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Genomic functions of developmental pluripotency associated factor 4 (Dppa4) in pluripotent stem cells and cancer

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ABSTRACT

Developmental pluripotency associated factor 4 (*Dppa4*) is a highly specific marker of pluripotent cells, and is also overexpressed in certain cancers, but its function in either of these contexts is poorly understood. In this study, we use ChIP-Seq to identify Dppa4 binding genome-wide in three distinct cell types: mouse embryonic stem cells (mESC), embryonal carcinoma cells, and 3T3 fibroblasts ectopically expressing Dppa4. We find a core set of Dppa4 binding sites shared across cell types, and also a substantial number of sites unique to each cell type. Across cell types Dppa4 shows a preference for binding to regions with active chromatin signatures, and can influence chromatin modifications at target genes. In 3T3 fibroblasts with enforced Dppa4 expression, Dppa4 represses the cell cycle inhibitor Cdkn2c and activates Ets family transcription factor Etv4, leading to alterations in the cell cycle that likely contribute to the oncogenic phenotype. Dppa4 also directly regulates Etv4 in mESC but represses it in this context, and binds with Oct4 to a set of shared targets that are largely independent of Sox2 and Nanog, indicating that Dppa4 functions independently of the core pluripotency network in stem cells. Together these data provide novel insights into Dppa4 function in both pluripotent and oncogenic contexts.

1. Introduction

Maintenance of a pluripotent state requires complex and precise regulation at many levels, including transcriptionally through the interaction of pluripotency-specific transcription factors within the embryonic stem cell (ESC) chromatin landscape. While a few core pluripotency factors have been well characterized, many open questions remain and there are some pluripotency-specific factors whose functions are largely unknown. One of the best examples is *Developmental pluripotency associated factor 4* (*Dppa4*), which is a putative pluripotency factor that is selectively expressed in ESC compared to differentiated cells. Both it and its homologue *Dppa2* are also two of the best pluripotency markers used to validate induced pluripotent stem cells (IPSC) (Kang et al., 2015). However, Dppa4 and Dppa2 protein functions remain largely unknown.

Surprisingly, given its pluripotency-specific expression pattern and tight associations with other pluripotency factors like Oct4 (Chakravarthy et al., 2008; Sperger et al., 2003), Dppa4 has nonetheless been shown to be dispensable for ESC maintenance and for early murine embryonic development (Madan et al., 2009). Targeted disruption of *Dppa4*, *Dppa2*, or both in mice, did not produce the predicted early embryonic lethal phenotypes, but rather phenotypes manifested only much later in development in lung and other tissues where these factors are not expressed normally (Madan et al., 2009; Nakamura et al., 2011). Knockdown of Dppa4 in ES cells has also produced results that did not consistently define its role in pluripotency (Ivanova et al., 2006).

The mechanisms of potential Dppa4-mediated transcriptional regulation are also poorly understood. Dppa4 mainly associates with active, euchromatic domains as assessed by cytostaining (Masaki et al., 2007), but it is also a member of a non-canonical Polycomb repressive complex (Oliviero et al., 2015), and it represses transcription in GAL4 assays in vitro (Tung et al., 2013). Dppa4 contains an N-terminal SAP (SAF-A/B, Acinus and PIAS) domain, which is thought to mediate Dppa4 DNA binding, but it also associates with histone H3 through its C-terminal domain (Masaki et al., 2010). Recent work has shown that DPPA4 interacts with ERBB3 binding protein (ERB1) in human pluripotent stem cells, and that this interaction can attenuate DPPA4 mediated gene repression (Somanath et al., 2018), however, the extent to which Dppa4 acts as a transcriptional activator, repressor, or both at

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endogenous targets in pluripotent cells, and how it associates with chromatin to impact cell biology are major questions that have not been fully elucidated.

In addition to predicted roles in pluripotent stem cells, *Dppa4* and *Dppa2* are also oncogenic when overexpressed in somatic cells, and they are overexpressed in certain human cancers where they correlate with poor prognosis (Tung et al., 2013; John et al., 2008; Monk and Holding, 2001). Dppa4/2 increase proliferation through upregulation of cyclins and other G1/S transition genes, and induce foci formation and anchorage independent growth (Tung et al., 2013). While several direct transcriptional targets of Dppa4 have been identified using a candidate approach, global, unbiased characterization of Dppa4 direct targets genome-wide in stem cells and cancer cells has not been reported. Such studies would provide a better understanding of the mechanisms of Dppa4 transcriptional regulation and its biological impact.

Here we defined the genomic functions of Dppa4 in both ESC and an oncogenic context. We profiled Dppa4 binding genome-wide by ChIP-Seq in three cell types: E14 ESCs, 3T3 fibroblasts with enforced Dppa4 expression, and P19 embryonal carcinoma cells (ECCs). Comparing Dppa4 binding across cell types, there was substantial overlap of Dppa4-bound targets between the three cell types, particularly strong overlap in P19 and E14 cells, and a shared preference for active chromatin signatures. We in addition identified Dppa4-dependent changes in specific chromatin modifications at a subset of the genes it activates and represses. We also found that some Dppa4-bound target genes can be regulated by Dppa4 in opposing directions in different cell types, suggesting that cell type-specific differences influence the actions of Dppa4 in regulation of its targets. For example, we found that expression of the novel Dppa4 target gene *Etv4* was increased both with ectopic *Dppa4* expression in fibroblasts and, conversely, by *Dppa4* knockout in mESCs. Our studies also implicate repression of *Cdkn2c* and the activation of *Etv4* as an important downstream effector of Dppa4 biological functions including proliferation in an oncogenic context. Our data also support a specific co-regulatory role for Oct4 and Dppa4 in ESC outside of the conventional Oct4-Sox2-Nanog regulatory context. Overall, our data define roles for direct Dppa4-mediated gene regulation in pluripotent stem cells and in an oncogenic context, and suggest specific epigenomic mechanisms of function.

2. Materials and methods

2.1. ChIP

ChIP was performed largely as described previously (O'Geen et al., 2011). Briefly, cells were crosslinked with 1% formaldehyde, lysed, and sonicated to an average fragment length of 500 bp before being immunoprecipitated with selected antibodies. The resulting chromatin was used for qPCR or library preparation for ChIP-Seq. For each ChIP, 20–50 μg of sonicated chromatin was used, with magnetic Dynabeads (Invitrogen) for immunoprecipitation. For ChIP-qPCR experiments, enrichment was calculated relative to the IgG negative control and then further normalized to an intergenic negative control region. The following antibodies were used: Rabbit IgG (Santa Cruz sc-2027), Goat IgG (Santa Cruz sc2028), H3K27ac (Abcam ab4729), H3K4me3 (Millipore 04–745), Dppa4 (R&D Systems AF3730), OCT4 (Abcam ab19857). HDAC1 (Abcam ab31263), HDAC2 (Abcam ab12169). Primers are listed in Supplemental Table 1.

2.2. ChIP-Seq

Two replicates of Dppa4 ChIP were performed in each of the following cell lines: E14, 3T3, and P19 cells. An input control was also sequenced for each cell line for normalization. Libraries were prepared with the Nextera library prep kit and sequenced on the Illumina Hi-Seq 2500 with fifty base pair single-end sequencing. Bases were called with Casava 1.8 (bcl2fastq 1.8). Raw sequencing data and processed peaks can be accessed with GEO accession number: GSE95055. Gene expression microarray data on Dppa4 overexpression fibroblasts can be accessed with GEO number: GSE58709.

2.3. Bioinformatics

Dppa4 ChIP-Seq reads were aligned to the genome using the Burrows-Wheeler Aligner (BWA), version 0.7.13-r1126 (Li and Durbin, 2010). MACS (version 1.4.2) (Zhang et al., 2008) was used to call peaks, with input samples used as the background control and an FDR of 0.05. Only peaks that overlapped between replicates were used for further analysis. For histone modification and Dppa2 ChIP-Seq, raw data was obtained from ENCODE and GEO, and analyzed using BWA and MACS to be more comparable with our Dppa4 data. DAVID was used for gene ontology analysis (Huang Da et al., 2009; Sherman et al., 2007). Galaxy (Giardine et al., 2005; Goecks et al., 2010) and Cistrome (Liu et al., 2011) were used for all other downstream analysis.

2.4. qPCR

For gene expression analysis, cDNA was prepared from 200 ng of RNA using the iScript cDNA kit, and RT-PCR was performed using Thermo Absolute Blue SYBR Green ROX (Catalog number AB-4162) on the LightCycler 480 (Roche). Mouse PP1A was used as the internal normalization control. RNA was extracted from cells using the Macherey Nagel Nucleospin RNA kit (Catalog number 740955).

For qPCR following ChIP, chromatin was diluted 1:10 and RT-PCR was performed using Thermo Absolute Blue SYBR Green ROX (Catalog number AB-4162) on the LightCycler 480 (Roche). Percent input values were calculated for each sample after subtracting IgG signal, and all values were then normalized to a negative control chromatin region (Crisp3).

Primer sequences in Supplemental Table 1.

2.5. Cell culture, transfections, and transductions

3T3 cells and NT2 clone D1 cells (supplied by Shiro Urayama) were cultured in DMEM supplemented with 10% FBS and 1% Glutamine. E14 cells were cultured under feeder-free conditions in 2i media with 2% FBS on gelatin-coated plates. P19 cells were cultured in DMEM supplemented with 10% FBS and 1% Glutamine. Four siRNA targeting Etv4 (Qiagen GS18612) were pooled and transfected into WT and Dppa4 3T3 cells at a concentration of 25 nM using Lipofectamine RNAi Max (Thermo Fisher Scientific) according to the manufacturer's directions. Cells were collected 48 h after transfection and assayed for knockdown by qPCR and cell cycle stage by propidium iodide staining and flow cytometry. Full length mouse Cdkn2c and Etv4 were cloned into the pBABE vector and transfected into platE cells to generate virus. WT and Dppa4 overexpressing 3T3 cells were transduced with virus containing media collected from platE cells for each construct. Transduction of cells with virus generated from an empty pBABE vector was used as a control. Cells were selected with hygromycin (100μg/mL) for 7 days, until all 3T3 cells in an untransfected control plate treated with 100μg/mL hygromycin had died.

2.6. TUNEL staining

Cells were seeded onto coverslips and collected after 48 h. The DeadEnd™ Fluorometric TUNEL system (Promega) was used for TUNEL staining. A total of six images (3 images from each of two slides) were collected for each 3T3 cell line (WT empty vector control, WT + Cdkn2c, WT + Etv4, Dppa4 empty vector control, Dppa4 + Cdkn2c, Dppa4 + Etv4). DNase I treatment was used as the positive control.