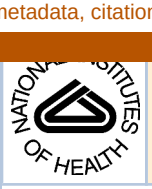

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Transcriptional role of cyclin D1 in development revealed by a "genetic-proteomic" screen

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Abstract

Cyclin D1 belongs to the core cell cycle machinery, and it is frequently overexpressed in human cancers[1,2]. The full repertoire of cyclin D1 functions in normal development and in oncogenesis is currently unclear. Here we developed FLAG- and HA-tagged cyclin D1 knock-in mouse strains that allowed high-throughput mass spectrometry approach to search for cyclin D1-binding proteins in different mouse organs. In addition to cell cycle partners, we observed several proteins involved in transcription. Genome-wide location (ChIP-chip) analyses revealed that during mouse development cyclin D1 occupies promoters of abundantly expressed genes. In particular, we found that in developing mouse retinas – an organ that critically requires cyclin D1 function[3,4] – cyclin D1 binds the upstream regulatory region of the *Notch1* gene where it serves to recruit CBP histone acetyltransferase. Genetic ablation of cyclin D1 resulted in decreased CBP recruitment, decreased histone acetylation of the *Notch1* promoter region, and led to decreased levels of the Notch transcript and protein in cyclin D1-null retinas. Transduction of an activated allele of Notch1 into cyclin $D1^{-/-}$ retinas increased proliferation of retinal progenitor cells, indicating that upregulating Notch1 signaling alleviates the phenotype of cyclin D1-deficiency. These studies reveal that in addition to its well-established cell cycle roles, cyclin D1 plays an *in vivo* transcriptional function in mouse

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Author Contributions F.B. and PS designed the study, analyzed the data and wrote the manuscript. F.B. performed the experiments with the help of co-authors as detailed below. S.J. performed protein purifications. J.E.E. performed and together with S.P.G. analyzed and interpreted mass spectrometry analyses. C.A.M and X.S.L. contributed biocomputational analyses, K.M and C.L. C. contributed *in vivo* transduction of D1-null retinas with Notch1, A.M, G.M.F, M.F.C, D.O and R.A.Y. contributed to analyses of ChIP-chip and gene expression data, J.O., Y.G., A.Z. and M.J. helped with the experiments. P.S. directed the study.

Author Information The complete ChIP-chip and expression datasets have been submitted to the online data repository GEO, record GSE13636 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=zvuhbiaakuwumxa&acc=GSE13636). Reprints and permissions information is available at www.nature.com/reprints.

development. Our approach, which we term "genetic-proteomic" can be used to study the *in vivo* function of essentially any protein.

To study the molecular functions of cyclin D1 during development and in cancer formation, we generated knock-in mouse strains in which tandem (FLAG- and HA-) tags were inserted into the endogenous *cyclin D1* locus through homologous recombination in embryonal stem cells. Tags were introduced into N-terminus of cyclin D1 ($D1^{Ntag}$ allele) or into C-terminus ($D1^{Ctag}$) and homozygous $D1^{Ntag/Ntag}$ and $D1^{Ctag/Ctag}$ mice were obtained (Supplementary Fig. 1). We reasoned that tagged knock-in mice would allow us to use sequential immunoaffinity purifications with anti-FLAG and –HA antibodies, followed by repeated rounds of extensive, high-throughput mass spectrometry, to determine the repertoire of cyclin D1-interacting proteins in different mouse organs under normal conditions, or during tumorigenesis.

In tissues of knock-in animals, the expression of the tagged cyclin D1 mirrored the levels of wild-type D1 in control animals, and the tagged D1 retained the ability to interact with its several known protein partners and to activate Cdk-kinase activity (Supplementary Fig. 2). Moreover tagged cyclin D1 afforded normal development of D1-dependent compartments and restored normal breast cancer-susceptibility in homozygous $D1^{tag/tag}$ animals, revealing that the tagged protein is fully functional *in vivo* (Supplementary Fig. 3).

In proteomic analyses, we focused on embryonic brains, retinas, mammary glands of postpartum females and mammary carcinomas arising in *MMTV-ErbB2* females, as these compartments were shown to critically require cyclin D1 function[3–5]. We purified cyclin D1-containing complexes from these compartments (Fig. 1a), and the identities of D1-associated proteins were determined by exhaustive rounds of "shotgun" liquid chromatography and tandem mass spectrometry (LC-MS/MS).

Among cyclin D1 interactors, we detected known cell cycle partners of D1, including cyclin-dependent kinases Cdk4 and Cdk6, and cell cycle inhibitors from KIP/CIP and INK families. We also observed interaction with Cdk1, Cdk2, Cdk5, and Cdk11 and found that the quantitative composition of these cyclin D1-containing complexes varies between organs (Fig. 1b, Supplementary Tables 1–3, Supplementary Fig. 4a).

Screening the list of interactors for ontology categories revealed enrichment for cell cycle ($p= 0.025$), as expected, but also for transcriptional regulation ($p= 0.040$) and apoptosis ($p= 0.084$) (Fig. 1c). Indeed, based on *in vitro* and cell culture analyses, D-cyclins were proposed to play Cdk-kinase-independent functions in transcription by acting as molecular bridges between DNA-bound transcription factors and chromatin modifying enzymes[6–8]. Cyclin D1 was shown to interact with these proteins via domains that are distinct from the one required for Cdk-binding and activation[9–11]. Our proteomic analyses suggested that cyclin D1 may indeed play a transcriptional function *in vivo*, during mouse development. For this reason, we decided to further study the link between cyclin D1 and transcriptional machinery.

We employed chromatin immunoprecipitation coupled to DNA-microarray analysis (ChIP-chip) to study association of cyclin D1 with genomic DNA sequences. Since anti-FLAG antibodies have been successfully used for ChIP-chip in several systems including murine cells[12], mice expressing tagged cyclin D1 provided us with a tool to query association of D1 with the genome.

We immunoprecipitated cyclin D1, along with associated DNA sequences from tagged E14.5 knock-in embryos using anti-FLAG antibodies, and hybridized immunoprecipitated DNA onto arrays. We detected binding of cyclin D1 to promoter regions (over 900 at highest-stringency

threshold $p< 1 \times 10^{-4}$; Fig. 2a, Supplementary Fig. 5a,b, Supplementary Tables 4, 5). Analyses of the exact location of cyclin D1 binding events revealed that D1 interacts with DNA in close proximity to transcription start sites (in 79% of cases within 1 kb) (Fig. 2b, c). Bioinformatics search for conserved DNA sequence motifs among D1-bound genomic fragments identified six enriched ($p<0.0005$) motifs that correspond to DNA-recognition sequences for transcription factors NF-Y, STAT, CREB2, ELK1, ZNF423 and CUX1 (Fig. 2d). Physical interaction of cyclin D1 with these transcription factors was verified using immunoprecipitation-Western blotting (Supplementary Fig. 4b).

To study the transcriptional function of cyclin D1 at a mechanistic level, and to test its biological significance, we focused on developing retinas. This organ critically requires cyclin D1 function, as evidenced by severe retinal hypoplasia in D1-null mice[3,4]. We found an 80% overlap between D1 targets identified in the whole embryo ChIP-chip versus in retinas (Supplementary Fig. 5c, data not shown).

Gene expression in developing mouse retinas had previously been profiled at multiple time-points using serial analysis of gene expression (SAGE), thereby providing a quantitative measure of the levels of particular transcripts[13]. We queried retinal SAGE libraries (http://cepko.med.harvard.edu/) against our ChIP-chip data, to test the correlation between binding of cyclin D1 to gene promoters versus the abundance of these transcripts. We found that genes whose promoters are bound by D1 belong to the abundantly expressed genes ($p < 10^{-15}$) (Fig. 2e).

Genes that are highly expressed in many tissues were shown to display high content of CpG dinucleotides in their promoter regions[14]. We performed a computational comparison of CpG content within cyclin D1-bound versus all other promoters. D1-bound promoters were highly enriched for CpG dinucleotide ($p < 1 \times 10^{-15}$) (Supplementary Fig. 6a), further strengthening the notion that cyclin D1 occupies promoters of abundantly expressed genes.

We next asked whether in retinal cells cyclin D1 is brought to DNA via sequence-specific transcription factors. As mentioned above, we observed enrichment of NF-Y DNA recognition sequences among cyclin D1-bound genomic fragments (Fig. 2d), and verified physical association of D1 with NF-Y (Supplementary Fig. 4b). We knocked-down NF-YA subunit in *in vitro* cultured rat retinal precursor R28 cells and found that this diminished recruitment of cyclin D1 to several NF-Y target promoters (Supplementary Fig. 7). These findings suggest that cyclin D1 interacts with DNA through DNA-bound transcription factors.

To determine whether cyclin D1 functioned to positively or negatively regulate transcription of target genes *in vivo*, we compared gene expression profiles between wild-type and D1-knockout[4] retinas using microarrays (Supplementary Fig. 6b, Supplementary Table 6), and overlaid the data with ChIP-chip promoter-occupancy results. Among D1-bound genes, we observed genes with increased as well as decreased levels in $D1^{-/-}$ retinas (Fig. 3a, Supplementary Fig. 8, Supplementary Table 7), suggesting that cyclin D1 can serve both to activate as well as to downregulate gene expression.

Inspection of the list of genes bound by cyclin D1 and showing altered expression in D1-null retinas, revealed the presence of *Notch1* – an essential regulator of retinal progenitor cell proliferation[15,16] – along with several transcriptional regulators that likely play role in this process (*Id3*, *Id1*, *Mrg1*, *Tcf4*)[17–19]. We verified binding of D1 to upstream regulatory regions of these genes (Fig. 3b, Supplementary Fig. 9) and their altered expression in D1-null retinas (Fig. 3c, Supplemental Fig. 6c).

The rate-limiting role of Notch1 in retinal development is well-established, and retinal-specific ablation of *Notch1* gene leads to a proliferative failure that resembles the D1-null

phenotype[3,4,20]. Despite overall hypoplasia, *D1*$^{-/-}$ retinas display excess of photoreceptor cells, with reduction of early-born horizontal and amacrine cells, again resembling fate-specification defects seen in Notch1-knockout retinas[20,21]. For this reason we further investigated the cyclin D1-Notch1 connection.

Activation of Notch1 during retinal development leads to upregulation of Hes5, which in turn represses the expression of proneural bHLH genes Math5 and Neurod1. Consequently, in Notch1-knockout retinas, Hes5 is downregulated, while Math5 and Neurod1 genes are de-repressed[22,23]. We found that in *D1*$^{-/-}$ retinas Hes5 transcript levels were decreased, while Neurod1 levels were elevated, suggesting that Notch signaling is compromised in D1-knockout retinas (Fig. 3d). We also found that the levels of Notch1 protein were significantly decreased in D1-null retinas (Fig. 3e). Knockdown of cyclin D1 in R28 cells decreased the levels of Notch1 transcripts and protein, while D1-overexpression had an opposite effect (Fig. 3f). Collectively, these observations indicate that cyclin D1 transcriptionally upregulates *Notch1* gene during retinal development. Consequently, Notch1 transcript and protein levels, as well as Notch-dependent signaling pathways are compromised in D1-null retinas.

We next asked whether increasing Notch1 signaling in *D1*$^{-/-}$ retinas would enhance progenitor cell proliferation. We injected a retroviral construct encoding the Notch intracellular domain (NICD, a constitutively activated allele of Notch1, Supplemental Fig. 10), into subretinal space of postnatal day 0 cyclin *D1*$^{-/-}$ pups, and gauged the response after ten days. Expression of NICD in *D1*$^{-/-}$ retinas significantly increased *in vivo* proliferation of retinal progenitor cells (Fig. 4a–c). Hence, restoring Notch1 signaling in D1-null progenitor cells alleviates the phenotype of cyclin D1-deficiency.

We asked how mechanistically cyclin D1 acts on the *Notch1* promoter to increase Notch1 expression. Cyclin D1 was previously shown to physically bind histone acetyltransferases, and postulated to bring them to target promoters[7,24,25]. Indeed, our mass spectrometry analyses detected CBP acetyltransferase among cyclin D1 protein partners in developing retinas (Supplementary Tables 1 and 2); physical interaction of D1 with CBP was confirmed by immunoprecipitation-Western blotting (Fig. 4d). We determined, using targeted ChIP with anti-CBP antibodies that in developing retinas CBP binds the same upstream regulatory region of the *Notch1* gene as does cyclin D1 (Fig. 4e, g, Supplementary Fig. 9). Co-localization of cyclin D1 and CBP on the *Notch1* gene regulatory region was also verified by ChIP with anti-FLAG antibody (to bring down D1) followed by re-ChIP with anti-CBP (Fig. 4e).

Knock-down of cyclin D1 in R28 cells decreased association of CBP with the *Notch1* gene. Conversely, overexpression of D1 increased binding of CBP (Fig. 4f). These results suggest that cyclin D1 functions to recruit CBP histone acetyltransferase to the *Notch1* promoter. Cdk4/6-inhibitor PD0332991 had no effect on this process, suggesting a Cdk-independent function of cyclin D1 (Supplementary Fig. 11).

CBP activates gene expression by catalyzing acetylation of histone residues[26]. We tested how manipulating cyclin D1 levels affects histone acetylation of the *Notch1* promoter. Knock-down of cyclin D1 in R28 cells decreased acetylation of H3K9,14 and H4K5 residues, whereas overexpression of D1 increased their acetylation (Fig. 4f, Supplementary Fig. 9).

Lastly, we examined molecular events within the *Notch1* gene in retinas of D1-deficient animals. We found that recruitment of CBP to the *Notch1* gene regulatory region and acetylation of histone H4K5 on the *Notch1* promoter were crippled in D1-knockout retinas (Fig. 4g). Collectively, these analyses indicate that cyclin D1 controls expression of Notch1 in retinal progenitor cells by recruiting CBP to the *Notch1* upstream regulatory region. In the absence of cyclin D1, CBP recruitment is reduced, leading to impaired histone acetylation of

the *Notch1* promoter region, and to decreased expression of the *Notch1* gene. This, in turn contributes to decreased retinal cell proliferation in D1-null animals.

The major finding of this work is demonstration that cyclin D1 plays a transcriptional function in normal mouse development by acting at gene promoters. While our mechanistic analyses focused on retinas, it remains to be seen if this transcriptional function contributes to development of other D1-dependent compartments, such as mammary glands. It will be also of interest to determine whether this function of D1 contributes to cancer formation. Notch1 can function as an oncogene, and several oncogenic pathways upregulate cyclin D1[27,28]. Our results indicate that cyclin D1 can serve not only as a downstream cell cycle recipient of oncogenic pathways, but also as an oncogene-activator. Of note, cyclin D1 was shown to upregulate Notch1 expression in ErbB2-positive breast cancer cells[29].

In this study we designed a novel system to study molecular functions of cyclin D1 in the living mouse. We call this approach "genetic-proteomic", because it combines genetic manipulations in the mouse germline with proteomic mass spectrometry analyses. In the future this system can be applied to study tissue-specific functions of essentially any protein in mice and other model systems such as zebrafish or *Drosophila*, at any physiological or pathological state.

METHODS SUMMARY

Generation of tagged cyclin D1 knock-in mouse strains is described in Full Methods. For one purification round of cyclin D1-containing complexes, we used pooled 15 brains dissected from E14.5 knock-in embryos, 40 eyes from P0 neonates, 5–6 mammary glands collected 1 day after delivery of pups, or 1–2 mammary adenocarcinomas arising in *MMTV-ErbB2* females. Same number of organs from wild-type mice was used for mock purifications. Details of protein purification, mass spectrometry and bioinformatics analyses are described in Full Methods. For ChIP-chip or targeted ChIP, cyclin D1 was immunoprecipitated using anti-FLAG antibody (M2 Sigma) from 1/5 of 10 pooled E14.5 knock-in (or for control wild-type) embryos, or from 1/5 of 200 pooled retinas dissected from P0 neonates, and used for real-time PCR, or hybridized onto BCBC 5A array (Beta Cell Biology Consortium) or hybridized onto tiled Agilent promoter arrays. Pooled retinas dissected from P0 wild-type or $D1^{-/-}$ neonates were used for targeted ChIP. Details and PCR primer sequences are described in Full Methods. For expression analyses, retinas were microdissected from P0 $D1^{-/-}$ or wild-type littermates. Total RNA was extracted from pooled 20 retinas using RNeasy Mini Kit (Qiagen); biotinylated cRNA probes were prepared according to standard protocols, and hybridized to Affymetrix GeneChip Mouse Expression Set 430 2.0 arrays. Hybridizations were performed in triplicate. RT - real-time PCR to quantify transcript levels was performed as described in Supplemental Methods. Rat retinal precursor cell line R28 was cultured as described[30]. *In vivo* retroviral infections were done as before[20] (see Full Methods).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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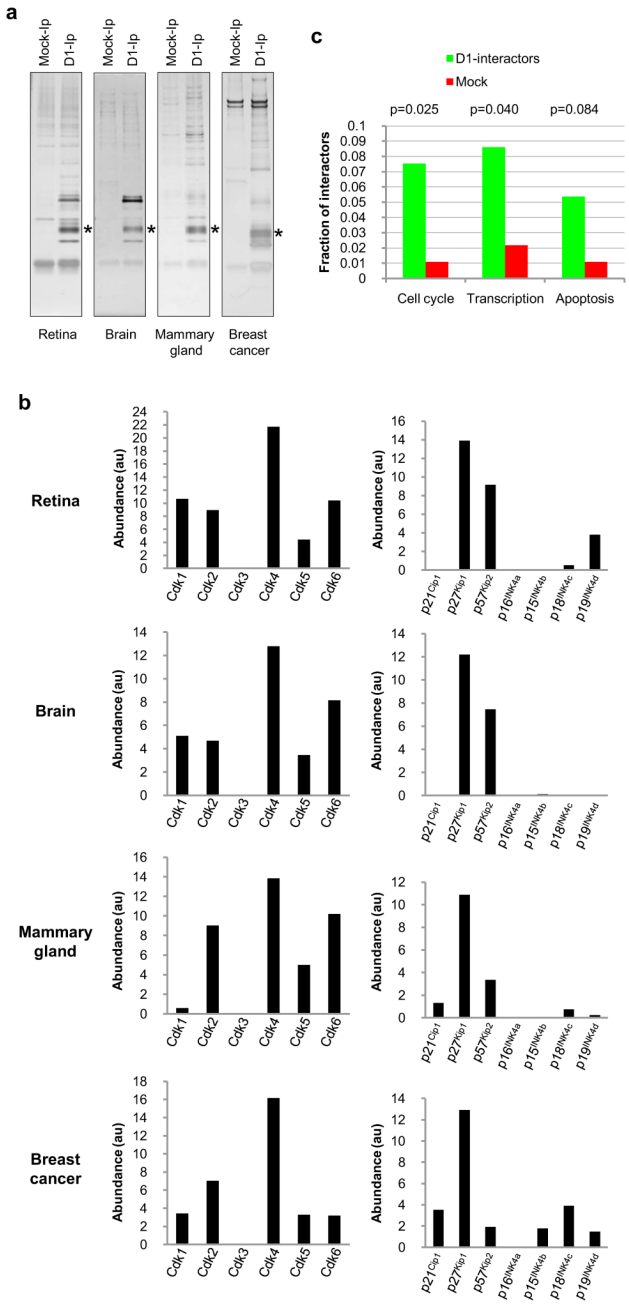

Figure 1. Proteomic analyses of cyclin D1-associated proteins

a , Silver-stained gels with cyclin D1-containing complexes purified from indicated organs (D1-Ip). Mock-Ip, mock purification from organs of wild-type mice. Cyclin D1 is marked by an asterisk. **b**, Relative abundance (arbitrary units, au) of Cdks and cell cycle inhibitors in cyclin D1-containing complexes in different compartments. **c**, Fraction of proteins among high-confidence cyclin D1-intactors and among “mock” purified proteins classified to indicated gene ontology categories.

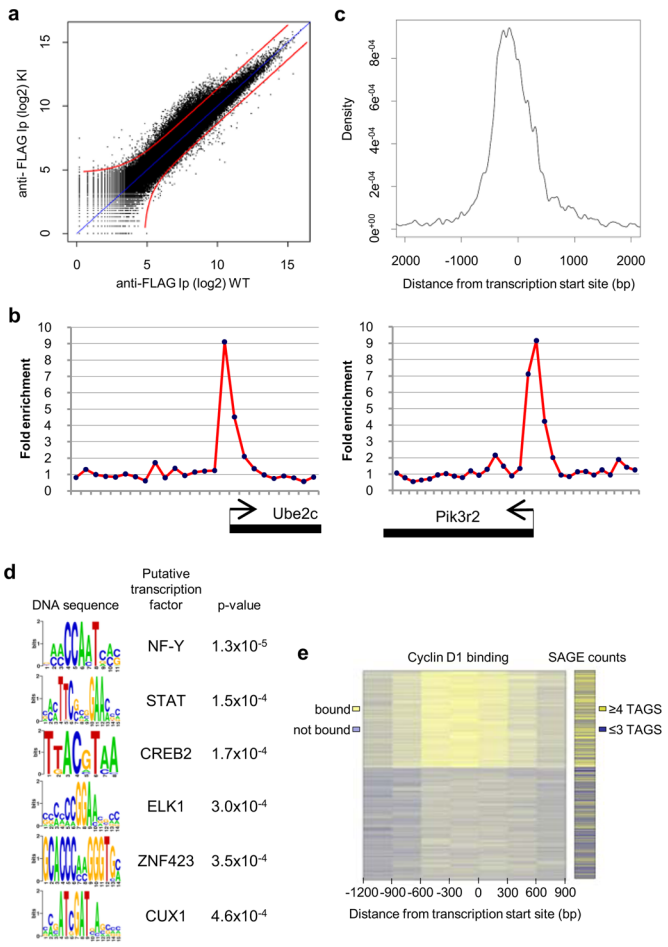

Figure 2. Analyses of cyclin D1 interaction with the mouse genome

a, Scatterplot of chromatin immunoprecipitation (Ip) with anti-FLAG antibody. Log_2 intensity values of Ip from knock-in embryos are plotted against values from wild-type embryos. **b**, Examples of cyclin D1-bound regions. Plots display unprocessed ChIP-enrichment ratios for all probes within a genomic region. **c**, Location of cyclin D1 binding sites relative to transcription start sites of RefSeq genes. **d**, Conserved DNA sequence motifs identified among cyclin D1-bound regions. **e**, Left panel: Unsupervised clustering of cyclin D1-binding events, identified in whole-embryo ChIP-chip. Each horizontal line represents one gene, centered around transcriptional start site. Yellow and blue depict bound and unbound probes. Right panel: Number of tags observed for a given transcript (yellow: ≥4 tags; blue: ≤3 tags).

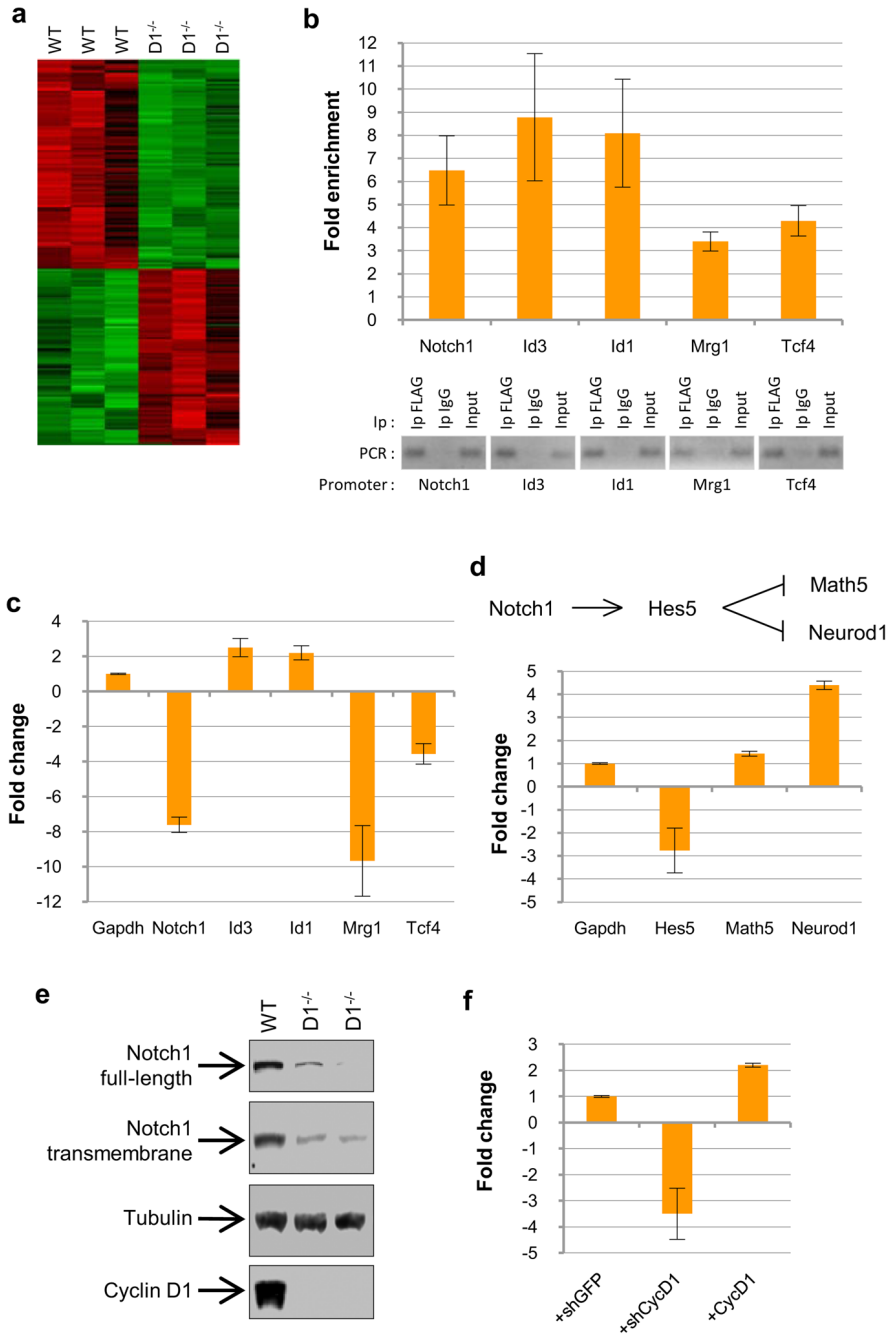

Figure 3. Analyses of cyclin D1 transcriptional function in retina

a, Expression pattern of genes whose promoter regions were bound by cyclin D1, and which showed altered levels in *D1*$^{-/-}$ retinas. **b**, Binding of cyclin D1 to regulatory regions of indicated genes verified by targeted ChIP with anti-FLAG antibodies (to bring down cyclin D1) using postnatal day 0 retinas. **c, d**, Expression levels of indicated genes quantified in wild-type and *D1*$^{-/-}$ retinas by RT-PCR. Shown is fold-difference between wild-type versus *D1*$^{-/-}$ retinas. **e**, Levels of Notch1 protein in retinas, determined by immunoblotting. **f**, Levels of Notch1 transcripts in R28 cells, quantified by RT-PCR following knock-down (+shCycD) or overexpression of cyclin D1 (+CycD1). Shown is fold-difference compared to cells infected with control vector. Error bars, SD.

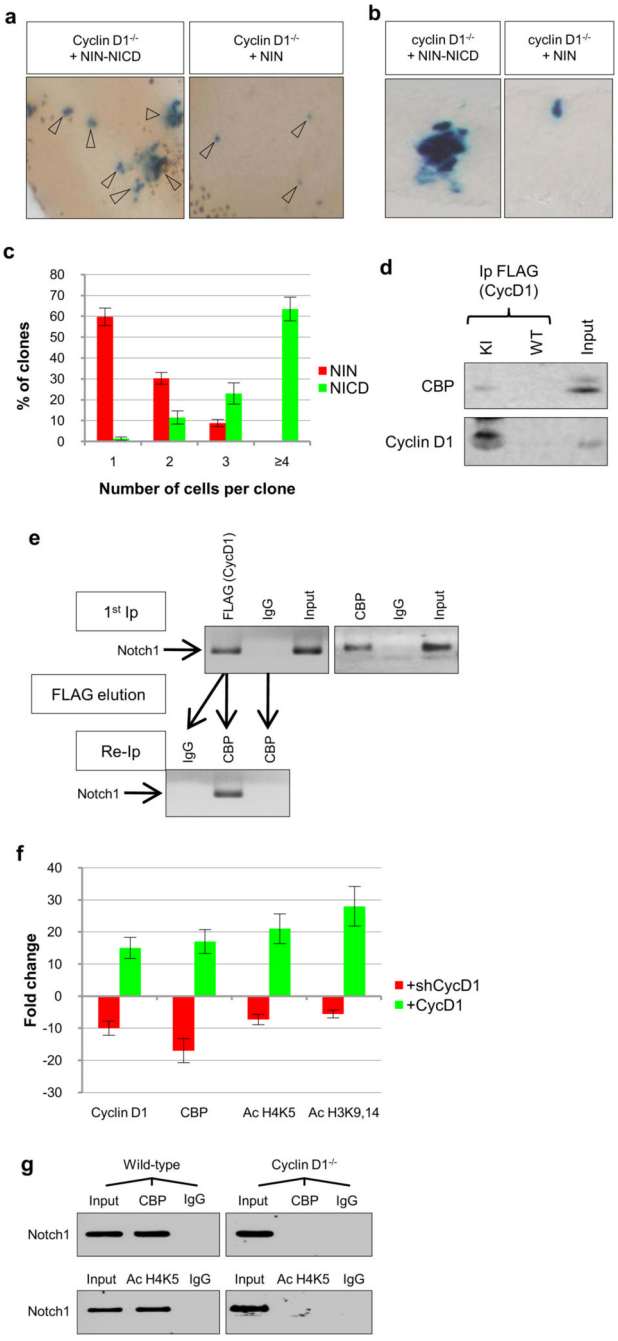

Figure 4. *In vivo* and molecular analyses of cyclin D1 – Notch1 connection

a, Whole mounts of *D1*$^{-/-}$ retinas infected with viruses encoding activated Notch and β-galactosidase (NIN-NICD, see Supplemental Fig. 10) or with vectors encoding only β-galactosidase (NIN), stained with X-gal to visualize clones of infected cells (arrowheads). **b,** higher magnifications of **a. c**, percentage of β-galactosidase-positive clones composed of 1, 2, 3 and ≥4 cells. **d**, Cyclin D1 was immunoprecipitated from P0 retinas and probed with indicated antibodies. **e,** Targeted ChIP on P0 retinas using anti-FLAG (to bring down cyclin D1) or with anti-CBP antibodies, followed by real-time PCR with *Notch1*-specific primers. Lower panel: ChIP with anti-FLAG followed by re-ChIP with anti-CBP antibodies and real-time PCR. **f**, Cyclin D1 was knocked-down (+shCycD1) or overexpressed (+CycD1) in R28 cells. ChIP

with anti-cyclin D1, anti-CBP, anti-acetylated histone H4K5 (AcH4K5) or anti-acetylated histone H3K9,14 (AcH3K9,14) antibodies was followed by real-time PCR with *Notch1*-specific primers. The results show fold-difference compared to cells transduced with empty vectors. **g**, ChIP using anti-CBP and anti-AcH4K5 antibodies followed by real-time PCR with Notch1-specific primers using wild-type and $D1^{-/-}$ retinas. Error bars, SD.