LETTERS

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

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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 oncogenesis is unclear at present. Here we developed Flag- and haemagglutinin-tagged cyclin D1 knock-in mouse strains that allowed a 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 analyses (chromatin immunoprecipitation coupled to DNA microarray; ChIP-chip) showed 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 CREB binding protein (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 Notch1 transcript and protein in cyclin D1-null (*Ccnd1*$^{-/-}$) retinas. Transduction of an activated allele of Notch1 into *Ccnd1*$^{-/-}$ retinas increased proliferation of retinal progenitor cells, indicating that upregulation of Notch1 signalling alleviates the phenotype of cyclin D1-deficiency. These studies show that in addition to its well-established cell cycle roles, cyclin D1 has an *in vivo* transcriptional function in mouse 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 haemagglutinin (HA)) tags were inserted into the endogenous cyclin D1 (*Ccnd1*) locus through homologous recombination in embryonic stem cells. Tags were introduced into the amino terminus (*Ccnd1*^{Ntag} allele) or the carboxy terminus (*Ccnd1*^{Ctag}) of cyclin D1, and homozygous *Ccnd1*$^{Ntag/Ntag}$ and *Ccnd1*$^{Ctag/Ctag}$ 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 anti-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 cyclin D1 in control animals, and the tagged cyclin D1 retained the ability to interact with its known protein partners and to activate Cdk-kinase activity (Supplementary Fig. 2). Moreover, tagged cyclin D1 afforded normal development of cyclin D1-dependent compartments and restored normal breast cancer susceptibility in homozygous *Ccnd1*$^{tag/tag}$ animals, showing 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 cyclin 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 cyclin D1, including the 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 and Supplementary Fig. 4a).

Screening the list of interactors for ontology categories showed 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, on the basis of *in vitro* and cell culture analyses, D-cyclins were postulated to have 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 through 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 study the link between cyclin D1 and transcriptional machinery further.

We used chromatin immunoprecipitation coupled to DNA microarray analysis (ChIP-chip) to study the association of cyclin D1 with genomic DNA sequences. Because 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 the association of cyclin D1 with the genome.

We immunoprecipitated cyclin D1, along with associated DNA sequences from embryonic day (E)14.5 tagged knock-in embryos using anti-Flag antibodies, and hybridized immunoprecipitated DNA onto

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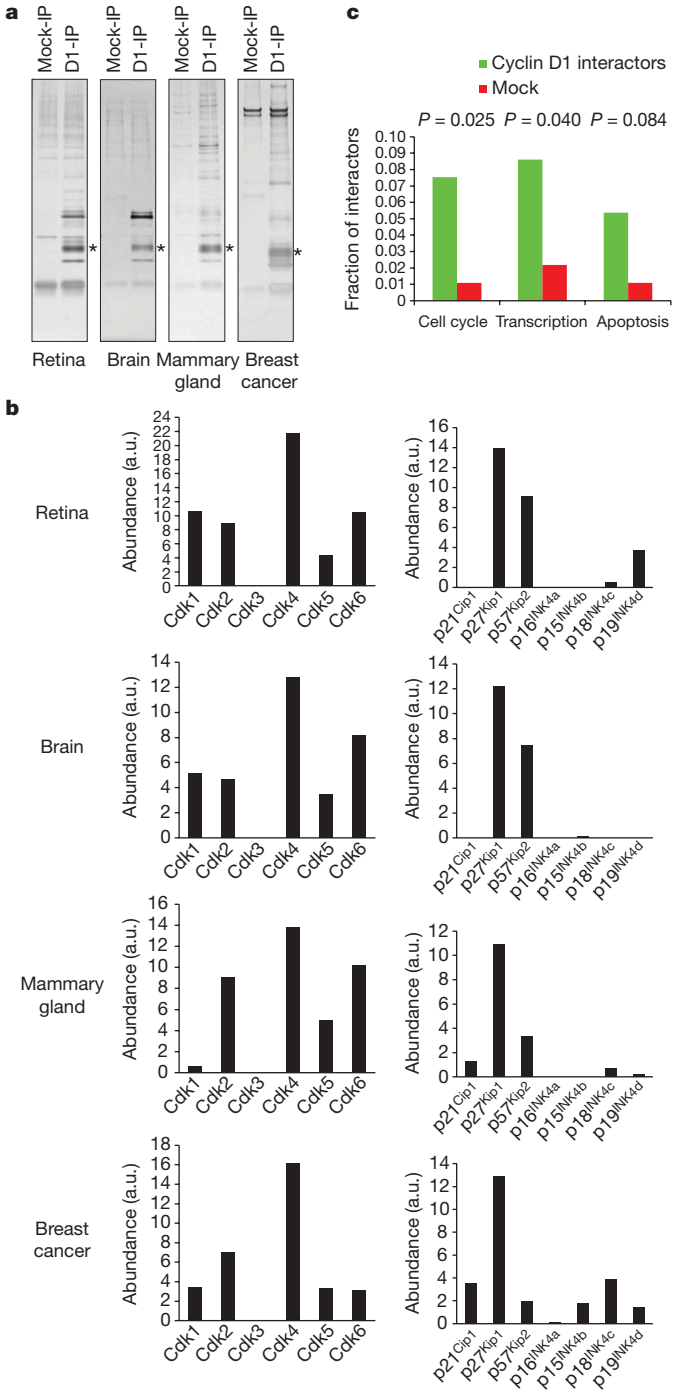

Figure 1 | Proteomic analyses of cyclin D1-associated proteins. **a**, Silver-stained gels with cyclin D1-containing complexes purified from indicated organs (D1-IP). Mock-IP, mock purification from organs of wild-type mice. Bands corresponding to cyclin D1 are marked by an asterisk. **b**, Relative abundance (arbitrary units, a.u.) of Cdks and cell cycle inhibitors in cyclin D1-containing complexes in different compartments. **c**, Fraction of proteins among cyclin D1 interactors and among 'mock' purified proteins classified to indicated Gene Ontology categories.

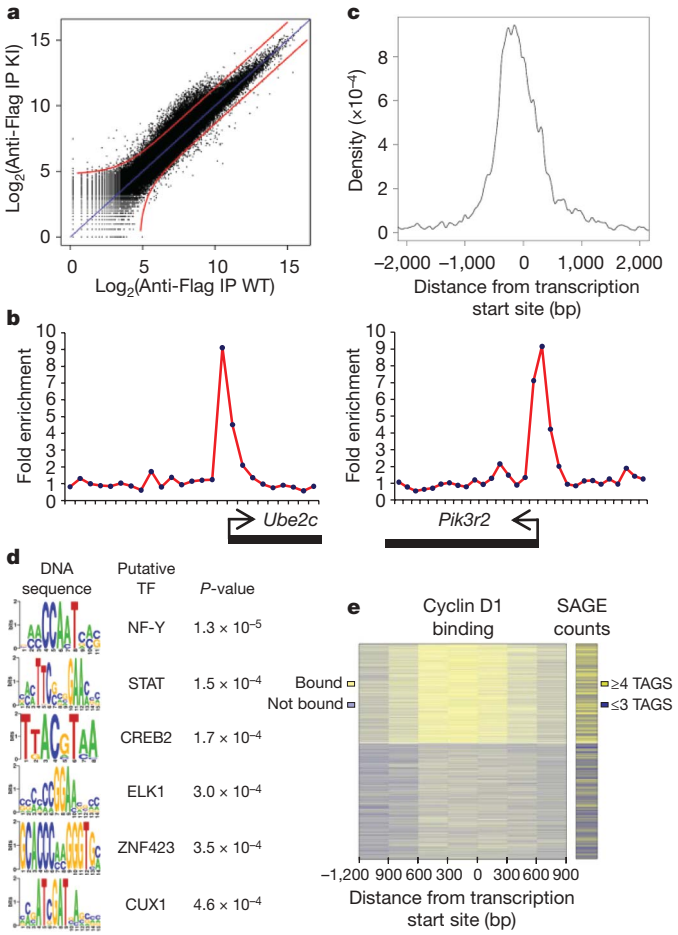

Figure 2 | Analyses of cyclin D1 interaction with the mouse genome. **a**, Scatterplot of chromatin immunoprecipitation with an anti-Flag antibody. Log_2 intensity values of immunoprecipitation from knock-in (KI) embryos are plotted against values from wild-type (WT) embryos. **b**, Examples of cyclin D1-bound regions. Plots show 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, unsupervised clustering of cyclin D1-binding events, identified in whole-embryo ChIP-chip. Each horizontal line represents one gene, centred around the transcriptional start site. Yellow and blue depict bound and not bound probes, respectively. Right, the number of tags observed for a given transcript (yellow: ≥4 tags; blue: ≤3 tags).

arrays. We detected binding of cyclin D1 to promoter regions (more than 900 at highest-stringency threshold, $P < 1 \times 10^{-4}$; Fig. 2a, Supplementary Fig. 5a, b and Supplementary Tables 4 and 5). Analyses of the exact location of cyclin D1 binding events showed that cyclin D1 interacts with DNA in close proximity to transcription start sites (in 79% of cases within 1 kb) (Fig. 2b, c). A bioinformatics search for conserved DNA sequence motifs among cyclin 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 cyclin D1-null mice[3,4]. We found an 80% overlap between cyclin D1 targets identified in the whole-embryo ChIP-chip versus targets identified in retinas (Supplementary Fig. 5c and data not shown).

Gene expression in developing mouse retinas had previously been profiled at several 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 the binding of cyclin D1 to gene promoters and the abundance of these transcripts. We found that genes whose promoters are bound by cyclin D1 belong to the abundantly expressed genes ($P < 1 \times 10^{-15}$) (Fig. 2e).

Genes that are highly expressed in many tissues were shown to have a high content of CpG dinucleotides in their promoter regions[14]. We

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performed a computational comparison of CpG content within cyclin D1-bound versus all other promoters. Cyclin 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 by sequence-specific transcription factors. As mentioned earlier, we observed the enrichment of NF-Y DNA recognition sequences among cyclin D1-bound genomic fragments (Fig. 2d), and verified physical association of cyclin D1 with Nf-y (Supplementary Fig. 4b). We knocked-down the Nf-ya subunit in *in vitro* cultured rat retinal precursor R28 cells and found that this diminished the recruitment of cyclin D1 to several Nf-y target promoters (Supplementary Fig. 7). These findings indicate 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 cyclin D1-knockout[4] retinas using microarrays (Supplementary Fig. 6b and Supplementary Table 6), and overlaid the data with ChIP-chip promoter-occupancy results. Among cyclin D1-bound genes, we observed genes with increased as well as decreased levels in *Ccnd1*$^{-/-}$ retinas (Fig. 3a, Supplementary Fig. 8 and Supplementary Table 7), suggesting that cyclin D1 can serve both to activate and downregulate gene expression.

Inspection of the list of genes bound by cyclin D1 and showing altered expression in cyclin D1-null retinas, revealed the presence of *Notch1*—an essential regulator of retinal progenitor cell proliferation[15,16]—along with several transcriptional regulators that probably contribute in this process (*Id3*, *Id1*, *Mrg1* (also known as *Meis2*) and *Tcf4*)[17–19]. We verified binding of cyclin D1 to upstream regulatory regions of these genes (Fig. 3b and Supplementary Fig. 9) and their altered expression in cyclin D1-null retinas (Fig. 3c and Supplementary Fig. 6c).

The rate-limiting role of Notch1 in retinal development is well-established, and retinal-specific ablation of the *Notch1* gene leads to a proliferative failure that resembles the cyclin D1-null phenotype[3,4,20]. Despite overall hypoplasia, *Ccnd1*$^{-/-}$ retinas display an 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 investigated further 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* (also known as *Atoh7*) and *Neurod1*. Consequently, in Notch1-knockout retinas, *Hes5* is downregulated, whereas *Math5* and *Neurod1* genes are de-repressed[22,23]. We found that in *Ccnd1*$^{-/-}$ retinas *Hes5* transcript levels were decreased, whereas *Neurod1* levels were increased, suggesting that Notch signalling is compromised in cyclin D1-knockout retinas (Fig. 3d). We also found that the levels of Notch1 protein were significantly decreased in cyclin D1-null retinas (Fig. 3e). Knockdown of cyclin D1 in rat retinal precursor R28 cells decreased the levels of Notch1 transcripts and protein, whereas cyclin D1 overexpression had the opposite effect (Fig. 3f). Collectively, these observations indicate that cyclin D1 transcriptionally upregulates the *Notch1* gene during retinal development. Consequently, Notch1 transcript and protein levels, as well as Notch-dependent signalling pathways are compromised in cyclin D1-null retinas.

We next asked whether increasing Notch1 signalling in *Ccnd1*$^{-/-}$ retinas would enhance progenitor cell proliferation. We injected a retroviral construct encoding the Notch intracellular domain (NICD, a constitutively activated allele of Notch1; Supplementary Fig. 10), into subretinal space of postnatal day (P) 0 cyclin *Ccnd1*$^{-/-}$ pups, and gauged the response after ten days. Expression of NICD in *Ccnd1*$^{-/-}$ retinas significantly increased *in vivo* proliferation of retinal progenitor cells (Fig. 4a–c). Hence, restoring Notch1 signalling in cyclin D1-null progenitor cells alleviates the phenotype of cyclin D1-deficiency.

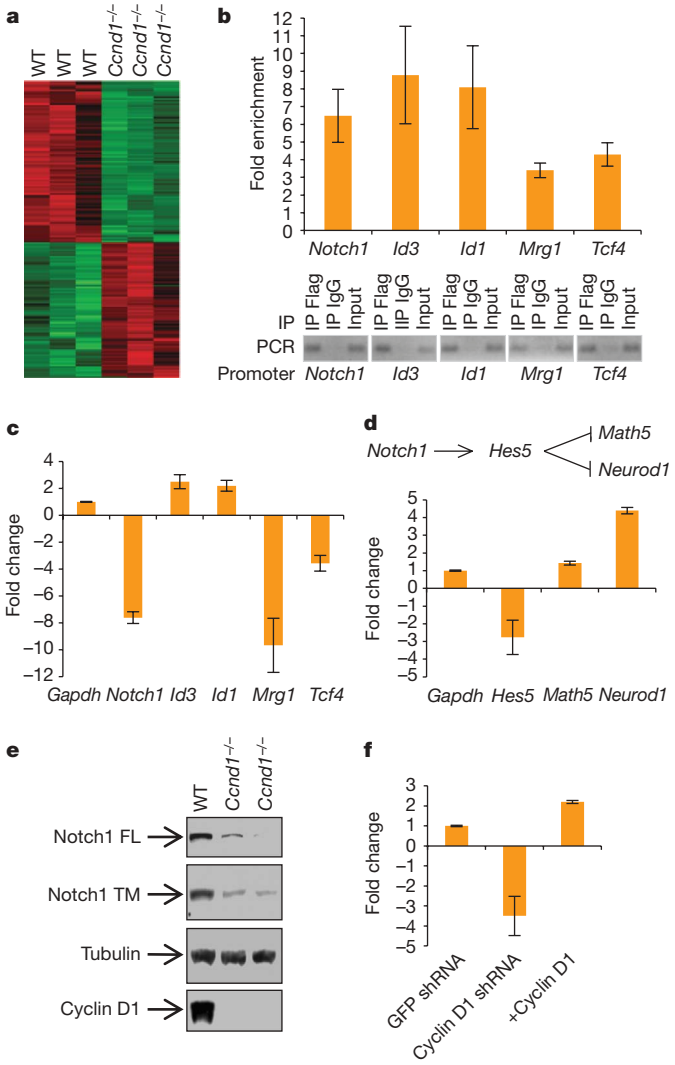

Figure 3 | Analyses of cyclin D1 transcriptional function in retina. **a**, Expression pattern of genes whose promoter regions were bound by cyclin D1, and which showed altered levels in *Ccnd1*$^{-/-}$ retinas. **b**, Binding of cyclin D1 to regulatory regions of indicated genes verified by targeted ChIP with anti-Flag antibodies (to bring down cyclin D1) using P0 retinas. **c**, **d**, Expression levels of indicated genes quantified in wild-type and *Ccnd1*$^{-/-}$ retinas by RT–PCR. Shown is the fold difference between wild-type and *Ccnd1*$^{-/-}$ retinas. **e**, Levels of Notch1 protein in retinas, determined by immunoblotting. FL, full length; TM, transmembrane. **f**, Levels of Notch1 transcripts in R28 cells, quantified by RT–PCR after knockdown of cyclin D1 (using short-hairpin RNA (shRNA)) or after overexpression of cyclin D1 (+cyclin D1). Shown is the fold difference compared to cells infected with control vector. GFP, green fluorescent protein. Error bars, s.d.

We addressed 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 cyclin D1 with CBP was confirmed by immunoprecipitation–western blotting (Fig. 4d). Using targeted ChIP with anti-CBP antibodies, we determined that in developing retinas CBP binds the same upstream regulatory region of the *Notch1* gene as cyclin D1 (Fig. 4e, g and Supplementary Fig. 9). Co-localization of cyclin D1 and CBP on the *Notch1* gene regulatory region was also verified by ChIP with an anti-Flag antibody (to bring down cyclin D1) followed by re-ChIP with an anti-CBP antibody (Fig. 4e).

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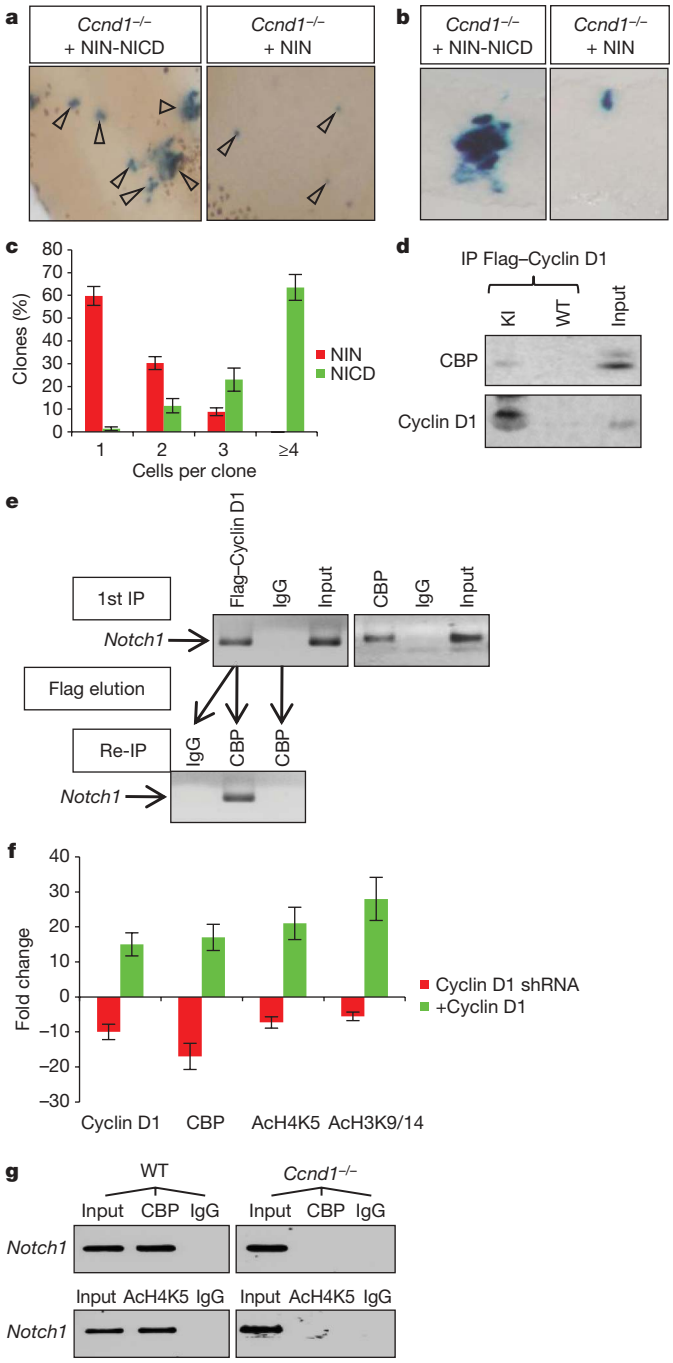

Figure 4 | *In vivo* and molecular analyses of the cyclin D1–Notch1 connection. **a**, Whole mounts of *Ccnd1*$^{-/-}$ retinas infected with viruses encoding activated Notch1 and β-galactosidase (NIN-NICD, see Supplementary Fig. 10) or with viruses encoding only β-galactosidase (NIN), stained with X-gal to visualize clones of infected cells (arrowheads). Original magnification, ×8. **b**, Higher magnifications (×20) of **a**. **c**, The percentage of β-galactosidase-positive clones composed of 1, 2, 3 and ≥4 cells. **d**, Cyclin D1 was immunoprecipitated using an anti-Flag antibody from P0 retinas and probed with indicated antibodies. **e**, Targeted ChIP on P0 retinas using anti-Flag (to bring down cyclin D1) or anti-CBP antibodies, followed by real-time PCR with *Notch1*-specific primers. Bottom panel, ChIP with anti-Flag followed by re-ChIP with anti-CBP antibodies and real-time PCR. **f**, Cyclin D1 was knocked down (cyclin D1 shRNA) or overexpressed (+cyclin D1) in R28 cells. ChIP with anti-cyclin D1, anti-CBP, anti-acetylated H4K5 (AcH4K5) or anti-AcH3K9/14 antibodies was followed by real-time PCR with *Notch1*-specific primers. The results show the 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 *Ccnd1*$^{-/-}$ retinas. Error bars, s.d.

Knockdown of cyclin D1 in R28 cells decreased association of CBP with the *Notch1* gene. Conversely, overexpression of cyclin D1 increased binding of CBP (Fig. 4f). These results indicate that cyclin D1 functions to recruit CBP histone acetyltransferase to the *Notch1* upstream regulatory region. The Cdk4/6-inhibitor PD 0332991 had no effect on this process, suggesting a Cdk-independent function of cyclin D1 (Supplementary Fig. 11).

CBP activates gene expression by catalysing acetylation of histone residues[26]. We tested how manipulating cyclin D1 levels affects histone acetylation of the *Notch1* promoter. Knockdown of cyclin D1 in R28 cells decreased acetylation of histone H3 Lys 9/14 (H3K9/14), and H4K5 residues, whereas cyclin D1 overexpression increased their acetylation (Fig. 4f and Supplementary Fig. 9).

Furthermore, we examined molecular events within the *Notch1* gene in retinas of cyclin D1-deficient animals. We found that the recruitment of CBP to the *Notch1* gene regulatory region and acetylation of histone H4K5 on the *Notch1* promoter were diminished in cyclin 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 cyclin D1-null animals.

The main finding of this work is the demonstration that cyclin D1 plays a transcriptional function in normal mouse development by acting at gene promoters. Although our mechanistic analyses focused on retinas, it remains to be seen whether this transcriptional function contributes to development of other cyclin D1-dependent compartments, such as mammary glands. It will be also of interest to determine whether this function of cyclin D1 contributes to cancer formation. Notch1 can function as an oncogene and several oncogenic pathways upregulate cyclin D1 (refs 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 new system to study molecular functions of cyclin D1 in the mouse. We call this approach 'genetic–proteomic', because it combines genetic manipulations in the mouse germ line 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 the Supplementary Methods. For one purification round of cyclin D1-containing complexes, we used pooled 15 brains dissected 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. The 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 the Supplementary Methods. For ChIP-chip or targeted ChIP, cyclin D1 was immunoprecipitated using an anti-Flag antibody (M2 Sigma) from one-fifth of ten pooled E14.5 knock-in (or for control wild-type) embryos, or from one-fifth of 200 pooled retinas dissected from P0 neonates, and used for real-time PCR, or hybridized onto promoter BCBC 5A arrays (Beta Cell Biology Consortium) or hybridized onto tiled Agilent promoter arrays. Pooled retinas dissected from P0 wild-type or *Ccnd1*$^{-/-}$ neonates were used for targeted ChIP. Details and PCR primer sequences are described in the Supplementary Methods. For expression analyses, retinas were microdissected from P0 *Ccnd1*$^{-/-}$ or wild-type littermates. Total RNA was extracted from pooled 20 retinas using an RNeasy Mini Kit (Qiagen); biotinylated complementary RNA probes were prepared according to standard protocols, and hybridized to Affymetrix GeneChip Mouse Expression Set 430 2.0 arrays. Hybridizations were performed in triplicate. Reverse transcription real-time PCR (RT–PCR) to quantify transcript levels was performed as described in Supplementary Methods. The rat retinal precursor

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cell line R28 was cultured as described[30]. *In vivo* retroviral infections were done as before[20] (see Supplementary Methods).

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions F.B. and P.S. designed the study, analysed 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. analysed 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 cyclin D1-null retinas with Notch1, A.M., G.M.F., M.F.C., D.T.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 Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/), under accession GSE13636. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to P.S. (Peter_Sicinski@dfci.harvard.edu).

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