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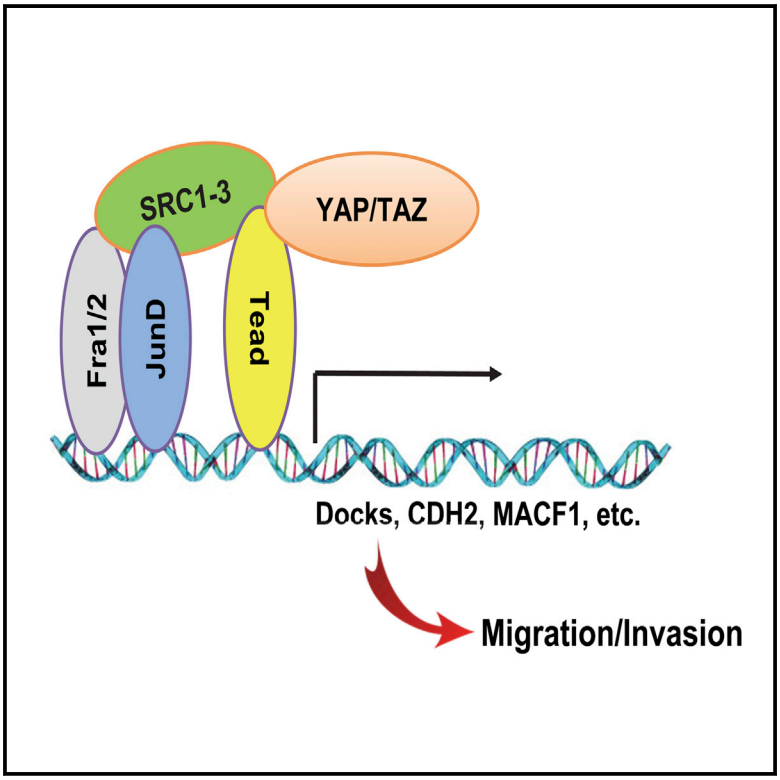
Cell Reports

Tead and AP1 Coordinate Transcription and Motility

Graphical Abstract



Authors

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In Brief

Tead and AP1 are two families of transcription factors involved in tumorigenesis. Here, Liu et al. show Tead-AP1 co-occupancy on active enhancer or promoter regions in diverse cancer cells. This Tead-AP1 cooperation engages SRC1–3 co-activators and drives a core set of downstream target genes to coordinate cancer cell migration and invasion.

Highlights

- Tead and AP1 coordinate downstream transcription in diverse cancer cells
- JNK-independent Tead-AP1 interaction engages SRC1–3 co-activators
- Tead and AP1 regulate the activity of Dock-Rac/CDC42 module
- Tead-AP1 cooperation controls migration and invasion through a core set of target genes

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Tead and AP1 Coordinate Transcription and Motility

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SUMMARY

The Tead family transcription factors are the major intracellular mediators of the Hippo-Yap pathway. Despite the importance of Hippo signaling in tumorigenesis, Tead-dependent downstream oncogenic programs and target genes in cancer cells remain poorly understood. Here, we characterize Tead4-mediated transcriptional networks in a diverse range of cancer cells, including neuroblastoma, colorectal, lung, and endometrial carcinomas. By intersecting genome-wide chromatin occupancy analyses of Tead4, JunD, and Fra1/2, we find that Tead4 cooperates with AP1 transcription factors to coordinate target gene transcription. We find that Tead-AP1 interaction is JNK independent but engages the SRC1–3 co-activators to promote downstream transcription. Furthermore, we show that Tead-AP1 cooperation regulates the activity of the Dock-Rac/CDC42 module and drives the expression of a unique core set of target genes, thereby directing cell migration and invasion. Together, our data unveil a critical regulatory mechanism underlying Tead- and AP1-controlled transcriptional and functional outputs in cancer cells.

INTRODUCTION

The Tead family transcription factors are a family of evolutionary conserved proteins carrying a TEA DNA binding domain that recognizes the 5′-GGAATG-3′ consensus sequence (Kaneko and DePamphilis, 1998; Pobbati and Hong, 2013). Scalloped (Sd) is the only Tead family protein in *Drosophila* (Halder et al., 1998; Wu et al., 2008a). In mammals, four Tead family members, Tead1–4, were originally identified by their various roles in early embryonic development (Chen et al., 1994; Nishioka et al., 2008; Sawada et al., 2008). Tead proteins require additional transcriptional co-activators to activate transcription, and recent studies have established the Yes-associated protein (YAP) family transcriptional regulators (Yki in fly and YAP/TAZ in mammals) as the major co-activator for Tead proteins (Nishioka et al., 2008; Wu et al., 2008a; Zhang et al., 2009a; Zhao et al., 2008), although other Tead upstream regulators have been reported (Gupta et al., 1997; Halder et al., 1998; Pobbati et al., 2012). YAP and TAZ are the key intracellular effectors of Hippo signaling, and dysregulation of the Hippo-YAP/TAZ pathway has been implicated in a variety of human cancers (Halder and Camargo, 2013; Hong and Guan, 2012; Moroishi et al., 2015; Pan, 2010). Despite the potential importance of Tead proteins in tumorigenesis, the molecular mechanism underlying Tead-mediated transcriptional regulation is not well understood and the Tead-controlled downstream target network in cancer cells remains poorly characterized.

RESULTS

Functional Requirement and Genomic Occupancy of Tead Proteins in Neuroblastoma, Lung, Colon, and Endometrial Cancer Cells

To gain insight into Tead-dependent oncogenic programs, we first examined the expression of Tead proteins in four distinct types of human cancers: lung adenocarcinoma, colorectal carcinoma, endometrial cancer, and neuroblastoma. Immunohistochemistry (IHC) revealed that nuclear Tead4 expression was readily detected in all four cancer types (Figure 1A). Although mis-regulation of the Hippo-YAP pathway in lung, colon, and endometrial cancers has been previously reported (Moroishi et al., 2015; Tsujiura et al., 2014), its connection to neuroblastoma, a common infant and childhood tumor arising from the neural crest lineage (Louis and Shohet, 2015), was not known. We found that Tead4 was highly expressed in the majority of human neuroblastoma samples we examined, in comparison to low or no expression in normal peripheral nerve tissues (Figure 1A; Figure S1), pointing to a potential Tead involvement in neuroblastoma pathogenesis. Tead4 and overall Tead proteins, detected by the Tead4 and pan-Tead antibodies, respectively, exhibited distinct expression patterns in human A549 (lung adenocarcinoma), HCT116 (colon cancer), SK-N-SH (neuroblastoma), and ECC1 (endometrial cancer) cells (Figure 1B), suggesting potential functional redundancy among Tead proteins in cancer cells. To block the activity of all Tead proteins, we generated lentiviral-based constructs, Tead1–4 knockdown/knockout

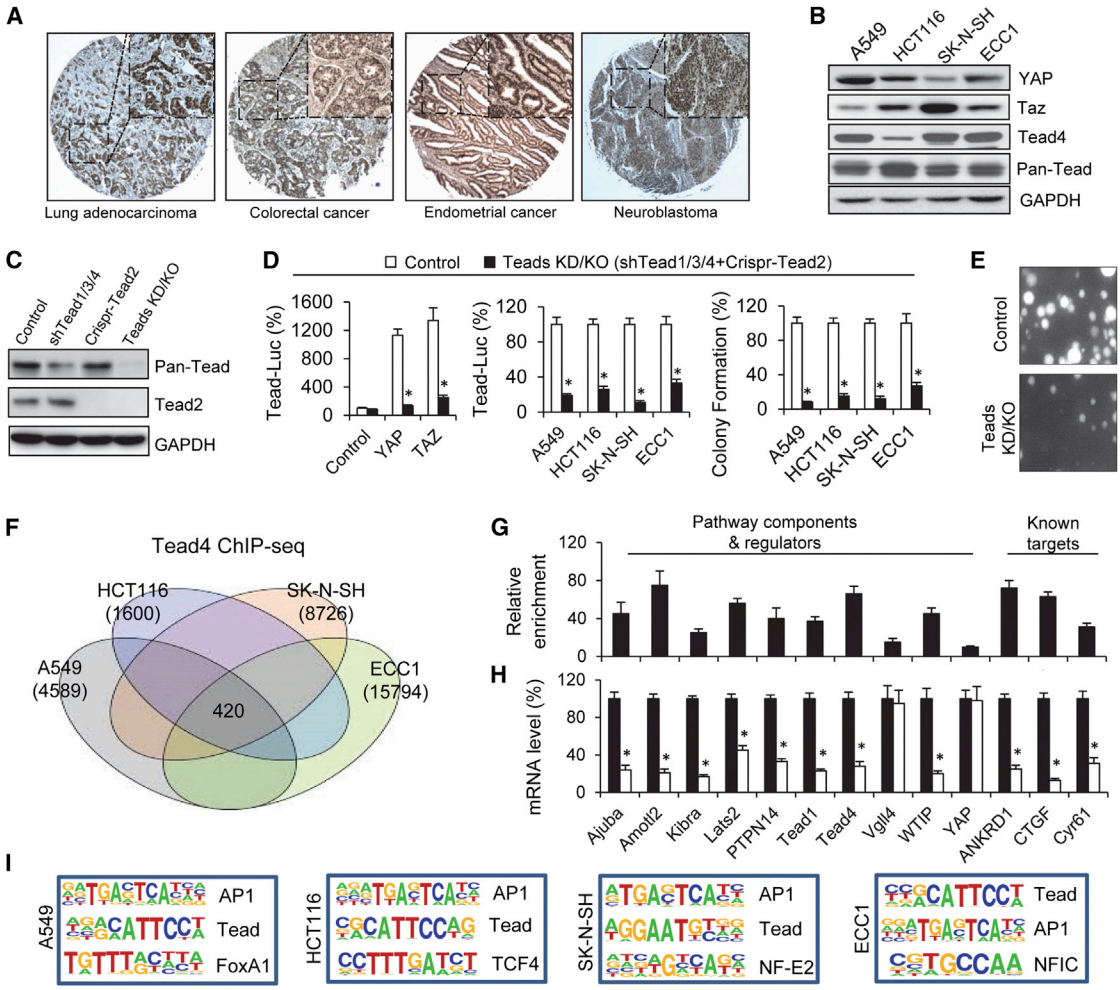

Figure 1. Functional Requirement and Genomic Occupancy of Tead Proteins in A549, HCT116, SK-N-SH, and ECC1 Cancer Cells

(A) Representative IHC images of Tead4 staining showing nuclear expression of Tead4 proteins in human lung adenocarcinoma, colorectal carcinoma, endometrial cancer, and neuroblastoma.

(B) Expression of YAP, TAZ, and Tead factors in A549, HCT116, SK-N-SH, and ECC1 cells. Immunoblot analysis of YAP, TAZ, Tead4, and overall Tead (pan-Tead) protein expression used the antibodies against YAP, TAZ, Tead4, and pan-Tead.

(C) Immunoblot analysis of overall Tead (pan-Tead) protein and Tead2 expression in HCT116 cells expressing shRNA against Tead1, Tead3, and Tead4 (shTead1/3/4); Crispr-mediated Tead2 KO construct (Crispr-Tead2); or both (Teads KD/KO).

(D) Teads KD/KO blocks YAP- or TAZ-induced Tead-Luc reporter activity in HEK293T cells and Tead-dependent transcriptional activity and colony formation in A549, HCT116, SK-N-SH, and ECC1 cells.

(E) Representative images of anchorage-independent colony formation in control and Teads KD/KO-expressing HCT116 cells.

(F) Venn diagram showing overlapping of Tead4 binding sites in A549, HCT116, SK-N-SH, and ECC1 cells identified by Tead4 ChIP-seq.

(G) ChIP-qPCR analysis of selected Tead4 binding sites in the known target genes and the genes involved in pathway feedback regulation. Mean fold enrichment in ChIP is expressed relative to a control β-actin genomic region. Sites are named according to the nearest locus.

(H) The qPCR analysis of the known YAP target genes, ANKRD1, CTGF, and Cyr61, as well as the target genes involved in pathway feedback regulation in HCT116 cells with and without Teads KD/KO.

(I) Enrichment of AP1 motif on Tead4-occupied *cis*-regulatory regions in the genomes of A549, HCT116, SK-N-SH, and ECC1 cells. De novo motif analysis of Tead4 binding sites revealed the presence of the two most enriched motifs of Tead and AP1 in all four genomes.

*$p < 0.01$, error bars indicate mean ± SD. See also Figures S1 and S2 and Table S1.

(Teads KD/KO), which enable both shRNA-mediated knockdown of human Tead1, Tead3, and Tead4 (Zhao et al., 2008) and Crispr-mediated knockout (KO) of human Tead2 (Figure 1C; Figure S1). Furthermore, we showed that Teads KD/KO effectively blocked YAP/TAZ-induced transcriptional activation and inhibited the ability of A549, HCT116, SK-N-SH, and ECC1 cells to form an anchorage-independent colony (Figures 1D and 1E), highlighting the critical functional requirement for Tead proteins in these cancer cells.

Next, we performed the analysis of genome-wide Tead4 chromatin immunoprecipitation sequencing (ChIP-seq) datasets of A549, HCT116, SK-N-SH, and ECC1 cells that are available

at the ENCODE project (http://genome.ucsc.edu/ENCODE/downloads.html). After intersecting the Tead4 ChIP-seq data from these four cancer cell lines (Figure 1F; Table S1), we found that in addition to the known direct YAP target genes, CTGF, Cyr61, and ANKRD1 (Dupont et al., 2011; Lai et al., 2011; Zhao et al., 2008), many genes carrying the Tead4 binding peaks encoded the Hippo pathway components and regulators, such as AmotL2, Ajuba, Tead1, and Tead4 (Figure 1G). Transcription of many of these genes could also be modulated by Tead inhibition (Figure 1H), suggesting an active feedback regulation of the Hippo pathway in cancer cells.

To identify the regulatory mechanism of Tead-mediated transcription, we performed de novo motif analysis of the Tead4 binding regions identified in our ChIP-seq data analysis. In addition to the core Tead motif, the activating protein-1 (AP1) motif was always among the two most enriched sequences within the Tead4 peaks in all four genomes (Figure 1I; Figure S2), suggesting a possible engagement of AP1 in Tead-regulated transcription.

AP1 and Tead4 Co-occupancy at *cis*-Regulatory Regions of Cancer Genomes

The AP1 family transcription factor is a collection of dimeric complexes composed of members of the Jun, Fos, activating transcription factor, and Jun dimerization protein families and has been implicated in a range of diseases, including cancers (Meixner et al., 2010; Sarkar et al., 2011; Schonthaler et al., 2011; Verde et al., 2007). However, the molecular mechanism underlying AP1 activity in tumor cells remains poorly understood. Immunofluorescence staining and western blot analysis showed that many AP1 proteins, including JunD, c-Jun, Fos, Fra1, and Fra2, were expressed in the cancer cells (Figures 2A and 2B) (data not shown), suggesting potential redundancy among AP1 proteins. Consistent with this idea, we showed that Crispr-mediated KO of JunD and c-Jun only partially blocked the activity of an AP1-controlled luciferase (AP1-Luc) reporter (Figures 2C and 2D); however, DN-JunD was able to effectively inhibit AP1-Luc reporter activation (Figure 2D). Dominant negative JunD (DN-JunD) is a dominant repressor form of JunD that lacks the N-terminal transcriptional activation domain but retains the DNA binding domain and the ability to dimerize with other AP1 proteins, thereby inhibiting AP1 activation (Figure S1).

To explore AP1-mediated transcriptional programs in cancer cells, we performed analysis of the JunD, Fra1, and Fra2 ChIP-seq datasets of the A549, HCT116, and SK-N-SH genomes from the ENCODE project (Tables S2 and S3). We then intersected these data with the Tead4 ChIP-seq datasets and found significant overlaps of Tead4 peaks with AP1 (JunD, Fra1, and Fra2) peaks in three cell lines (Figures 2E–2G). Statistical analysis of Tead4 peaks overlapping JunD or Fra1/2 peaks or random genomic regions showed significant enrichment of the AP1 overlapping peaks with the empirical p values at 0 in all cases. More strikingly, more than half of the Tead4 binding regions overlapped with the JunD- or Fra1/2-occupied sites in all three genomes (Figures 2E–2G). Furthermore, we performed de novo motif analysis of the JunD and Fra1/2 peaks identified in three cell lines and showed that the Tead motif was also among the top enriched motifs within the JunD or Fra1/2 binding regions (Figure S2). Thus, our analyses of both Tead4 and AP1 genome-wide ChIP-seq data identified the intensive interactions between Tead4- and AP1-controlled transcriptional networks in a range of human cancer cells.

To further characterize Tead4 and AP1 genomic co-occupancy, we examined the histone modification status of Tead4/AP1 peaks in A549 cells by analysis of genome-wide H3K4me1, H3K4me3, and H3K27ac ChIP-seq data obtained from the ENCODE project. The Tead-AP1 peaks with histone H3 monomethylation at lysine 4 (H3K4me1) were considered as enhancer regions. Active enhancers were defined by the co-presence of H3K4me1 and H3K27ac (histone H3 acetylation at lysine 27), and promoter regions were defined as H3K4me3 (histone H3 trimethylation at lysine 4) and H3K27ac overlapping peaks that have minimal overlapping of 1,000 bp and are close to a transcriptional start site (2,000 bp upstream to 500 bp downstream). Our analysis revealed that most Tead4-AP1 co-occupied peaks were the active enhancer regions (86%), and 12% of them were active promoters (Figures 2H and 2I). In addition, the bimodal distribution of H3K4me1 signal around the peak center (Figure 2J) supported the notion that these Tead-AP1 peaks were in an active state. Further analysis showed that the Tead and AP1 binding motifs were located close to the peak summit (Figure 2J), and the median space between them was about 70 bp. Taken together, these data suggest that Tead and AP1 factors may operate closely in active enhancer or promoter regions to regulate downstream transcription in cancer cells.

Tead and AP1 Coordinate Downstream Gene Transcription

To examine Tead-AP1-mediated co-regulation of downstream transcription, we dissected in detail the Tead4 or AP1 binding *cis*-regulatory elements identified in the ANKRD1, Dock9, and Tead4 loci (Figure 3A). Tead4 is a core Hippo component likely involved in pathway feedback regulation (Figure 1H), and ANKRD1 is a known YAP target gene (Dupont et al., 2011; Lei et al., 2015); however, it was not clear whether Dock9 can be transcriptionally regulated by Tead or AP1. The analysis of histone modification status revealed that these Tead1 or JunD binding sites were located within the active promoter (ANKRD1 and Tead4) or active enhancer (Dock9) regions (Figure 3A). The lack of AP1 occupancy in the peak of the Tead4 locus suggested that its expression may be independent of AP1 (Figure 3A). We found that YAP and TAZ short hairpin RNA (shRNA) knockdown, but not DN-JunD expression, decreased Tead4 expression in HCT116 cells (Figures 3C and 3D). In contrast, ANKRD1 and Dock9 expression was significantly decreased by inhibition of both Tead and AP1 activity (Figure 3B). Furthermore, we generated the luciferase reporter constructs driven by the promoter or enhancer peaks identified in the ANKRD1, Dock9, and Tead4 loci (ANK-Luc, Dock9-Luc, and Tead4-Luc, respectively). We showed that although Tead4-Luc was only responsive to YAP activation (Figure 3G), YAP and JunD co-expression synergistically induced reporter activation of ANK-Luc and Dock9-Luc (Figures 3E and 3F). Together, these data suggest that YAP/Tead-AP1 cooperation on the distal or proximal regulatory regions regulates a subset of target gene expression.

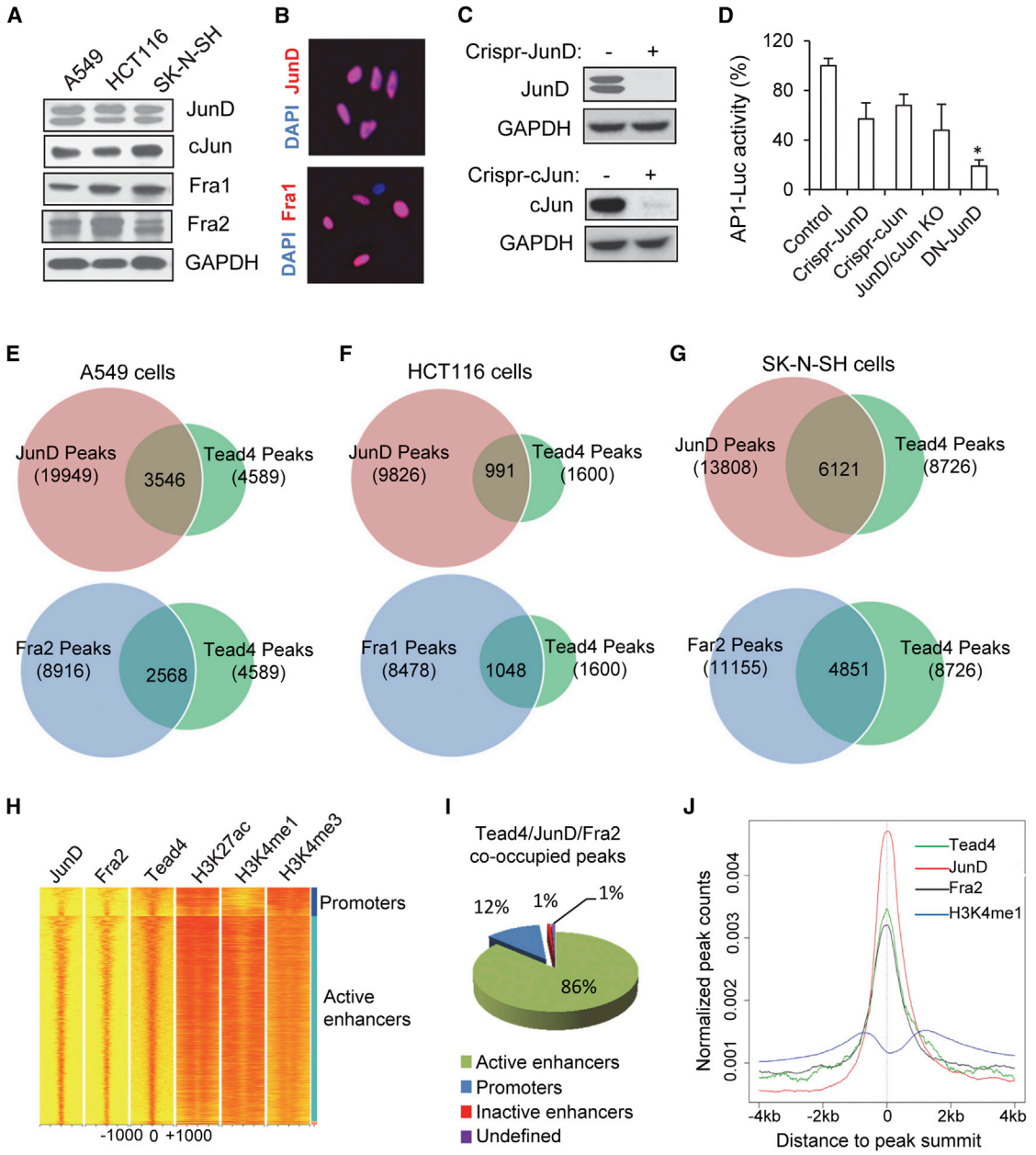

Figure 2. AP1 and Tead4 Co-occupancy at *cis*-Regulatory Regions of Cancer Genomes

(A) Immunoblot analysis of JunD, c-Jun, Fra1, and Fra2 protein expression in A549, HCT116, and SK-N-SH cells.

(B) Immunofluorescence staining of JunD and Fra1 in HCT116 cells. DAPI labels the nuclei.

(C) Immunoblot analysis of JunD and c-Jun expression in HCT116 cells with and without expression of Crispr KO constructs against JunD or c-Jun.

(D) Reporter activity of AP1-Luc in HCT116 cells with the Crispr KO constructs against JunD and c-Jun or the expression construct of DN-JunD.

(E–G) Intersection of Tead4, JunD, and Fra1/2 ChIP-seq in the A549 (E), HCT116 (F), and SK-N-SH (G) genomes, showing significant co-occupancy of Tead4 and JunD/Fra1/2 in all three cell lines.

(H) Heatmap representing Tead4/JunD/Fra2 co-occupied peaks located within promoter or enhancer regions of the A549 genome. The heatmap is sorted by density of Tead4 signals in each category. 0, peak center; ±1,000, 1 kb upstream or downstream of the center.

(I) Percentage of Tead4/JunD/Fra2 co-occupied peaks in the categories of active promoters (H3K4me3^{+};H3K27ac^{+}), active enhancers (H3K4me1^{+};H3K27ac^{+}), and inactive enhancers (H3K4me1^{+};H3K27ac^{-}).

(J) Bimodal distribution of the H3K4me1 signal around the summit of the Tead4/JunD/Fra2 co-occupied peaks.

*$p < 0.01$, error bars indicate mean ± SD. See also Figures S1 and S2 and Tables S2 and S3.

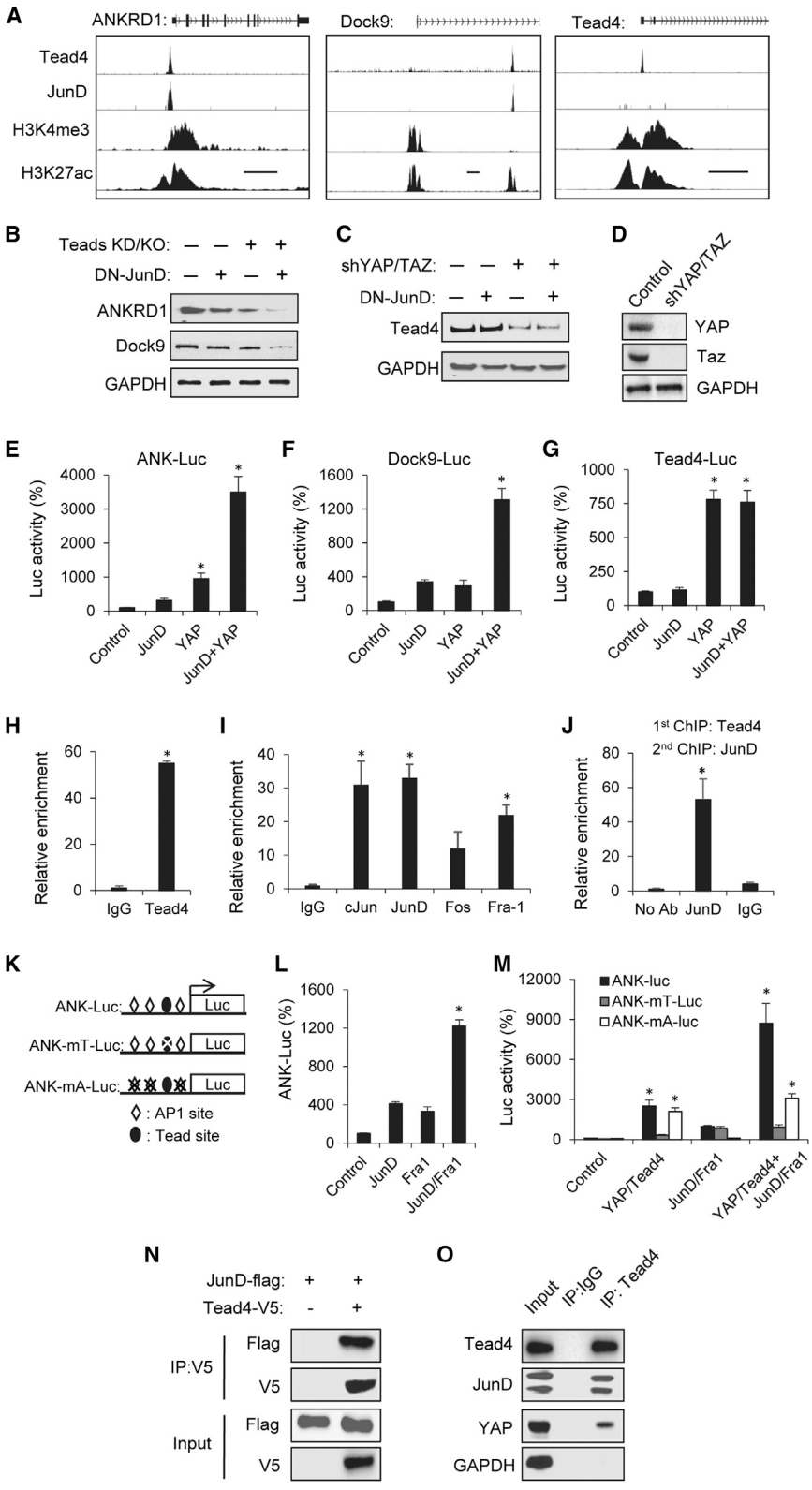

Figure 3. Tead and AP1 Cooperation on Downstream Gene Transcription

(A) Diagram showing the Tead4, JunD, H3K4me3, and H3K27ac peaks in the promoter or enhancer regions of the ANKRD1, Dock9, and Tead4 loci of the HCT116 genome. Scale bar, 2 kb.

(B) Immunoblot analysis of ANKRD1 and Dock9 expression in HCT116 cells with Teads KD/KO or AP1 inhibition by DN-JunD.

(C) Immunoblot analysis of Tead4 expression in HCT116 cells with shRNA knockdown against YAP and TAZ (shYAP/TAZ) or AP1 inhibition by DN-JunD.

(D) Immunoblot analysis of YAP and TAZ expression in HCT116 cells expressing shYAP/TAZ.

(E–G) Luciferase reporters driven by the Tead4 peaks from the ANKRD1, Dock9, and Tead4 loci were generated, and the reporter activity of ANK-Luc (E), Dock9-Luc (F), and Tead4-Luc (G) in HEK293T cells was measured with or without ectopic expression of JunD and YAP.

(H and I) ChIP in HCT116 cells was performed with control immunoglobulin G (IgG), Tead4, c-Jun, JunD, Fos, and Fra1 antibodies as indicated. The enrichment of the ANKRD1 promoter region was calculated based upon qPCR relative to the IgG control.

(J) Sequential ChIP with antibody against Tead4 followed by antibody against JunD confirms the presence of both transcription factors on the ANKRD1 promoter. Enrichment is calculated based upon qPCR relative to the no antibody (No Ab) or IgG control.

(K) Diagrams of the wild-type ANKRD1 luciferase (ANK-Luc) reporter and the luciferase reporters driven by the mutated peak lacking the Tead motif (ANK-mT-Luc) or the AP1 motifs (ANK-mA-Luc).

(L) Activity of the ANK-Luc reporter was measured in HCT116 cells with JunD, Fra1, or both.

(M) Activity of ANK-Luc, ANK-mT-Luc, and ANK-mA-Luc reporters was measured with YAP/Tead4, JunD/Fra1, or both.

(N and O) JunD binds both exogenous and endogenous Tead4 in cells. (N) Indicated plasmids were co-transfected into HEK293T cells, and Tead4 was immunoprecipitated with anti-V5 antibody. Immunoblot analysis shows coIP of JunD detected by anti-FLAG antibody. (O) Tead4 binds to endogenous JunD in HCT116 cells. Endogenous Tead4 was immunoprecipitated with anti-Tead4 antibody, and coIP of JunD and YAP was shown by anti-JunD and anti-YAP immunoblots. A control IgG was used as the negative control for immunoprecipitation.

*$p < 0.01$, error bars indicate mean ± SD.

Our ChIP-qPCR analysis showed that Tead4 and various AP1 proteins, including JunD, c-Jun, Fos, and Fra1, were able to bind to the peak located in the ANKRD1 promoter (Figures 3H and 3I). The co-occupancy of Tead4 and AP1 was confirmed by sequential re-ChIP experiments (Figure 3J). Furthermore, we generated the ANK-Luc-based reporter constructs with a mutated Tead or AP1 binding site (ANK-mT-Luc or ANK-mA-Luc, respectively) (Figure 3K). When JunD and Fra1 were co-expressed, the JunD/Fra1 heterodimer strongly induced ANK-Luc reporter activity (Figure 3L). However, expression of Tead4 alone with JunD/Fra1 did not further enhance reporter activity (data not shown), suggesting that unlike YAP, AP1 is not able to activate Tead proteins. In contrast, when Tead4 was activated by YAP, it acted synergistically with JunD/Fra1 to promote wild-type ANK-Luc reporter activity (Figure 3M). Furthermore, although YAP/Tead4 or JunD/Fra1 was capable of activating AP1 site-mutated or Tead site-mutated luciferase reporters, respectively, YAP/Tead4 and JunD/Fra1 lost the ability to synergize with each other (Figure 3M), suggesting that YAP/Tead and AP1 proteins do not rely on each other to bind to the *cis*-regulatory region but rather act synergistically to achieve maximum transcriptional output.

We also detected JunD and Tead4 protein-protein interactions exogenously in transfected HEK293T cells (Figure 3N) and endogenously in HCT116 cells (Figure 3O). Our co-immunoprecipitation (coIP) assays detected Tead4 binding to endogenous YAP or AP1 proteins, including JunD, c-Jun, and to a lesser degree, Fos (Figure 3O) (data not shown). However, we could not detect strong interaction between YAP and AP1 proteins by YAP and AP1 coIP (data not shown), further suggesting the interaction occurs at the level of the Tead and AP1 transcription factors.

Tead and AP1 Interaction Is JNK Independent

To explore the molecular mechanism underlying Tead-AP1 interaction, we first examined the possible involvement of c-Jun amino-terminal kinases (JNKs). The activity of AP1 proteins can be regulated via N-terminal phosphorylation by upstream JNKs (Davis, 2000). Recent reports also showed that JNK can modulate Hippo/YAP signal transduction in certain contexts (Lee and Yonehara, 2012; Sun and Irvine, 2013; Tomlinson et al., 2010). Therefore, we sought to test whether Tead-AP1 cooperation depends on JNK activity in cancer cells. We used a recently developed, highly specific JNK inhibitor, JNK-IN-8 (Zhang et al., 2012), and showed that JNK-IN-8 treatment of HCT116 or A549 cells was able to effectively block c-Jun phosphorylation but not large tumor suppressor kinase (Lats)-mediated YAP phosphorylation (Figure 4A) (data not shown). JNK inhibition also did not alter YAP cellular localization or the expression of the YAP target genes, CTGF and ANKRD1 (Figure 4B; Figure S3). Furthermore, JNK inhibition or activation by overexpression of a MKK7-JNK construct did not affect Tead-AP1 interaction or transcriptional cooperation, measured by Tead/JunD coIP and ANK-Luc reporter activity (Figure 4C; Figure S3). In addition, we showed that c-Jun4A, a mutant form of c-Jun with all four N-terminal JNK phosphorylation sites mutated, was as efficient to induce ANK-Luc reporter activation as wild-type c-Jun (Figure S3). These results indicated that JNK phosphorylation does not likely play a major role in regulating Tead-AP1 functional interaction, and either an additional or a different mechanism is involved.

Tead-AP1 Cooperation Engages SRC1–3 Co-activators

The p160 family of steroid receptor co-activators, SRC1–3 (also known as NCOA1–3), were originally identified as nuclear hormone co-activators (Xu et al., 2009), although it was later discovered that they can interact with a range of other transcriptional factors, including AP1 proteins, to regulate gene transcription (Lee et al., 1998; Qin et al., 2014; Xu et al., 2009; Yan et al., 2006). A previous report identified all three SRCs as Tead binding partners through a yeast two-hybrid screen and showed they can potentiate Tead-mediated transcription (Belandia and Parker, 2000). However, the cellular context and function of this interaction were not clear. Our immunofluorescence and immunoblot analyses revealed that SRC proteins were expressed in A549, HCT116, SK-N-SH, and ECC1 cancer cells (Figures 4D and 4E) (data not shown). More importantly, we demonstrated the binding of endogenous SRC3, JunD, and Tead4 in HCT116 cells (Figure 4F). To further characterize the interactions among these proteins, we generated the expression constructs of three truncated mutants of SRC3 fused with a C-terminal V5 tag: SRC3-N, SRC3-M, and SRC3-C (Figure 4G). We then examined their ability to bind to endogenous Tead4 and JunD proteins using the coIP assay. Consistent with the previous report (Belandia and Parker, 2000), we showed that SRC3 interacted with Tead4 through its N-terminal basic-helix-loop-helix (bHLH)-PAS domain (Figure 4H). We found that the SRC3 domain responsible for JunD binding was mainly located in its C terminus (Figure 4H). These data raised an intriguing possibility that SRC factors bridge the interaction between Tead and AP1, thereby mediating their cooperation.

Consistent with this idea, our SRC3 ChIP-qPCR analysis revealed that SRC3 was significantly more enriched at the ANKRD1 peak with Tead and AP1 co-occupancy than at the Tead4 peak with only Tead occupancy (Figure 4I). To further examine the importance of SRC proteins in Tead-AP1 cooperation, we generated the Crispr-based constructs to knock out all three SRC proteins in HCT116 cells (Figure 4J). In addition, we used a recently identified SRC1/3 specific inhibitor, Bufalin (Figure 4K) (Wang et al., 2014). We found that inhibition of SRC function by SRC1–3 Crispr KO or Bufalin treatment did not affect Tead4 protein expression (Figure 4K) or Tead-Luc reporter activity induced by YAP5SA, a constitutively active form of YAP (Figure 4L) (Zhao et al., 2008). However, we found that SRC inhibition significantly blocked the endogenous Tead-JunD interaction (Figure 4M) and inhibited the synergistic effect on ANK-Luc reporter activation by YAP/Tead-JunD/Fra1 co-expression (Figure 4N). Taken together, these studies suggested that Tead-AP1 cooperation is mediated at least in part by the SRC1–3 co-activators.

Tead-AP1 Drives a Core Set of Target Genes to Regulate Migration and Invasion

Our analysis of Tead4 ChIP-seq in a range of cancer cells identified the previously known YAP/Tead targets, including CTGF, Cyr61, Axl, Birc5, and AREG, involved in regulation of proliferation and apoptosis (Dong et al., 2007; Lai et al., 2011;

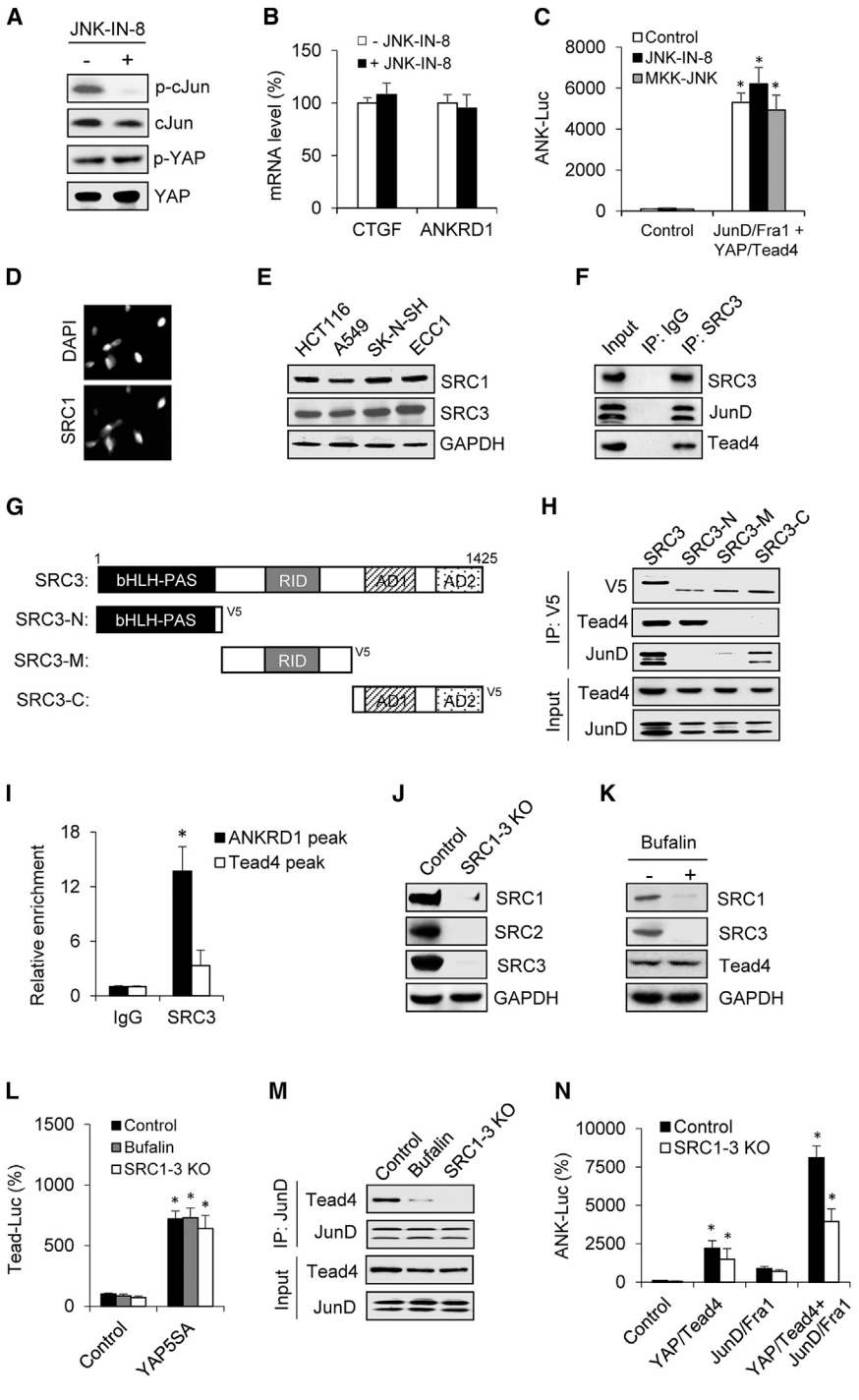

Figure 4. JNK-Independent AP1-Tead Interaction Engages SRC1–3 Co-activators

(A) Immunoblot analysis of c-Jun, phosphorylated c-Jun (p-cJun), YAP, and phosphorylated YAP (p-YAP) in HCT116 cells with or without the JNK inhibitor JNK-IN-8.

(B) A qPCR analysis of CTGF and ANKRD1 transcription in HCT116 cells with or without JNK-IN-8.

(C) JNK inhibition or activation does not affect Tead/AP1 cooperation. Activity of the ANK-Luc reporter induced by JunD/Fra1 and YAP/Tead4 was measured with the presence of JNK-IN-8 or the expression of MKK7-JNK.

(D) Immunofluorescence staining of SRC1 in HCT116 cells. DAPI labels the nuclei of HCT116 cells.

(E) Expression of SRC1 and SRC3 proteins in A549, HCT116, SK-N-SH, and ECC1 cells, assayed by immunoblotting.

(F) SRC3 binds to endogenous JunD and Tead4 in HCT116 cells. Endogenous SRC3 was immunoprecipitated with anti-SRC3 antibody, and coIP of JunD and Tead4 was shown by anti-JunD and anti-Tead4 immunoblots. A control immunoglobulin G (IgG) was used as the negative control for immunoprecipitation.

(G) Diagrams of wild-type and truncated SRC3 constructs with a C-terminal V5 tag. Indicated are the bHLH-PAS domain, the nuclear receptor interacting domain (RID), and activation domains 1 and 2 (AD1 and AD2, respectively) in SRC3, SRC3-N, SRC3-M, and SRC3-C.

(H) In HCT116 cells expressing wild-type and truncated SRC3 proteins, immunoprecipitation was performed with anti-V5 antibody, and coIP of endogenous Tead4 and JunD was shown by anti-Tead4 and anti-JunD immunoblots.

(I) ChIP from HCT116 cells was performed with control IgG or the SRC3 antibody as indicated. The enrichment of the ANKRD1 and Tead4 peak was calculated based upon qPCR relative to the IgG control.

(J) Immunoblot analysis of SRC1, SRC2, and SRC3 in HCT116 cells with or without expression of Crispr KO constructs against SRC1, SRC2, and SRC3 (SRC1–3 KO).

(K) Immunoblot analysis of SRC1, SRC3, and Tead4 in HCT116 cells with or without the SRC1/3 inhibitor, Bufalin.

(L) Activity of the Tead-Luc reporter induced by YAP5SA in HEK293T cells with or without SRC1–3 Crispr KO or Bufalin treatment.

(M) In HCT116 cells with SRC1–3 Crispr KO or Bufalin treatment, endogenous JunD was immunoprecipitated with anti-JunD antibody and coIP of endogenous Tead4 was shown by anti-Tead4 immunoblots.

(N) In HCT116 cells expressing SRC1–3 KO, reporter activity of ANK-Luc was measured with YAP/Tead4, JunD/Fra1, or both.

*$p < 0.05$, **$p < 0.01$, error bars indicate mean ± SD. See also Figure S3.

Xu et al., 2011; Zhang et al., 2009b; Zhao et al., 2008). However, it was not clear whether the cooperation between YAP/Tead and AP1 drives additional function or distinct targets in cancer cells.

To explore potential downstream transcriptional programs controlled by Tead and AP1, we performed functional clustering analysis of the overlapping genes with Tead4, JunD, and Fra1/2 peaks from A549, HCT116, and SK-N-SH cells (Figure 5A). By

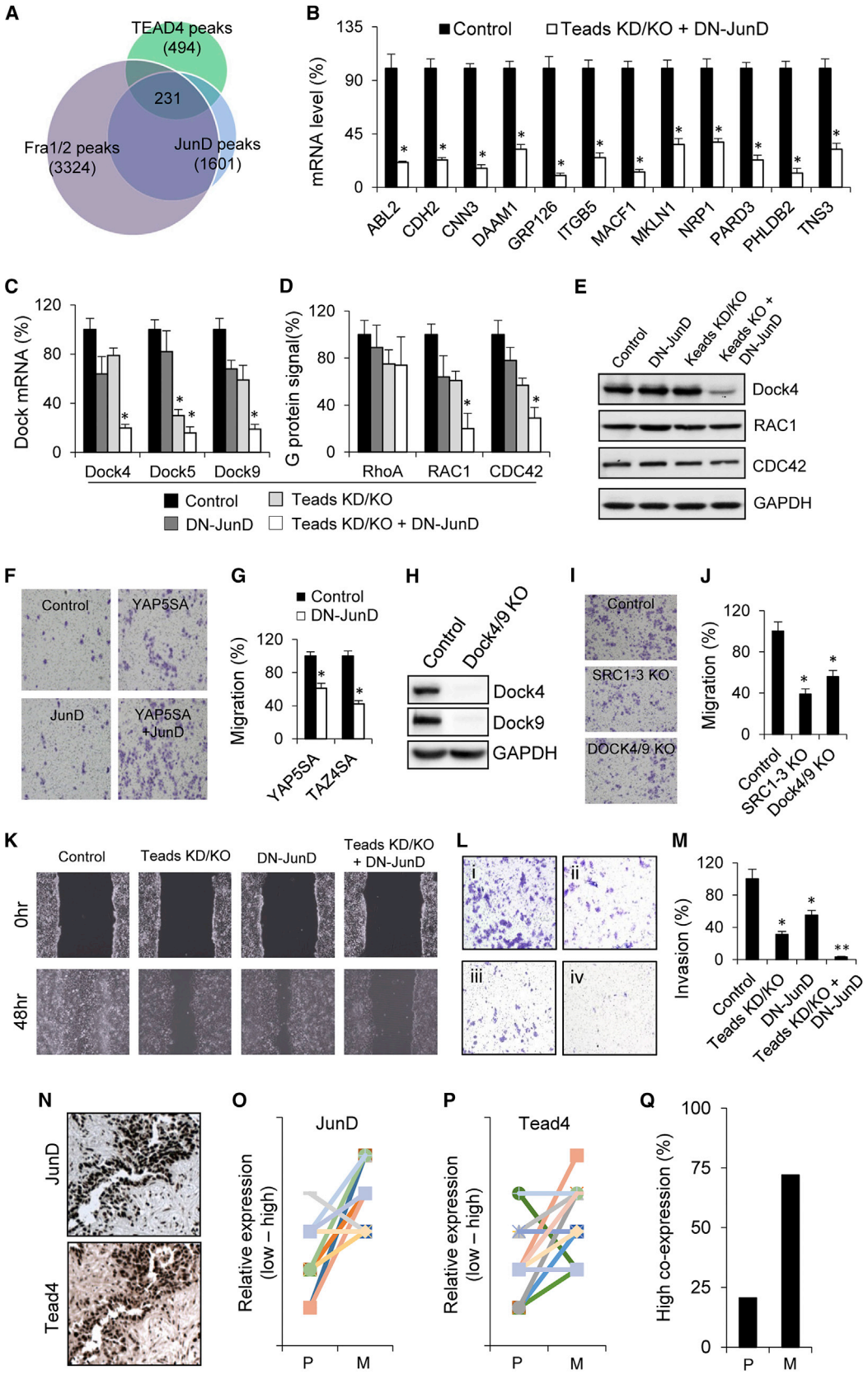

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Table 1. Functional Clustering of the Overlapping Genes Carrying Tead4 Peaks or Tead/AP1 Co-occupied Peaks from A549, HCT116, and SK-N-SH Cells

Tead4 Peaks	Tead-AP1 Peaks
blood vessel morphogenesis	blood vessel morphogenesis
transcription activator activity	focal adhesion[a]
regulation of transcription	regulation of cell adhesion[a]
regulation of kinase activity	cell-cell adhesion[a]
regulation of cell motion[a]	cell migration[a]
pattern specification process	regulation of kinase activity
lung development	regulation of protein modification process
focal adhesion[a]	cytoskeletal protein binding
chordate embryonic development	regulation of cell motion[a]
regulation of protein modification process	regulation of cell growth
regulation of RNA metabolic process	contractile fiber[a]
protein amino acid phosphorylation	protein amino acid phosphorylation
transforming growth factor β receptor binding	actin cytoskeleton organization[a]
growth factor binding	guanosine triphosphatase activation
regulation of cell growth	embryonic morphogenesis

[a]Functional clusters associated with cell motility.

comparing the functional clustering data between the genes with the Tead4 peaks and the genes with Tead4/AP1 co-occupied peaks, we found significant enrichment of the clusters associated with cell adhesion, motility, and migration within the genes carrying the Tead/AP1 co-occupied peaks (Table 1), suggesting a potential regulation of cell migration and invasion by Tead-AP1. In addition, Tead4, JunD, and Fra1/2 often bound to the distal or proximal regulatory regions of the genes encoding the Dock proteins (Tables S1, S2, S3, and S4). Dock is a family of 11 related proteins functioning as the specific guanine nucleotide exchange factors for the small G proteins Rac and Cdc42 that play a pivotal role in orchestrating cell adhesion and movement (Laurin and Côté, 2014). Our qPCR and immunoblot analyses in HCT116 cells showed that transcription and expression of many Dock factors, including Dock4, Dock5, and Dock9, were downregulated by inhibition of both AP1 and Tead activation (Figures 3B, 5C, and 5E; Figure S4). Moreover, we found that the activity of Rac1 and CDC42, but not the protein levels, was significantly decreased following Tead and AP1 inhibition (Figures 5D and 5E). In contrast, the activity and expression of RhoA, the other member of the Rho small G protein family, were largely unaffected (Figures 5D and 5E). These data suggested that Tead and AP1 act as important upstream regulators for the Dock-Rac/CDC42 functional module in cancer cells.

In addition to the Dock-Rac/CDC42 axis, our functional annotation revealed that more than 50 of the 300 genes with Tead and AP1 co-occupied peaks were directly implicated in regulation of cell migration and invasion (Table S4). Among them, we found that transcription of ABL2, CDH2, CNN3, DAAM1, GRP126, ITBG5, MACF1, MKLN1, NRP1, PARD3, PHLDB2, and TNS3 was inhibited by blocking Tead and AP1 activity in HCT116 cells (Figure 5B; Figure S4), suggesting that these genes represent a core set of new direct targets of Tead and AP1 in cancer cells. Moreover, these results support the idea that a key functional output of YAP/Tead-AP1 cooperation is to regulate cancer cell migration and invasion.

To test this hypothesis, we ectopically expressed YAP5SA and JunD in the HT29 cells, a relatively less invasive colon cancer cell line (de Both et al., 1999), and found that YAP5SA and JunD co-expression significantly promoted migration of HT29 cells, as measured by transwell migration assay (Figure 5F). In addition, we showed that migration induced by YAP5SA or TAZ4SA (an active form of TAZ) was partially inhibited by DN-JunD (Figure 5G). Furthermore, we found that Crispr-mediated SRC1–3 KO or Dock4 and Dock9 KO inhibited YAP5SA/JunD-dependent

Figure 5. Tead and AP1 Drive a Core Set of Downstream Targets to Regulate Cell Migration and Invasion

(A) Venn diagram showing the overlapping Tead4, JunD, and Fra1/2 peaks identified by ChIP-seq in A549, HCT116, and SK-N-SH genomes.
(B) A qPCR analysis of the transcription of selected target genes involved in the regulation of cell migration and invasion in HCT116 cells with Teads KD/KO and AP1 inhibition by DN-JunD.
(C) A qPCR analysis of the transcription of Dock4, Dock5, and Dock9 in HCT116 cells with Teads KD/KO or AP1 inhibition by DN-JunD.
(D) Measurement of the activity of the Rho family small G proteins, RhoA, RAC1, and CDC42, in HCT116 cells with Teads KD/KO or AP1 inhibition by DN-JunD.
(E) Immunoblot analysis of DOCK4, RAC1, and CDC42 in HCT116 cells expressing DN-JunD, Teads KD/KO, or both.
(F) Representative images of transwell migration of HT29 cells with ectopic expression of JunD, YAP5SA, or both.
(G) Relative migration activity of HT29 cells expressing YAP5SA or TAZ4SA with or without DN-JunD.
(H) Immunoblot analysis of Dock4 and Dock9 in HCT116 cells expressing Crispr KO constructs against Dock4 and Dock9 (Dock4/9 KO).
(I) Representative images of transwell migration of YAP5SA/JunD-expressing HT29 cells with SRC1–3 or Dock4/9 KO.
(J) Quantification of the transwell migration assay shown in (I).
(K) Representative images of the cell scratch assay of HCT116 cells expressing DN-JunD or Teads KD/KO at 0 or 48 hr.
(L) Representative images of the matrigel invasion assay in control (i) or HCT116 cells expressing DN-JunD (ii), Teads KD/KO (iii), or both (iv).
(M) Quantification of the matrigel invasion assay shown in (L).
(N–Q) Co-expression of JunD and Tead4 proteins in human lung adenocarcinomas (n = 30) and matched lymph node metastases (n = 30) by tissue microarray assays. (N) Representative IHC images of nuclear expression of JunD and Tead4 in lymph node metastases of lung adenocarcinoma. (O and P) Higher expression of JunD (O) and Tead4 (P) in lymph node metastases in comparison to matched primary lung adenocarcinoma samples. (Q) The percentage of co-expression of high-level JunD and Tead proteins in primary lung adenocarcinoma and matched lymph node metastases. P, primary lung adenocarcinoma; M, matched lymph node metastases.
*$p < 0.05$, **$p < 0.01$, error bars indicate mean ± SD. See also Figure S4 and Table S4.

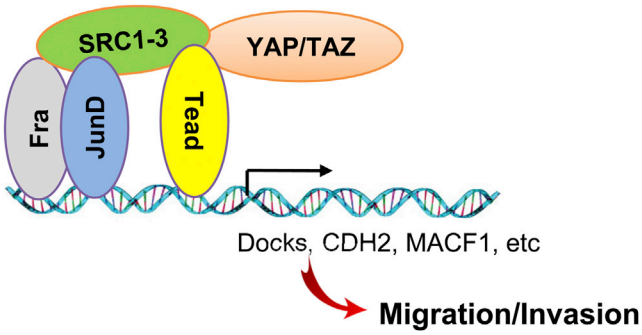

Figure 6. YAP/Tead-AP1 Cooperation on *cis*-Regulatory Regions Regulates Migration and Invasion

A schematic model showing YAP/Tead-AP1 cooperation on *cis*-regulatory regions engages SRC1–3 co-activators and drives downstream gene expression to regulate cancer cell migration and invasion.

migration in HT29 cells (Figures 5H–5J), highlighting the functional importance of these proteins in YAP/Tead-AP1 cooperation. Next, we demonstrated that the combined inhibition of Tead and AP1 activity in the highly invasive HCT116 colon cancer cells (de Both et al., 1999) led to effective inhibition of cell migration and invasion, measured by cell scratch and matrigel invasion assays (Figures 5K–5M). Moreover, we analyzed the expression of JunD and Tead4 in 62 human lung adenocarcinomas and their matched lymph node metastases and found that JunD and Tead4 proteins were expressed significantly higher in the lymph node metastases than in the matched primary cancers (Figures 5N–5Q), suggesting the potential involvement of Tead-AP1 cooperation in promoting tumor invasion and metastasis.

DISCUSSION

By intersecting the transcriptional networks employed by both Tead4 and AP1 proteins in diverse cancer cells, we uncovered a critical mechanism underlying Tead- and AP1-mediated oncogenic regulation. Our data support a model that Tead and AP1 interaction at the active *cis*-regulatory genomic regions promotes or maximizes transcription output of target genes (Figure 6). The Tead-AP1 cooperation we described here is distinct from the recently reported Tead-independent Fos regulation by Kras and YAP in pancreatic cancer cells (Shao et al., 2014). However, our data are consistent with a recent report of Tead and AP1 co-occupancy on *cis*-regulatory regions in the invasive melanoma genome (Verfaillie et al., 2015). Furthermore, during the revision of this manuscript, a recent study described the genomic association between YAP/TAZ/Tead and AP1 in breast cancer cells that drives oncogenic growth (Zanconato et al., 2015). Our analyses of both AP1 and Tead4 genome-wide ChIP-seq data in a range of tumor cells, including neuroblastoma, colon, lung, and endometrial cancers, suggest that Tead-AP1 cooperation on transcription is a general mechanism used by both families of transcription factors during tumorigenesis.

Recent reports showed the possible JNK regulation of Hippo signaling via upstream Lats kinases or direct phosphorylation of YAP in fly and mammalian cells during apoptosis (Lee and Yonehara, 2012; Sun and Irvine, 2013; Tomlinson et al., 2010). However, our data indicate that Tead-AP1 cooperation in cancer cells does not appear to require JNK phosphorylation, highlighting the complex crosstalks between these pathways in different functional and cellular contexts. More importantly, our study revealed a key molecular mechanism underlying Tead-AP1 interaction that is mediated by the SRC1–3 transcriptional co-factors (Figure 6). The SRC1–3 family transcriptional co-activators, originally identified as nuclear receptor co-regulators, have been shown to interact with a variety of transcription factors. Our study suggest a model in which Tead and AP1 co-occupancy at the promoter and active enhancer regions engages SRC factors, which bind to Tead and AP1 through distinct N-terminal and C-terminal domains to bridge the Tead-AP1 interaction, thereby mediating at least in part their cooperation on downstream transcription in cancer cells (Figure 6).

Current knowledge of the YAP/Tead downstream targets in cancers has been largely focused on the genes, such as CTGF, Cyr61, Birc5, AXL, and AREG, that are involved in regulation of proliferation, apoptosis, and oncogenic growth (Dong et al., 2007; Lai et al., 2011; Xu et al., 2011; Zanconato et al., 2015; Zhang et al., 2009b; Zhao et al., 2008). Our results not only identified a core subset of downstream YAP/Tead target genes but also uncovered a critical functional output of Tead-AP1 in coordination of cell migration and invasion in diverse types of cancer cells (Figure 6). These Tead and AP1 downstream targets include members of the Dock family proteins and other genes such as CDH2, MACF1, ABL2, and TNS3, which all have been directly implicated in controlling different aspects of cell motility, including cytoskeleton organization, cell adhesion, and migration (Bradley and Koleske, 2009; Laurin and Côté, 2014; Qian et al., 2009; Wu et al., 2008b). Recent studies have reported that cell attachment, cell-matrix interaction, and mechanical forces can influence upstream Hippo signaling converging on YAP/TAZ regulation (Chang et al., 2015; Dupont et al., 2011; Halder et al., 2012; Zhao et al., 2012). Our study suggests the importance of the interplay and possible feedback regulation between Tead-AP1 activation and cell adhesion, migration, and invasion during tumorigenesis and metastasis.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Lentiviral Infection

HCT116, SK-N-SH, ECC1, FET, DLD1, LS174T, and HEK293T cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). A549 cells were cultured in F-12K medium supplemented with 10% FBS. HT29 cells were cultured in McCoy 5A medium supplemented with 10% FBS. Caco2 and RKO cells were cultured in Eagle's minimal essential medium supplemented with 20% FBS. SW48 cells were cultured in L15 medium supplemented with 10% FBS. Detailed information of lentiviral expression vectors, Crispr KO or shRNA knockdown constructs, and luciferase reporter constructs are described in Supplemental Experimental Procedures.

Cell and Biochemical Assays

For IHC, human cancer tissue microarray samples were purchased from Biomax, Genvelop, or the University of Massachusetts Cancer Center Tissue Bank. Antibody information for IHC, immunofluorescence, and immunoblot analyses are shown in Supplemental Experimental Procedures. Detailed protocols for assaying cell migration and invasion, anchorage-independent

colony formation, ChIP, protein immunoprecipitation, and small G protein activation are also described in Supplemental Experimental Procedures.

ChIP-Seq, De Novo Motif Discovery, and Functional Clustering Analyses

Tead4, JunD, Fra1, Fra2, H3K4me1, H3K4me3, and H3K27ac ChIP-seq data were obtained from the ENCODE project, and data analysis and de novo motif discovery were performed using Homer software (http://homer.salk.edu/homer/). Functional clustering analysis was done using DAVID v.6.7 (https://david.ncifcrf.gov/). Additional details are provided in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.12.104.

AUTHOR CONTRIBUTIONS

X.L., H.L., M.R., Q.L., J.L.C., and H.L.G. conducted the experiments and analyzed the data. J.O., L.J.Z., and J.-S.P. performed bioinformatics analyses. A.M.M., R.J.D., and J.M. designed the experiment. J.M. supervised the project and wrote the manuscript.

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