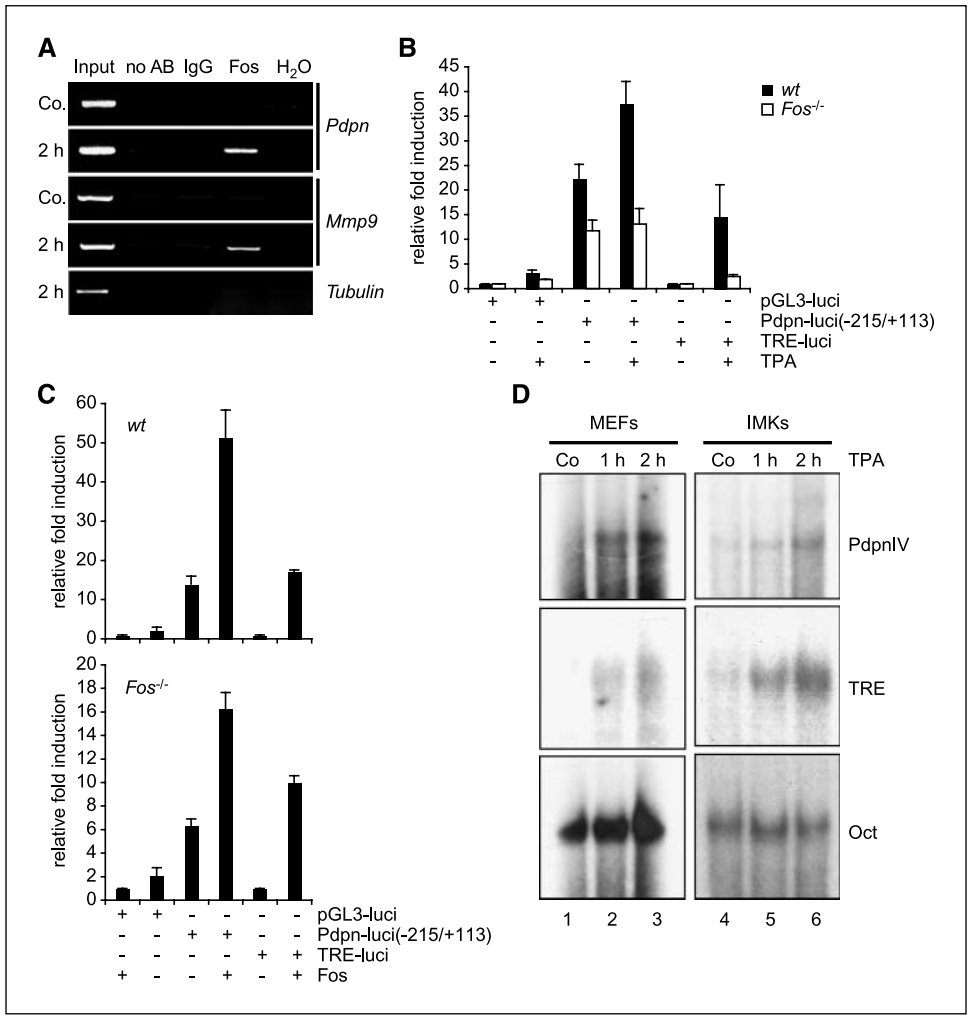

Figure 4. Pdpn is a direct Fos target gene. *A,* ChIP experiments revealed Fos binding to the *Pdpn* promoter in IMK cells 2 h post-TPA treatment but not in control-treated cells (*Co*). A PCR reaction with primers specific for the *Mmp9* promoter served as positive control and primers specific for genomic β-*Tubulin* DNA served as control for specificity. *B,* transient transfection of *wt* (*black bars*) and *Fos*$^{-/-}$ MEFs (*white bars*) with a control plasmid (*pGL3-luci*) or a *Firefly* luciferase reporter plasmid with the proximal *Pdpn* promoter [*Pdpn-luci(−215/+113)*]. *Firefly* luciferase activity was measured from transfected cells that were acetone (*control*)- or TPA-treated. A TRE-luci reporter plasmid served as control for TPA-induced Fos/AP-1 activity. *C, wt* (*top*) and *Fos*$^{-/-}$ MEFs (*bottom*) were transfected with *Firefly* luciferase reporter plasmids as described in *B*. In addition, cells were cotransfected with mock or Fos expression plasmids. *D,* EMSA with nuclear extracts of acetone- and TPA-treated MEFs (*lane 1–3*) and IMKs (*lane 4–6*) revealed induced DNA-binding activity at the PdpnIV oligonucleotide sharing a TRE-like motif (*top*). An oligonucleotide with a conserved TRE motif (*middle*) served as a positive control for induced AP-1 DNA-binding, and bandshifts with an Oct oligonucleotide served as control for quality and quantity of nuclear extracts.

recent data[6] described Fos-regulated *Pdpn* expression in osteoblasts and in osteosarcomas, which further supported our findings and suggested a more common function of the Fos-Pdpn axis during cancer development of epithelial and bone tissues. Thus, it will be a major challenge for the future to elucidate the role of the Pdpn protein in processes of Fos-dependent neoplastic transformation and malignant progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

[6] A. Kunita (University of Tokyo), personal communication.

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