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Identification of DPPA4 and DPPA2 as a novel family of pluripotency-related oncogenes

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Abstract

In order to identify novel pluripotency-related oncogenes, an expression screen for oncogenic foci-inducing genes within a retroviral human embryonic stem cell (hESC) cDNA library was conducted. From this screen, we identified not only known oncogenes but also intriguingly the key pluripotency factor, DPPA4 (developmental pluripotency-associated 4) that encodes a DNA binding SAP domain-containing protein. DPPA4 has not been previously identified as an oncogene, but is highly expressed in embryonal carcinomas, pluripotent germ cell tumors, and other cancers. DPPA4 is also mutated in some cancers. In direct transformation assays, we validated that DPPA4 is an oncogene in both mouse 3T3 cells and immortalized human dermal fibroblasts (HDFs). Overexpression of DPPA4 generates oncogenic foci (sarcoma cells) and causes anchorage-independent growth. The *in vitro* transformed cells also give rise to tumors in immuno-deficient mice. Furthermore, functional analyses indicate that both the DNA-binding SAP domain and the histone-binding C-terminal domain are critical for the oncogenic transformation activity of DPPA4. Down-regulation of DPPA4 in E14 mouse embryonic stem cells (mESCs) and P19 mouse embryonic carcinoma cells (mECCs) causes decreased cell proliferation in each case. In addition, DPPA4 overexpression induces cell proliferation through genes related to regulation of G1/S transition. Interestingly, we observed similar findings for family member DPPA2. Thus, we have identified a new family of pluripotency-related oncogenes consisting of DPPA2 and DPPA4. Our findings have important implications for stem cell biology and tumorigenesis.

Introduction

Embryonic stem cells (ESCs) are derived from the inner cell mass of mammalian blastocysts. Both human ESCs (hESCs) and mouse ESCs (mESCs) possess unlimited capacity for self-renewal and pluripotency[1]. These two unique features make hESCs one of the most promising resources for future regenerative medicine therapies[2]. Induced

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pluripotent stem cells (iPSCs) also have these two key properties, and have the additional unique potential for patient-specific therapies that would reduce possible immunogenicity issues. Over the past decade, the feasibility of stem cell-based therapeutic strategies has been validated *in vitro* and *in vivo*[3]. One of the most troubling roadblocks to pluripotent stem cell-based therapies is that both hESCs and iPSCs possess innate tumorigenicity[4]. Both hESCs and iPSCs are readily able to form teratoma[5], a usually benign tumor composed of tissues from all three germ layers. However, the underlying mechanisms that result in tumor formation from the pluripotent cells remain largely unknown.

The surprisingly close relationship between cancer and stem cells has been noted for years[6], a concept further reinforced by the realization that cancer stem cells (CSCs, also known as "tumor initiating cells") have important roles in not only hematopoietic cancers, but also many solid tumors[7, 8]. Within any given tumor, CSCs possess the unique ability to induce tumor recurrence[9]. Therefore, identification and therapeutic targeting of these CSCs through a better understanding of their molecular machinery, may lead to better clinical treatments[10].

The fact that the initial studies and understanding of ESCs were mainly founded on the earlier studies of embryonic carcinoma cells (ECCs) suggested that ESCs and ECCs may be related cell types[5], with both potentially related to CSCs. Further, it implied that ESCs may express pluripotency genes which are also oncogenes, a notion further supported by the fact that all genetic changes used to produce iPSCs are in addition either outright oncogenic or somehow related to cancer[11, 12]. For example, many of the factors used for iPSCs induction, such as Myc, KLF4, and SV Large T antigen, are oncogenes, while loss of p53 function is a common event in cancer[13] and stimulates iPSC formation. A transcriptome analysis of iPSCs and transformed cancer cells generated from the same parental MEFs also supports the idea that induced pluripotency and oncogenic transformation are similar processes on some levels[14]. In addition, many core pluripotency factors such as Nanog are highly expressed in cancers[15], but their oncogenic functions, if any, remain undefined.

To further test the notion that pluripotency-related genes may have key roles in tumorigenesis and to search for stem cell-related oncogenes, here we performed an expression screen of a novel retroviral hESC (H9) cDNA library. We transduced 3T3 cells with the hESC cDNA library and found robust oncogenic foci[16] formation by the library, suggesting the presence of an abundance of proto-oncogenes that are normally expressed in hESCs. In addition to several known oncogenes, one oncogenic factor that we identified was the core pluripotency factor, DPPA4[17]. While DPPA4 expression has been previously found to cluster with that of OCT4 in hESCs and pluripotent germ cell tumors[18], it has not been previously identified as an oncogene. Here we describe the validation of DPPA4 and its family member, DPPA2, as novel pluripotency-related oncogenes. Furthermore, our results from oncogenic focus formation and soft agar assays indicate that both the DNA binding SAP domain and the histone binding region of DPPA4 are important for its function as an oncogene. In addition, both DPPA4 and DPPA2 can induce the expression of several cyclins and G1/S transition genes, which can further lead to increased cell proliferation rates and elevated S phase cell numbers. Decreased cell proliferation in both E14 mESCs and P19 mECCs upon knockdown of DPPA4 further supports the idea that DPPA4 plays a role in cell cycle regulation in pluripotent cells. Our study therefore reports a new family of pluripotency-related oncogenes that can lead to oncogenic transformation by induction of several known cancer-related signaling pathways.

Materials and methods

(Also see supplemental methods section)

Generation of h9 hESC retroviral library

We generated the H9 hESC retroviral library using SMART cDNA Library construction kit (Clontech) following manufacturer's protocol. cDNA was synthesized from 1 μg/μl of total RNA isolated from H9 hESCs depleted from MEFs. dsDNA was generated by long-distance PCR (LD-PCR), and then ligated into pRetro-LIB retroviral plasmid. The obtained amplified library had a titer of 6 million cfu/ml = 18 million independent clones with average insert size of 400–2500 bp.

Focus formation assay with the h9 hESC retroviral library

Recombinant retroviruses were produced by transfection of the H9 hESC cDNA plasmid library into the packaging cell line PlatE and were used to transduce 3T3 cells in the presence of 6 μg/ml polybrene. After culturing for three weeks, oncogenic transformed foci were isolated, expanded, and subjected to genomic DNA extraction. The cDNA inserts were amplified by PCR using the primers originally used to generate the cDNA library and sequenced. Sequencing primers locations are as follows: 5' primer (1444–1463): 5'-AGCCCTCACTCCTTCTCTAG-3' and 3' primer (1785–1760): 5'-ACCTACAGGTGGGGTCTTTCATTCCC-3').

Expression microarrays and Quantitative RT-polymerase chain reaction

RNAs isolated from transformed 3T3s were submitted to the UC Davis Genome Center Expression Analysis Core for hybridization of MouseGW-6 beadchip microarrays (Illumina;v2). The microarray data were normalized by quantile normalization and analyzed using GenomeStudio (Illumina; v2010.1). Genes with expression changed by at least 1.5 fold and a p value <0.05 were considered differentially expressed. The gene lists were further analyzed by DAVID V6.7 Functional Annotation Bioinformatics Microarray Analysis software (http://david.abcc.ncifcrf.gov/).

Chromatin Immunoprecipitation (ChIP) and ChIP-qPCR

Chromatin samples were prepared as described[19]. 3 μg of each following antibody was added to 300–400 μg chromatin samples: anti-mDPPA4 aa125–296 (R&D System), anti-DPPA2 (ab91318, Abcam), or Goat IgG or Rabbit IgG. SYBR green-based qPCR was carried out in triplicates using Absolute Blue QPCR Master Mix (Thermo) using the following primers: *mTrp53* forward 5'-CCGTGTTGGTTCATCCCTGTA-3', reverse 5'-TTTTGGATTTTTAAGACAGAGTCTTTGTA-3'; *mCdkn1a* forward 5'-GCCTGGGCACGTCCTAGA-3', reverse 5'-CAGTTGTGGCGCGATTCTG-3'.

RNA interference

293FT cells were transfected with the pLKO.1 lentiviral constructs containing the shRNAs against mouse DPPA4 (Sigma Aldrich, St. Luis, MO) along with the packaging plasmids (pMD.G and Delta 8.9), XtremeHD DNA transfection reagent (Roche). Empty vector and scramble shRNA were used as controls. E14 mESCs and P19 mECCs were infected with the viral medium collected 48 hours after transfection in the presence of 6 μg/ml of polybrene. Transduced cells were selected with 1 μg/ml puromycin.

Results

Identification of novel pluripotency-related oncogenes by hESC cDNA library expression screening

In order to identify novel pluripotency-related oncogenes, we conducted an expression screen by producing an H9 hESC retroviral cDNA library and using it to transduce mouse fibroblast 3T3 cells (Figure 1A). The readout for the expression screen was oncogenic focus

formation. A total of 10^7 3T3 cells were transduced with virus encoding the library. After culturing for 3 weeks, hundreds of foci were apparent in the library-transduced 3T3 cells. No oncogenic focus formation was observed in the negative control cells transduced with the empty retrovirus (pRetroLIB), whereas hundreds of foci were generated in the positive control cells co-transduced with k-Ras and c-MycT58A (stabilized mutated form,[20], Figure 2A).

We selected 116 distinct oncogenic foci from the library plates for viral cDNA sequence identification. These foci were independently isolated and expanded to obtain genomic DNA. The cDNA inserts were amplified by PCR and sequenced. Two to five DNA inserts were recovered from each focus genome, indicating multiple gene insertions per cell and an effective multiplicity of infection of approximately 2–5. A total of 71 genes were identified from the foci. This pool of putative pluripotency-related oncogenes were analyzed by String 8.3[21] to determine possible protein-protein interaction networks suggested by published studies (Figure 1B). The functional protein association network mapping indicates that translation/ribosome protein complex is a main category of the potential oncogenes from the screen. Besides known oncogenes (*PIK3R1*[22] and *CDC42*[23]), it was notable that the pluripotency-related gene, DPPA4, was identified as a putative novel oncogene from the screen.

Validating the oncogenic potential of the novel putative oncogenes

Given the fact that multiple insertions were observed in each oncogenic foci, we performed individual validation studies of the oncogenic potential of 6 selected putative oncogenes from the screen (Sup Table. 1) by transducing cells with each separate gene: *LDHB, RPL41, RPL35A, RPS27A, PAFAH1B1, and DPPA4*. After 3 weeks of incubation, oncogenic foci formed in the cells separately transduced with DPPA4, LDHB, RPS27A, and RPL41, but not in the cells with RPL35A or PAFAH1B1 transduction (Sup Figure 1 and data not shown), validating the oncogenic transformation potential of DPPA4, LDHB, RPS27A, and RPL41. Cells transduced with both c-MycTA and k-Ras were included as a positive control.

Given its association with pluripotency, we decided to focus on DPPA4. To further characterize the oncogenic potential of DPPA4 in 3T3 cells, we conducted both *in vitro* soft agar anchorage-independent growth assays[24] as well as *in vivo* tumor formation assay in immunodeficient mice[25] using cells overexpressing DPPA4. We found that the cells transduced with either human or mouse DPPA4 formed multiple colonies in soft agar after 3 weeks of incubation, while none were observed in the cells transduced with the empty vector (pRetroLIB, Figure 2A top panels). Similarly, tumor formation in immunodeficient mice was readily observed for the DPPA4-transduced cells 4 weeks after subcutaneous injections, but not the empty vector-transduced cells (Figure 2A middle panels). Tumors formed at all injections sites in the mice injected with the mDPPA4 or hDPPA4-tramsformed cells and with the positive control cells (Figure 2A bottom panels). Masses were eventually observed in the mice injected with the negative control cells, but were much smaller and only evident at a minimum of 7 weeks post-injection. H&E stain of the tumor slides from DPPA4 tumors suggests that they were sarcomas as expected given the fibroblast 3T3 cells of origin utilized in the studies (data not shown). Taken together, these results demonstrate that DPPA4 can act as a single hit oncogene in 3T3 cells.

Examining the transformation activity of DPPA4 in human cells and cancers

To test whether DPPA4 can cause oncogenic transformation in human cells as well, human *DPPA4* (hDPPA4) was retrovirally transduced into hTERT immortalized human dermal fibroblasts (HDFs). The HDFs were previously made susceptible to ecotropic viral transduction by introduction of mouse solute carrier family 7 (cationic amino acid

transporter, y+ system) member 1 (*Slc7a1*) gene encoding the ecotropic retrovirus receptor, which enables ecotropic retrovirus transduction[26]. In anchorage-independent soft agar assays, significantly higher numbers of colonies formed from the hDPPA4-transduced cells compared to the control cells (p=3.9E-6, Figure 2B and 2C), indicating that hDPPA4 can induce oncogenic transformation in human cells. Importantly, the existing cDNA microarray data sets deposited in the *Oncomine* (www.oncomine.com, January 2013, Compendia Bioscience, Ann Arbor, MI)[27] indicate that *DPPA4* is highly expressed in testicular embryonal carcinoma[28] and in seminoma[29] compared to normal testis (Figure 2D), supporting a possible role for DPPA4 as an oncogene in human caners. To look for the aberrations of DPPA4 in cancer patience, we searched the COSMIC database (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/) and found that DPPA4 is indeed mutated in human cancers. There are 31 unique samples with mutations out of total 5208 unique samples.

Characterizing a panel of DPPA4 truncation mutants

To identify the role of specific DPPA4 functional domains in inducing tumorigenicity, a panel of truncated DPPA4 mutants (Figure 3A) was transduced into 3T3 cells. Wild-type (WT) DPPA4 is a 296 amino acid protein that has a nuclear SAP (SAF-A/B, Acinus and PIAS) domain[30], a novel DNA binding domain[31]. DPPA4 has recently been shown to directly bind to DNA (although not to a specifically defined sequence) through its N-terminal region containing the SAP domain and to histone H3 via its C terminus in vitro[32]. Importantly, both types of binding were reported to be necessary for regulation of chromatin structure and for the proper localization of DPPA4 in ESC nuclei.

Our panel of mutants contained forms of DPPA4 lacking specific domains including the SAP and c-terminal domains. We measured protein expression of WT and each mutant by Western blotting and immunocytochemistry using two different antibodies (see Methods), one against the C-terminus (Supp. Figure 2A and 2C) and the other against the N-terminus (Supp. Figure 2B and 2D). The expression of ΔSAP was similar to the full length, whereas the level of ΔC was only 50% compared of the full length. Surprisingly, the ΔN80 was barely detectable, even though ΔN115 had about 70% expression compared to the full length. Therefore, we also included the additional mutants ΔN70 and ΔN60, as well as internal deletions, ΔN60–70 and ΔN60–80. The expression of ΔN70 was similar to ΔN80, but ΔN60 had 65% expression compared to the full length, indicating that there could be a protein stability regulatory domain between N60–80. To investigate these mutants further, immunofluorescence staining was conducted (Supp. Figure 2C and 2D). Interestingly, Δ80 DPPA4 protein was present mostly in the cytosol, whereas ΔN60 was in the nucleus (Supp. Figure 2E), suggesting that there might be a nuclear localization signal (NLS) between in the first 80 residues of DPPA4. However, both ΔN60–70 and ΔN60–80 were localized in the nucleus and had expression levels around 50% compared to the WT, suggesting that the NLS is not within N60–80 and N60–80 only plays a partial role in regulating DPPA4 protein stability. The C-terminus also plays a role in nuclear localization of DPPA4 as well as ΔC had significant cytoplasmic localization.

Defining the functional domains within DPPA4 that confer oncogenicity in 3T3s

The oncogenicity of different mutant DPPA4 proteins was measured by *in vitro* focus formation assays and soft agar anchorage independent colony formation assays. After three weeks of incubation, the numbers of the oncogenic foci formed in the cells transduced with all the truncations were significantly lower than in the full-length DPPA4-transduced cells ($P<0.05$, N=3, Figure 3B). In addition, when compared to the empty vector-transduced control cells, only the cells transduced by the full length DPPA4 and ΔN60 generated significantly higher numbers of foci. ΔSAP DPPA4 exhibited a significant reduction in

colony formation indicating that the SAP domain is required for DPPA4-induced oncogenicity. Even though the protein expression level of ΔC DPPA4 was only about 50% compared to the full length, oncogenic foci formation by ΔC was more strongly impaired and foci numbers were similar to those in the negative control, suggesting that the C terminus also contributes substantially to the oncogenicity induced by DPPA4. In soft agar assays, after 6–8 weeks of incubation, the differences between the numbers of colonies formed in the cells transduced with the DPPA4 truncations and the WT DPPA4 were statistically significant (Figure 3C). However, the numbers of colonies formed by cells transduced with the ΔSAP and ΔC were not significantly different from the vector control. Overall, these data indicate that both the DNA-binding SAP domain and the histone-binding C-terminal domain are critical to the oncogenic transformation activity of mDPPA4 in 3T3 cells.

Characterizing the transcriptional function of specific DPPA4 domains

Although DPPA4 is a potential transcriptional factor containing a DNA binding SAP domain, the transcriptional function and DNA binding properties of DPPA4 are currently unknown. To address the question of whether DPPA4 is a transcriptional activator or repressor, we constructed a Gal4-mDPPA4 fusion protein to perform Gal4 Luciferase (Luc) reporter assays. The rationale for choosing the Gal4 assay is that the DNA binding preference of DPPA4 remains undefined at this time and the Gal4 assay allows for measurement of transcriptional function independent of knowing a factor's DNA sequence binding preference. The Gal4-Luc reporter activity in cells with Gal4-mDPPA4 was 125-fold lower compared to cells with Gal4 control (Figure 3D), indicating that DPPA4 can act as a potent transcriptional repressor. Luc reporter assays conducted using different DPPA4 truncations (the same panel as used in the transformation assay above), indicate that the ΔN115 truncation impaired transcriptional repression by DPPA4. Similar expression levels of the Gal4 fusion proteins were observed by Western blot using two different DPPA4 antibodies (Supp. Figure 3A; note that the proteins with the highest molecular weight of each sample were the predicted size of bands in each case). In addition, N80 showed similar luciferase signal compared to the full length DPPA4 (Supp. Figure 3B and 3C), indicating that the repression domain is located within N1–80. Gal4-DPPA4 N114–213, which showed no repression function, was a negative control in this assay (Supp. Figure 3C. Therefore, ΔN115 is important for DPPA4 to carry out its transcriptional repression function. Notably, however, there was no direct link between loss of repressive function and loss of tumorigenic function from studies of the panel of DPPA4 mutants.

Analyzing the transcriptional function of DPPA4 during oncogenic transformation

To analyze the potential transcriptional mechanisms by which DPPA4 causes cellular transformation, we conducted expression microarray studies of RNA isolated from (1) mDPPA4-transformed cell lines, (2) positive control Myc-transformed cells lines, (3) positive control SV40-transformed cells lines, and (4) control cells transduced with empty vector virus (Figure 4). We found 259 genes upregulated and 191 genes downregulated uniquely in DPPA4-transformed cells (Supp. Table. 2). The genes up- or down-regulated at least 1.5 fold in DPPA4-transformed cells (Supp. Table 3) were analyzed by gene ontology (GO) analysis using DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/). DPPA4 overexpression leads to up-regulation of many ontological clusters with highly statistically significant P values that are related to tumorigenesis, included the following: cell cycle (p = 1.51E-20), DNA replication (p = 2.14E-12), mitochondrion (p = 4.01E-14), chromosome (p = 5.63E-19), and p53 pathway (p = 1.61E-05; Supp. Table. 4A). Fitting with the functions of these clusters, DPPA4-transformed cells have a significantly higher proliferation rate compared with the control cells (p = 6.58E-16). As for DPPA4 down-regulated genes, the clusters were as a whole not as statistically significant (Supp. Table.

4B), but indicate that DPPA4 down-regulates differentiation-associated genes and genes involved in cell motility.

Interestingly, a substantial number of DPPA4 specific up- or down-regulated genes were also the novel oncogenes or their near family members, which we had previously identified from the original cDNA screen (Figure 1B), including *PI3KR1, UBB, ESD, FDPS, and MYL6*, suggesting that DPPA4 may play a central, previously unappreciated role in regulating cellular pathways that may further contribute to the tumorigenicity, including teratoma forming activity of ESC and iPSC.

DPPA4 transformation alters expression of factors in the p53 signaling pathway

Cluster analysis of the data indicates that DPPA4-transformed cells are more closely related to SV40-transformed cells than to cMyc-transformed cells (Figure 4A), suggesting that p53 pathway might be regulated by DPPA4. The expression array data also indicated that the levels of several major components in p53 signaling pathway, including *E2F1, MDM2, CDKN1A (p21), TRP63, BAX, APAF1*, and *GADD45A*, were altered in the DPPA4-transformed cells. The expression changes in *CDKN1A, MDM2, TRP63*, and *APAF1* identified by the array data were verified by quantitative RT-PCR (Figure 4B). The expression of *CDKN1A* was approximately 4.5-fold higher in the DPPA4-transformed cells compared to the control. In fact, the significantly increased expression of *CDKN1A* implies an increased transcriptional activity of p53, which was also confirmed by elevated p53 protein level in the DPPA4-transformed cells (Figure 4C), but this was not DPPA4 specific as it was observed in cells with Myc or SV40 as well. Importantly, senescence-associated β-galactosidase staining indicated no increased senescence occurred in the DPPA4-tranduced cells, while most of the positive control Ras-tranduced cells were undergoing cellular senescence on post-transduction day 7 (Figure 4D).

An important question is the extent to which gene expression changes in cells with DPPA4 overexpression are directly or indirectly mediated by DPPA4. To address this issue, ChIP-qPCR was conducted on two candidate targets arising from the array studies, *TRP53* and *CDKN1A.* In ChIP assays, we found that in the DPPA4- and DPPA2-transformed cells and the vector-transduced cells that DPPA4 binds to the promoter region of both *TRP53* and *CDKN1A* (Figure 4E).

Identification of DPPA2, a DPPA4 close relative, as an oncogene

The *Dppa2* and *Dppa4* genes are neighboring, highly related genes in the human and mouse genomes[30]. DPPA2 and DPPA4 proteins are also highly homologous. DPPA2 was first identified as embryo/cancer sequence A (ESCA)[33], whose expression is found in human embryos and reappears in human cancers. We tested the hypothesis that DPPA2 may also act as an oncogene much the same as its family member DPPA4. We found that cells transduced with DPPA2 generated oncogenic foci and also anchorage-independent growing colonies in soft agar (Supp. Figure 4), indicating that DPPA2 can also act as an oncogene. Notably, there are 30 unique samples with DPPA2 mutations out of total 5089 unique samples from the COSMID database.

To further investigate whether there is a difference between the oncogenicity induced by DPPA4 and DPPA2 and whether there is a synergistic effect, we carried out quantitative focus formation assay and soft agar assay in 3T3s transduced with either DPPA4 or DPPA2 alone, and with both DPPA4 and DPPA2 (Figure 5A and 5B). Western Blots were performed to determine expression levels (Figure 5C). The results show that DPPA4-tranduced cells generate more foci and more colonies compared to DPPA2-tranduced cells, suggesting that DPPA2 has apparently substantially lower oncogenic activity than DPPA4.

Interestingly, no synergistic effect was observed in the cells transduced with both DPPA4 and DPPA2. Additionally, coimmunoprecipitation showed that DPPA4 and DPPA2 interact (Figure 5D). It is also notable that DPPA4 overexpression consistently led to elevated DPPA2 protein levels, suggesting positive feedback regulation of DPPA2 by DPPA4 at a post-transcriptional level as increases in DPPA2 RNA by DPPA4 were not observed (Figure 6A). However, DPPA2 did not appear to increase DPPA4 levels.

Identification of cell cycle control as a mechanism of DPPA4 and DPPA2-induced transformation

We measured cell proliferation and performed cell cycle analysis in 3T3s transformed by either DPPA4 or DPPA2. We found that both the DPPA4- and the DPPA2-transformed cells have a significantly higher proliferation rate compared to control cells (P-Values were 6.58E-16 and 8.09E-12 respectively, Figure 5E), indicating that both DPPA4 and DPPA2 induce cell proliferation. In cell cycle studies, both the DPPA4- and the DPPA2-transformed cells had significantly increased proportions of S phase cells compared to the control (P-values were 0.007 and 0.003 respectively, Figure 5F). In order to further investigate whether DPPA4 and DPPA2 have synergistic functions, we also analyzed proliferation and cell cycle properties of cells transduced with both the genes. We found no detectable synergistic effect between DPPA4 and DPPA2. Notably, the cell proliferation rate of the DPPA2-transformed cells is lower than that of DPPA4-transformed cells, further suggesting that DPPA2 is a weaker oncogene than DPPA4 in 3T3s.

We also examined the expression levels of several cyclins and G1/S transition genes that were up-regulated in the microarray data of DPPA4-transformed cells by qRT-PCR. *CCND1* expression in the DPPA4-transformed cells increased approximately 2.5-fold compared to the vector control (Figure 6B). However, *CCND1* expression was not induced in the DPPA2-tranformed cells, indicating that there might be other mechanisms involved. The significantly higher expressions of other cyclin family members, including *CCNB1* and *CCNG1* (Figure 6C), were also further confirmed by qRT-PCR in the DPPA4-transformed cells. Further, expression levels of G1/S transition genes, *TAF10, E2F1, and MTBP* (Figure 6C and 6D), were also higher in the DPPA4-transformed cells. Taken together, the studies of the DPPA4-tranformed cells suggest that DPPA4 can induce cell proliferation through genes related to regulation of the G1/S transition, a potential mechanism contributing to oncogenic transformation by DPPA4.

Knockdown DPPA4 leads to decreased cell proliferation in both mouse ESCs and ECCs

To further study the involvement of DPPA4 in regulating cell proliferation in pluripotent cells, we conducted DPPA4 knockdown followed by cell proliferation assays in both E14 mESCs and P19 ECCs. Reduced DPPA4 protein levels in the shRNA transduced E14s and P19s were confirmed by Western Blots (Figure 7A and Figure 7C). Cell proliferation studies indicated that DPPA4-knockdown E14 mESCs have a significantly lower proliferation rate compared to the scramble control cells (Figure 7B, P-values <0.01). Same results were also observed in P19 mECCs (Figure 7D, P-value <0.01). These findings support the notion that DPPA4 is a positive regulator of cell proliferation in both mESCs and mECCs.

Discussion

Here we identified DPPA4 and its family member DPPA2 as novel oncogenes when overexpressed that also normally serve as pluripotency factors when present at endogenous levels. In terms of mechanisms, we demonstrated that DPPA4 stimulates cell proliferation in both mESCs and mECCs. It is important to note that DPPA4 transcriptional repressive function was not linked in our domain analysis to oncogenic transformation, suggesting an

alternate function for tumorigenesis stimulated by DPPA4, perhaps through its C-terminal histone H3 binding domain. Even though DPPA2 has not previously been reported to be an oncogene, its known functions from published studies suggest possible roles in cancer-related cellular functions. For example, knockdown of expression of DPPA2 in mESCs results in decreased cell proliferation and induced differentiation[34, 35]. Notably, in the study of identifying re-expressed human embryonic genes in cancer cells, the authors reported that embryo-cancer sequence A (*ESCA*), later named *DPPA2,* is expressed in human tumors but not normal somatic tissues[33]. The expression of DPPA2 in the non-small cell lung cancers (NSCLC) is restricted to a putative stem cell sub-population that has the ability to induce spontaneous immunogenicity in vivo[36]. Our data showing that DPPA2 acts as an oncogene and induces cell proliferation further supports the idea that DPPA2 as well as DPPA4 could be novel potential targets for cancer therapy.

Studies of DPPA4 in ESC self-renewal and differentiation indicate that it is a nuclear factor that preferentially associates with transcriptionally active chromatin[37]. In addition, both DPPA4 and DPPA2 co-loclized with euchromatic regions in the ESC nucleus[35]. Importantly, in the DPPA2 knockout ESCs, enrichment of dimethylation at H3K9 in the promoter regions of DPPA2's targets were observed, indicating that DPPA2 can maintain active epigenetic status in ESCs[35]. However, we demonstrated that DPPA4 is a very strong transcriptional repressor using the Gal4/UAS system (Figure 4D). These potentially conflicting results suggest a complexity to DPPA2/4 family effects on transcription and epigenetic states, perhaps not unexpected given the apparent lack of correlation between DPPA4 transcriptional repressive and oncogenic funtions in our studies.

The roles of DPPA4 in ESCs in vivo have been investigated in mice constitutively lacking DPPA4[38]. In contrast to predictions, DPPA4 is dispensable for ESCs identity and germ cell development. DPPA4 has been identified as an embryo-specific activation gene, but the role and the mechanisms by which DPPA4 acts as pluripotency- associated factor remain unclear. Microarray data from DPPA4-deficient and control ESCs has identified DPPA4 targets related to germ cell development and fertility[38]. However, as a chromatin modulator and a DNA binding protein, the specific regulatory functions of DPPA4 remain unknown. When we compared our microarray data from the DPPA4-transformed 3T3 cells to the data from DPPA4-deficient ESCs, we found little overlap. There are only a handful of genes that are both down-regulated in the DPPA4-deficient ESCs and up-regulated in the DPPA4-transformed cells, and vice versa, suggesting that the potential targets we identified during oncogenic transformation are generally distinct from targets in ESCs.

The functions of DPPA4 and DPPA2 specifically in cellular reprogramming and iPSCs remain unclear at this time. In the first Yamanaka iPSC paper, both DPPA4 and DPPA2 were included as the beginning 24 factors to be screened[39]. Taking out either one of them from the cocktail did not strongly affect the reprogramming efficiency in their study, suggesting that DPPA4 and DPPA2 are not essential. However, a more recent study[40] from implicates DPPA2 as a key indicator of stably reprogrammed cells. Therefore, DPPA2 and possibly DPPA4 have significant roles in induced pluripotency, but their specific actions and the mechanisms involved remain to be determined.

While we have presented substantial evidence here that DPPA4 and DPPA2 are bona fide oncogenes, there are some alternative possibilities that warrant discussion and future experiments will more definitively resolve these issues. First, although we have determined that there are mutations in DPPA4 and DPPA2 in human cancers via the COSMIC database, a key open question is whether these mutations are functionally important from a cancer standpoint. Future studies will determine whether these mutations change DPPA4/DPPA2 protein stability or functions in ways that could be oncogenic. Second, while we have

presented database findings that DPPA4 is highly expressed in germ cell tumors versus normal testes, at this time we do not know for certain if DPPA4 causes germ cell tumors. An alternative explanation is that DPPA4 expression is relatively high in germ cells, which are relatively rare in normal testes, but abundant in germ cell tumors. Future experiments on DPPA4 and DPPA2 in germ cell-related cancers should provide additional insight.

Besides having the common SAP domain, DPPA4 and DPPA2 have 32% identity in amino acid sequence and similar expression patterns[30]. The close relationship between DPPA4 and DPPA2 supports the hypothesis that they may have similar functions, but we found no synergistic effect in the cells transduced with both DPPA4 and DPPA2, suggesting that they may have redundant roles in the induction of oncogenic transformation. Interestingly, DPPA2/DPPA4 double mutant and the single DPPA4 mutant ESCs are both normal[38]. In addition, DPPA4 and DPPA2 proteins interact in ESCs[35] and in 3T3s (Figure 5D), indicating that they may cooperatively or antagonistically function. Notably, in the DPPA2/DPPA4 double knockout mice and the DPPA4 knockout mice, the lung defect was milder than what was observed in the DPPA2 knockout mice[35]. Given these published findings and our own data, we propose that the balance between DPPA4 and DPPA2 is important to their respective functions both as pluripotency genes and as oncogenes. Elucidating the roles of DPPA4 and DPPA2 as chromatin binding proteins[32], for example by future ChIP-Seq studies, will provide more insight into their normal and cancer-related functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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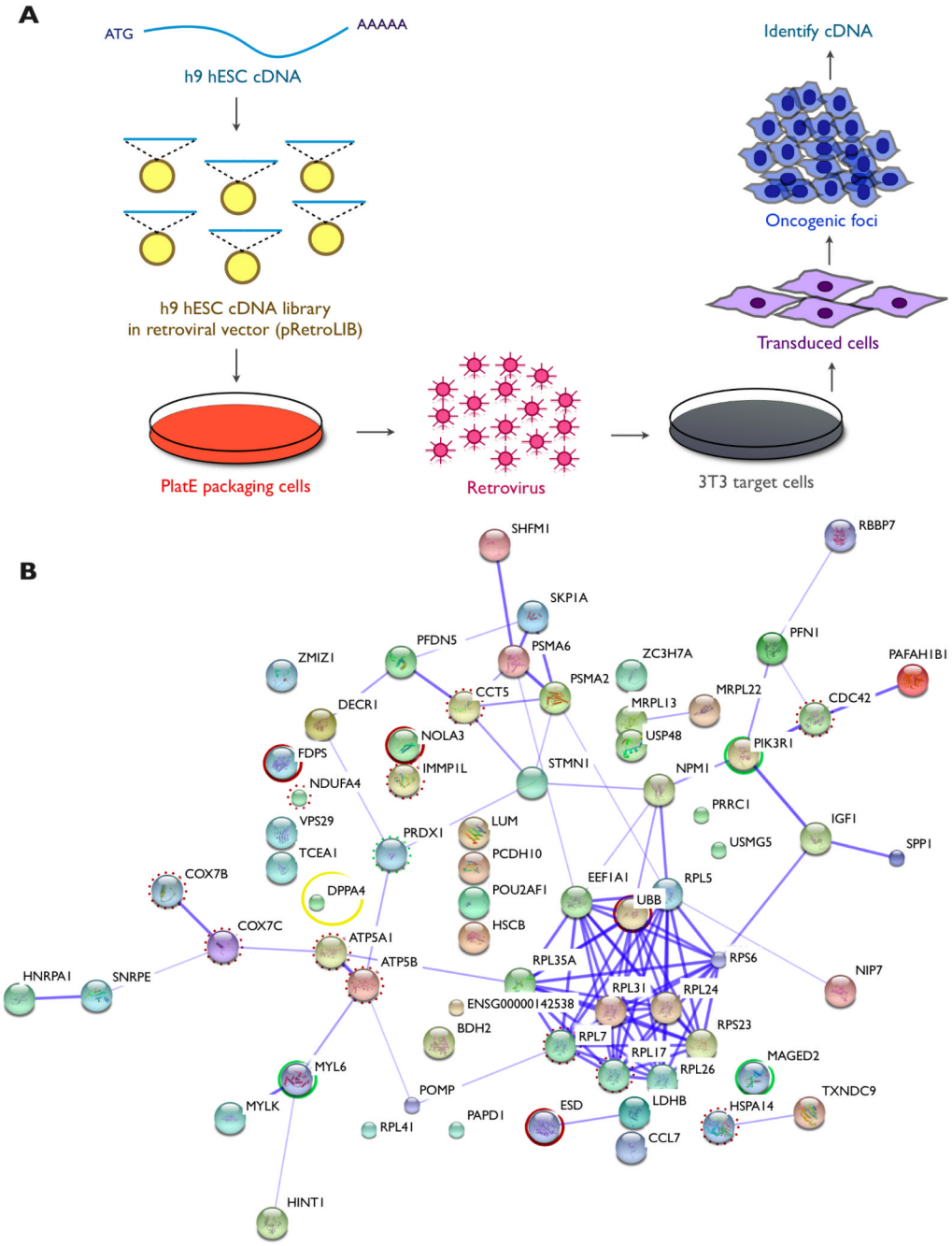

Figure 1. Retroviral cDNA library expression screen for pluripotency related oncogenes

(A) Packaging cells were transfected with the H9 hESC cDNA library to produce virus. A total of 10^7 3T3 cells were transduced with the virus from the packaging cells to give a strong likelihood that all the cDNAs present in the library would be expressed in the target cells. After culturing for 3 weeks, oncogenic foci were selected based on colony formation. (B) The 71 genes identified from the retroviral cDNA screen were analyzed using the String 8.3 database that displays protein-protein interactions from published literature. The red and green circles represent, respectively, the putative oncogenes that were also separately up- or down-regulated in the DPPA4-transformed 3T3 cells from the microarray data. The dotted

circles indicate the genes whose near family members were found differentially expressed in DPPA4-transformed cells.

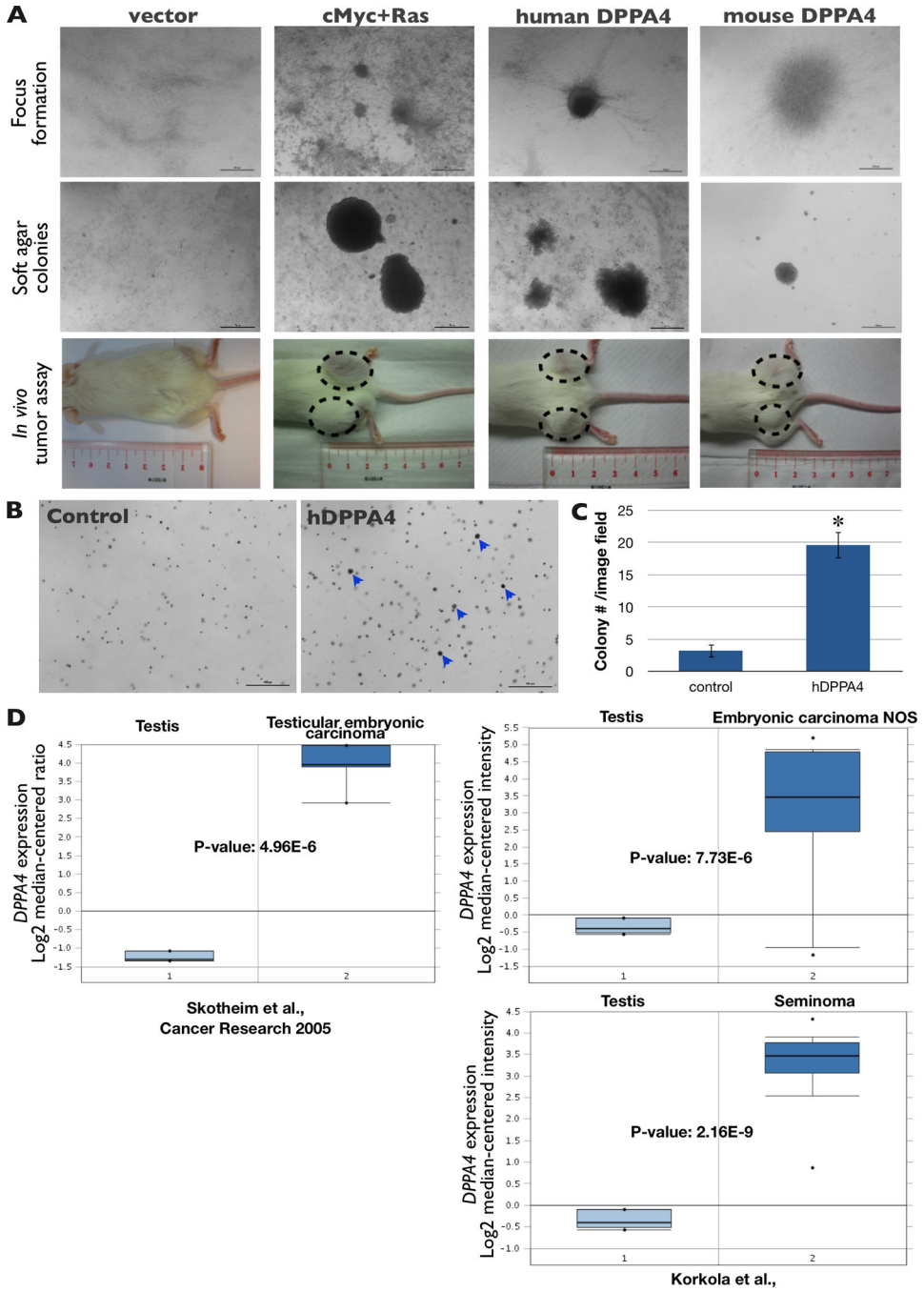

Figure 2. Oncogenic transformation activity of DPPA4 and its expression in human stem cell-related tumors

(A) 3T3 cells were co-transduced with viruses for c-MycTA (stabilized form) and k-Ras as a positive control, or with the empty retroviral vector as a negative control, or with either human or mouse DPPA4. The transduced cells were then cultured for 17 days for the analysis of focus formation (top panels; scale bar: 500 μm). The cells were also assayed for anchorage-independent growth in soft agar for over 21 days (Middle panels, scale bar: 500 μm) and for tumorigenicity in immunodeficient mice for 4 weeks (bottom panels). The black circles indicate the tumors formed after subcutaneous injections. (B) Soft agar assay using hTERT immortalized human dermal fibroblasts (HDFs) as a basis. After eight weeks, large

colonies (indicated by blue arrows) were scored as positive. (C) Colonies > 50 μm in diameter were counted in each image field. Plots of the colony numbers in each image field, with the average represented by the column and the standard deviations by the bar (☜p=3.9E-6). (D) Elevated expression of DPPA4 mRNA in tumors (testicular embryonic carcinoma and seminomas) compared to normal testis samples. The results are from the data-mining platform, Oncomine. The Y-axis is the mRNA expression levels, which have been log2, transformed and normalized to the median value of the dataset. The dots represent the high/low values, the box represent the 75%/25% values, the line in the box represents the median, and the error bars are the 90%/10% values.

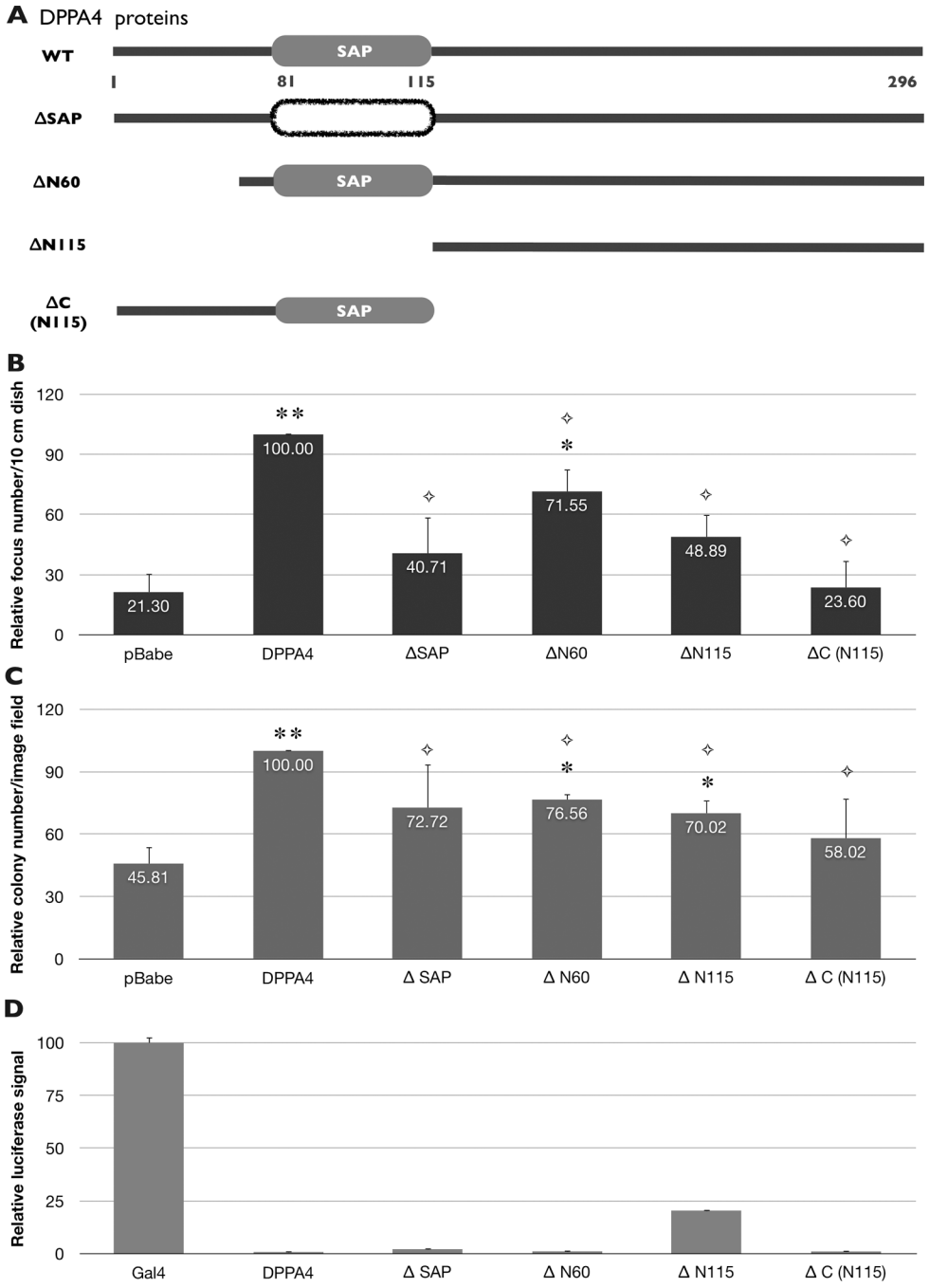

Figure 3. Functional domain analysis of DPPA4 as a novel oncogene and transcriptional repressor

(A) Schematic representation of wild type (WT) and different truncated DPPA4 proteins used in these studies. The gray box depicts the SAP domain (amino acid 81–115). The C-terminal region (amino acid 116–296) contains the chromatin-binding domain. (B) Focus formation assay was performed to quantify the oncogenic potential of different DPPA4 truncations in 3T3 cells. Mean numbers of oncogenic foci after incubation of 3 weeks of three independent experiments with standard deviations are presented. The numbers were normalized to the focus number of WT DPPA4 as 100. (C) Anchorage-independent growth of cells stably transduced with different DPPA4 truncations. After culturing in 0.35% soft

agar for 8 weeks, colonies were counted. Plots of the colony numbers from three experiments, with the average represented by the column and the range by the bar (☜p<0.05, ☜☜p<0.01 compared to pBabe; ☏p<0.05 compared to WT DPPA4). (D) 293FT cells were transfected separately with 0.1 μg of Gal4DBD, Gal4DBD-DPPA4, or Gal4DBD-DPPA4 truncations, together with 1 μg of Gal4 luciferase and 20 ng of Renilla luciferase. Luciferase values were normalized to Renilla luciferase activity to adjust for transfection efficiency and then standardized relative to Gal4DBD samples (defined as 100 relative luciferase unit, RLU).

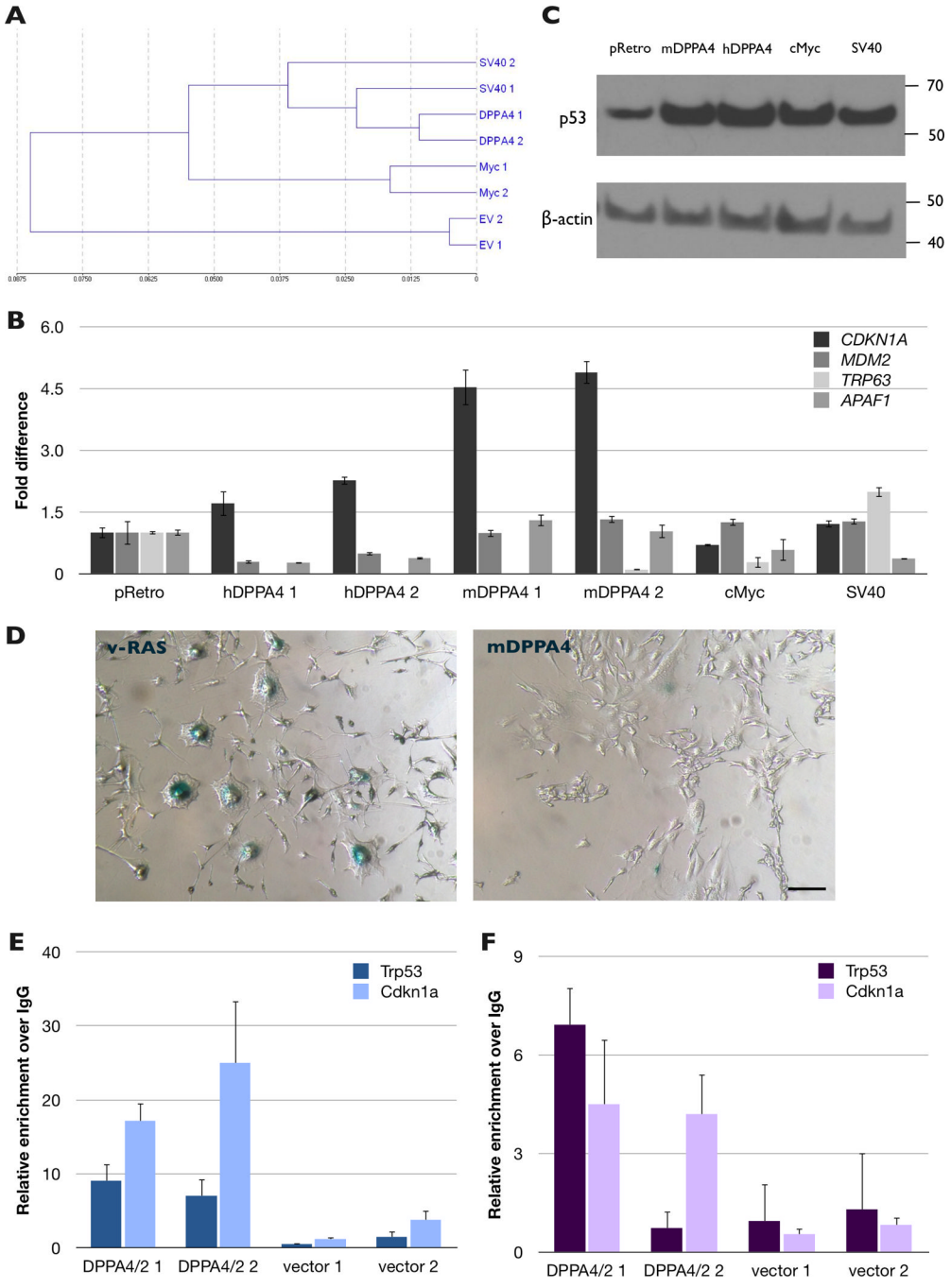

Figure 4. Characterization of the DPPA4-transduced cells and their transcriptome

(A) Schematic representation of the clustering according to the expression microarray. (B) qPCR was conducted on cDNA that was synthesized from non-transformed 3T3 cells or cells transformed by human DPPA4 (two clones), mouse DPPA4 (two clones), c-Myc, and SV40 large T antigen, respectively. Four p53 pathway genes (*CDKN1A*, *MDM2*, *TRP63*, and *APAF1*) were selected to validate the expression levels of genes obtained from the microarray. (C) Western blot analysis of extracts from the vector transduced cells and mDPPA4, hDPPA4, cMyc and SV40-transformed cells, using total p53 and β-actin antibodies. (D) Acidic β-galactosidase staining was performed on Ras- and DPPA4-

transduced cells on post-transduction day 7. Blue cells are senescent (scale bar: 50 μm). (E and F) ChIP samples were prepared in two transformed cell lines (DPPA4/2 1 and 2) and two vector-transduced control cell lines using mDPPA4 antibody and Goat IgG in (E) or mDPPA2 antibody and Rabbit IgG in (F). The enriched promoter region of *TRP53* and *CDKN1A* were ChIP'd and analyzed using SYBR qPCR.

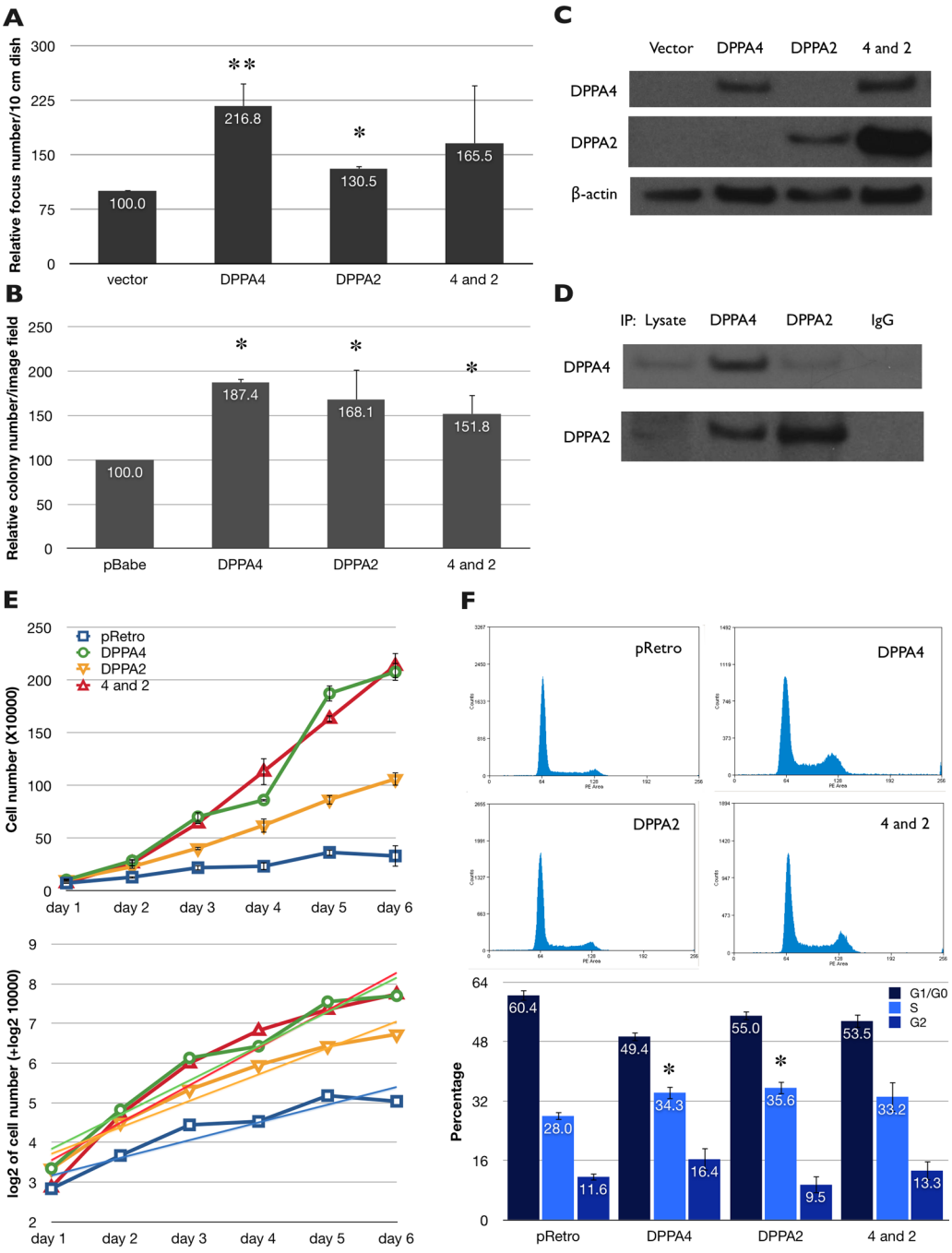

Figure 5. DPPA4 and DPPA2 interact and stimulate proliferation, but not synergistically

(A) Focus formation assays were performed to quantify the relative oncogenic potential of DPPA4 and DPPA2 in 3T3 cells and potential synergism. Mean numbers of oncogenic foci after incubation of 3 weeks of three independent experiments with standard deviations are presented. The numbers were normalized to the focus number of empty pBabe vector control as 100. (B) Anchorage-independent growth of cells stably transduced with either DPPA4 or DPPA2 alone or both together. After culturing in 0.35% soft agar for 8 weeks, colonies were counted and photographed. Plots of the colony numbers from three experiments, with the average represented by the column and the range by the bar

(☜p<0.05, ☜☜p<0.01 compared to pBabe). (C) Western blot analysis of cell lysates from the vector transduced cells and DPPA4, DPPA2, DPPA4 and DPPA2-transformed cells, using antibodies against DPPA4, DPPA2, and β-actin respectively. (D) Coimmunoprecipitation of DPPA4 and DPPA2 in cells transduced with both proteins. (E) 10^4 control cells or transformed cells were seeded into the 24-well tissue culture plates. Cell proliferation was measured by counting cell numbers each day up to 6 days. Data are presented as the mean ± SD of three independent experiments. P-values of log2 slope differences against vector control were all smaller than 0.01. (F) Control cells or transformed cells were used for cell cycle profile analysis by DNA content using Propidium iodide (PI) staining (top). Cell cycle profiles were analyzed using Winmdi and Cylchred. Plots of the percentage numbers from three experiments (☜p<0.05 compared to pBabe).

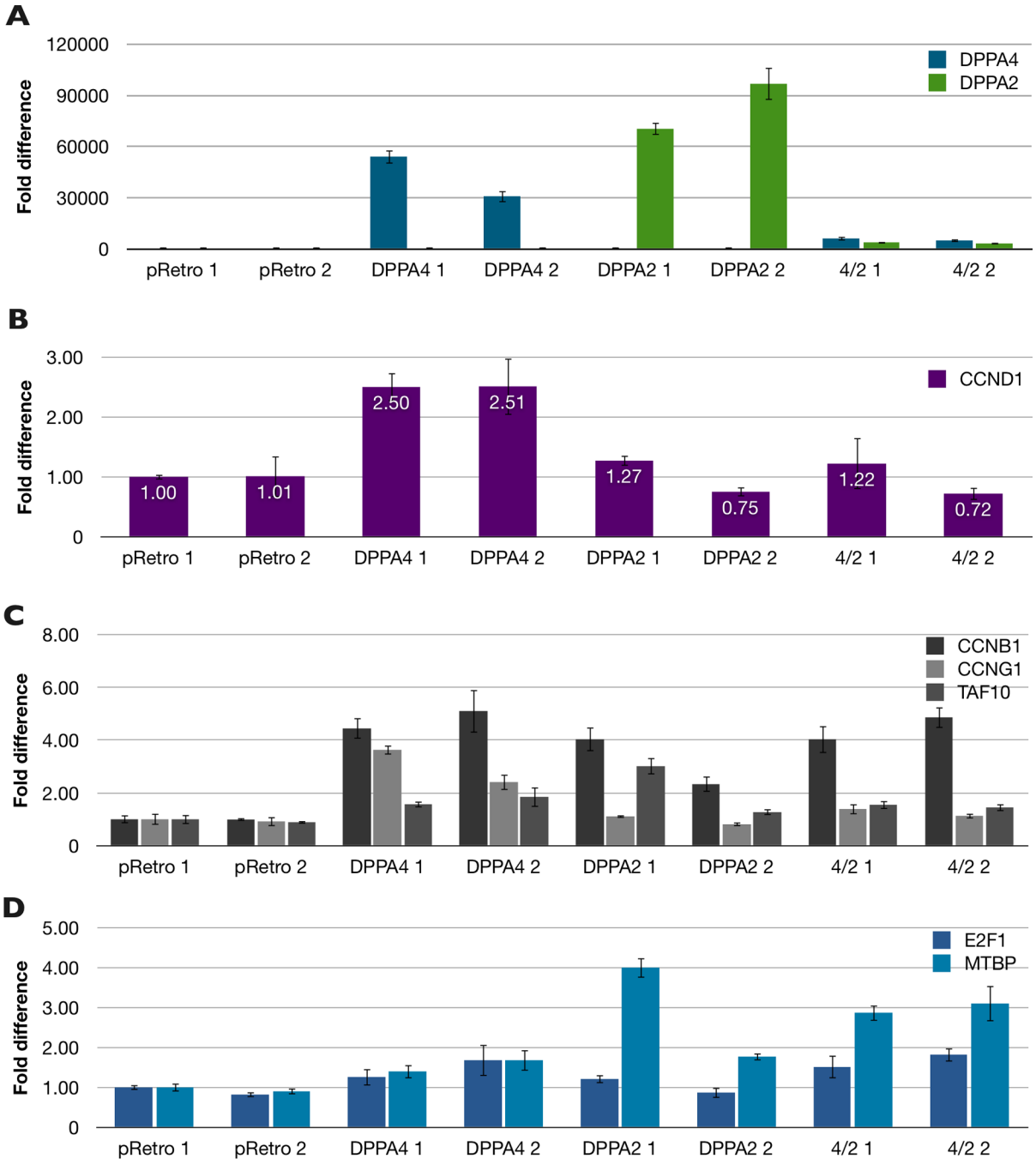

Figure 6. DPPA4 up-regulates genes that encode cell cycle regulatory factors

cDNA was synthesized from non-transformed 3T3 cells (pRetro) or cells transformed by DPPA4, DPPA2, and DPPA4 plus DPPA2 (4/2), respectively, and were then subjected to SYBR green qPCR for the indicated genes. (A) *DPPA2* and *DPPA4* expression. (B) *CCND1* expression. (C) Expression of two cyclin genes (*CCNB1*, and *CCNG1*) and *TAF10*. (D) Expression of other G1/S transition-related genes (*E2F1*, and *MTBP*). These genes were selected to validate based on their apparent altered expression levels from the microarray study of DPPA4-transformed cells.

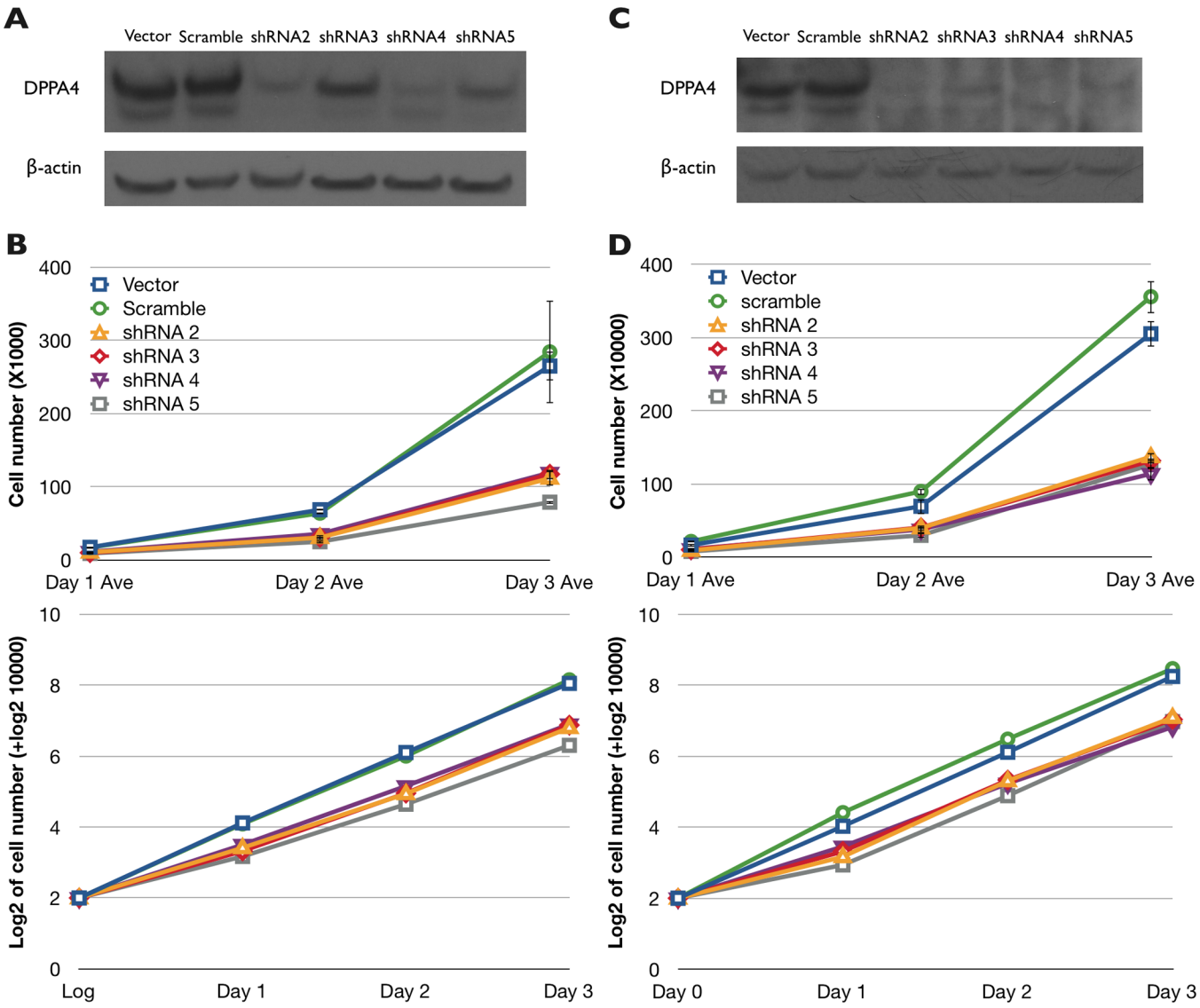

Figure 7. Decreased cell proliferation induced by DPPA4 shRNA knockdown in E14 ESCs and P19 ECCs

(A and B) Western blot analysis of extracts from cells transduced with pLKO1 vector, scramble shRNA, or different DPPA4 shRNAs using mDPPA4 and β-actin antibodies in E14s (A–B) and in P19s (C–D). (C) Control E14 cells and DPPA4-knockdown E14 cells were seeded into the 24-well tissue culture plates. Data are presented as the mean ± SD of three independent experiments. P-Values between the log slopes of scramble shRNA and DPPA4-specific shRNAs were all <0.01. (D) Control P19 cells and DPPA4-knockdown cells were subjected to cell proliferation assay as described in (C). P-Values between the log slopes of scramble shRNA and DPPA4-specific shRNAs were also all <0.01.