Supplemental Materials and Methods

The generation of MMTV-tTA and TetO-D1 transgenic strains:

The Flag-tagged Cyclin D1 (T286A) cDNA was cloned in front of an IRES-Luciferase cassette, which was subsequently cloned into the *Eco*RV site of the pTetSplice vector. Four transgenic founder lines were obtained following pronuclear injection, and two lines (10897 and 11233) were used for experiments described here. The MMTV-tTA transgene was generated by inserting the tTA cDNA into the blunted *Eco*R1 site of the Mmtv-Sv40-Bssk vector (provided by Dr. Muller, McGill). We obtained two founder lines (25754 and 25755) with identical expression pattern and used line 25755 for experiments described in this article.

Primer sequences to genotype the newly generated mouse strains:

Transgene	Forward and reverse primer	Size of amplicon
TetO-D1	5'-CCG TCA GAT CGC CTG GAG ACG-3' 5'-GGC GGA TGG TCT CCA CTT CGC-3'	380 bp
MMTV-tTA	5'-AGT GAT AGA GCT CTT GCC TAG C-3' 5'-GCC AAT ACA GTG TAG GCT GC-3'	364 bp

Quantitative imaging analysis to assess the expression of Cyclin D1/D3 in primary human breast cancers

Histological sections were deparaffinized and underwent antigen retrieval on the Dako PT module using low pH retrieval buffer (Dako) before immunostaining on the Dako Autolink Plus autostainer. Endogenous peroxidase activity was blocked using Dako FLEX Peroxidase block for 10 min; followed by serum-free protein block for 30 min. Slides were incubated for 20 min with either a mixture of target primary antibodies at optimized dilutions (Cyclin D1, 1:200; Cyclin D3, 1:200; Her2, 1:4000) with rabbit or mouse pancytokeratin antibodies as appropriate. Slides were then washed three times with Dako wash buffer and subsequently incubated with a mixture of secondary antibody which contained a horseradish peroxidase–conjugated antibody and another antibody conjugated to Alexa 488 (Molecular Probes). Slides were then washed three times with Dako wash buffer and subsequently incubated with Tyramide-Cy5 (Perkin-Elmer). Finally, all sections were stained with 4',6-diamidino-2-phenylindole (DAPI) for nuclear visualization. Automated quantitative analysis was performed using the AQUA/PM2000 Imaging Platform (HistoRx). Slides were scanned and images of each tissue were captured at 3 different channels detecting FITC/Alexa 488, Cy5, or DAPI. AQUA scores were generated based on the images acquired and the software program; finally, results are validated manually. The levels of Cyclin D1, D3, or ErbB2 were considered high when they were greater than the mean plus 2 SDs from the levels of normal breast epithelia.

Supplemental Figures

Fig. S1. Cyclin D1 deficiency in the FVB strain background causes male infertility

A. Immunofluorescence staining of Cyclin D1 in reproductive organs of an FVB wildtype male. Slides where counterstained with DAPI. **B**. Histological analysis of testis and epididymis from wildtype and Cyclin D1 knockout mice, bars represent 50 µm.

Fig. S2. Primary mammary epithelial cells (MECs) derived from MMTV-neu TetO-D1 *Cyclin D1*$^{-/-}$ females express luciferase following lentiviral-mediated expression the tetracycline-controlled transactivator (tTA).

Bioluminescence imaging on primary cells with and without (control) tTA prior to (**A**) and following orthotopic transplantation and engraftment into wildtype recipient mice (**B**).

Fig. S3. Primary antibodies against Cyclin D1 and Cyclin D3 have similar binding affinities

Both primary antibodies were recognized by the same secondary antibody under identical blotting conditions.

Fig. S4. Cyclin D1 and D3 are significantly upregulated in ErbB2-positive human breast cancers

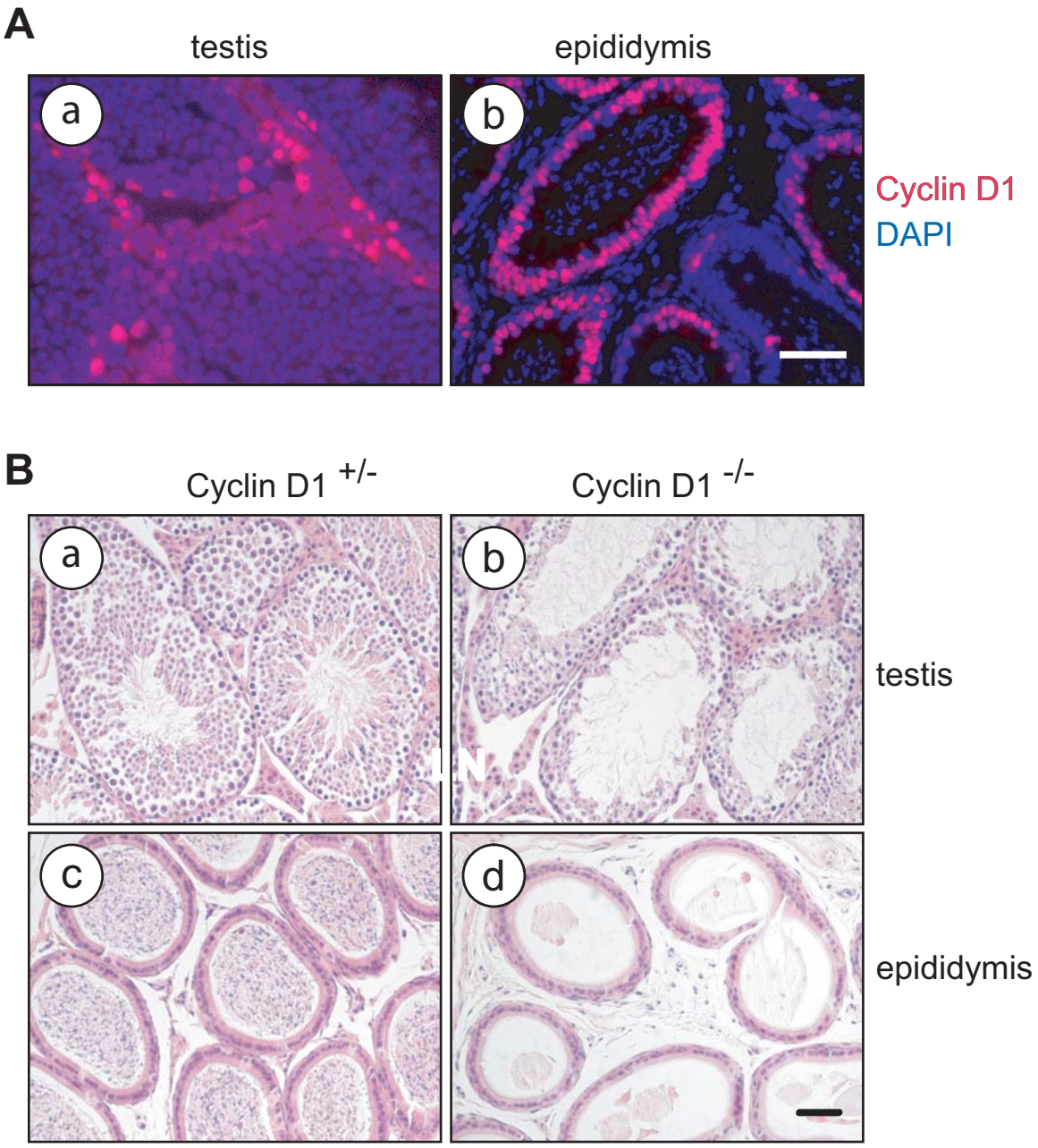
Representative images of three ErbB2-positive breast cancer cases that overexpress Cyclin D3 (case 1), Cyclin D1 (case 2), and both (case 3).

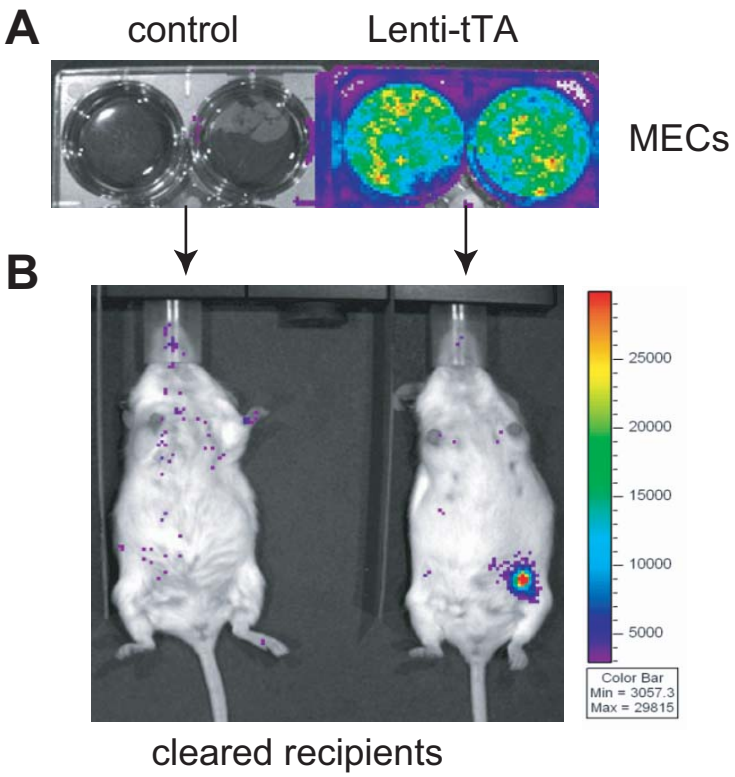
Fig. S5. Knockdown of Cyclin D3 leads to a compensatory upregulation of Cyclin D1 in a Her2/ErbB2-positive human breast cancer cell line.

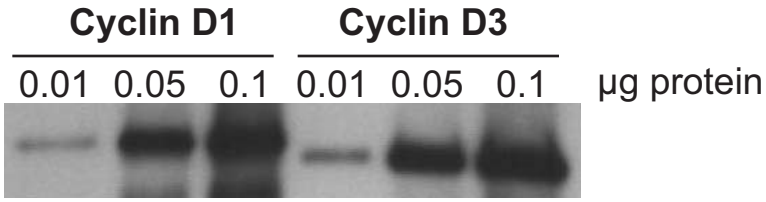
HCC1419 cells (ATCC) that express both Cyclin D1 and D3 were infected with a panel of published shRNA vectors or a control vector (pLKO1) and selected with puromycin. Although these vectors were successfully used to downregulate Cyclin D1 or D3 in a pancreatic cancer cell line (Radulovich, et al., 2010 PMID: 20113529), only one vector (shD3-2) was suitable to stably downregulate the expression of D3 in HCC1419 cells (lane 5). Note that these cells exhibit a compensatory upregulation of Cyclin D1.

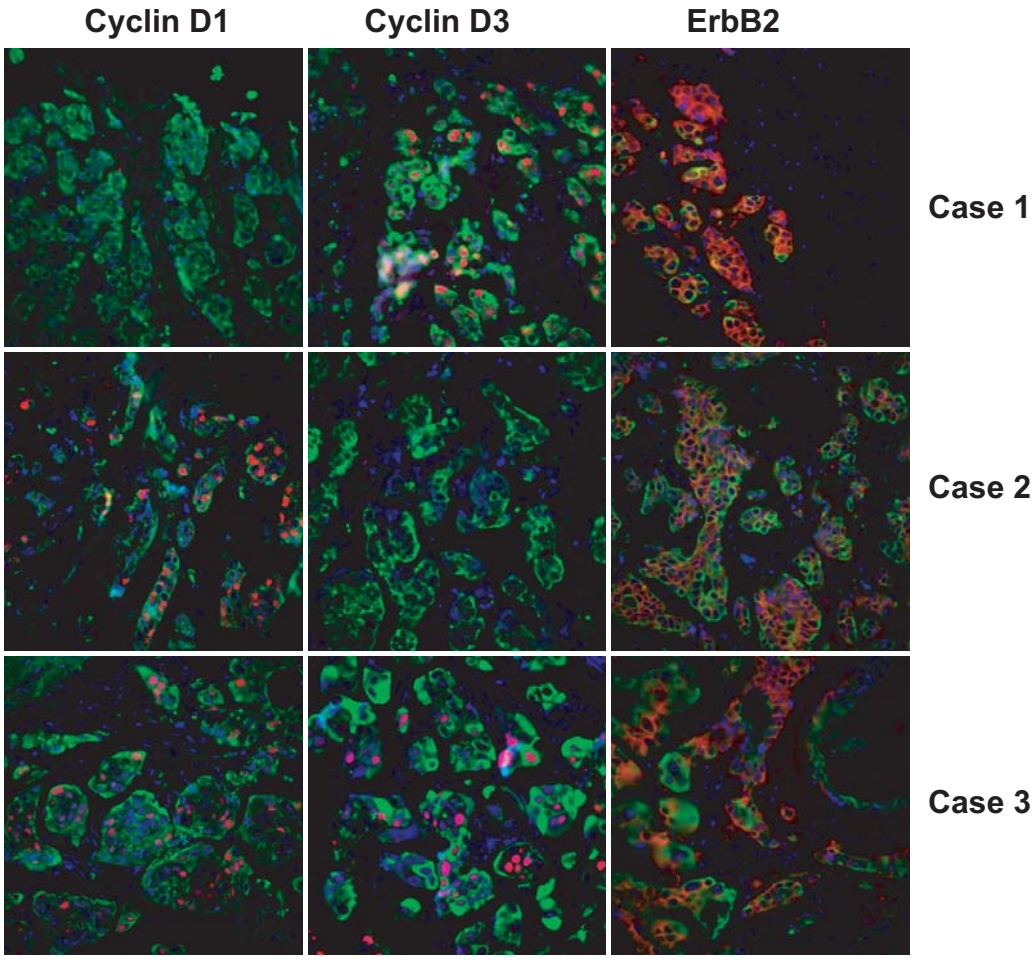
Fig. S6. Escapees that have regained expression of Cyclin D3 give rise to small tumors after a prolonged latency

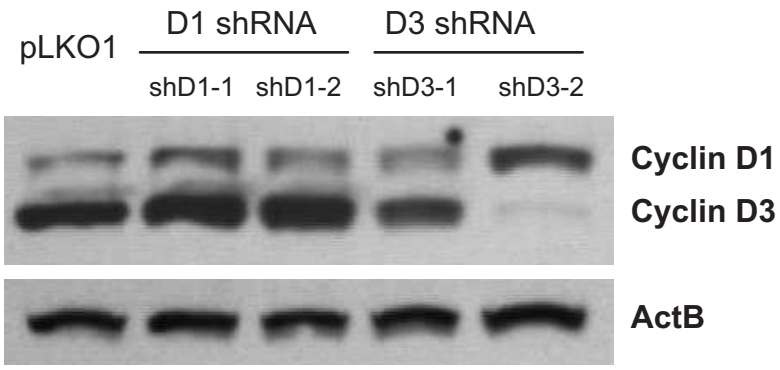
Western blot analysis to determine the expression of Cyclin D3 and Cyclin E in five ErbB2-indcued tumors that originated from cancer cells that carry an shRNA construct against Cyclin D3 and that lack endogenous Cyclin D1 expression. The control tumors originated from the same isogenic cancer cells that lack only Cyclin D1.

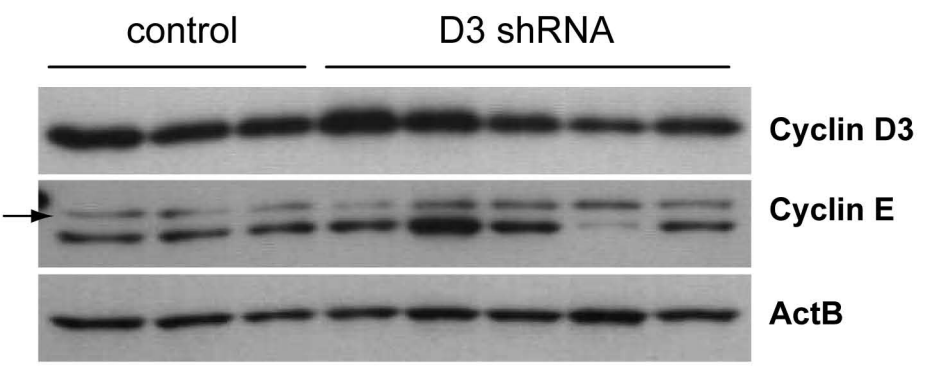

A
testis
epididymis
a
b
Cyclin D1
DAPI
B
Cyclin D1 +/-
Cyclin D1 -/-
a
b
testis
c
d
epididymis


A
control
Lenti-tTA
MECs
B
25000
20000
15000
10000
5000
Color Bar
Min = 3057.3
Max = 29815
cleared recipients


Cyclin D1
Cyclin D3
0.01 0.05 0.1 0.01 0.05 0.1
µg protein


Cyclin D1
Cyclin D3
ErbB2
Case 1
Case 2
Case 3


pLKO1
D1 shRNA
D3 shRNA
shD1-1
shD1-2
shD3-1
shD3-2
Cyclin D1
Cyclin D3
ActB


control
D3 shRNA
Cyclin D3
Cyclin E
ActB