Supplemental Material:

**A Chromatin Independent Role of Polycomb-Like 1 to Stabilize p53 and Promote Cellular Quiescence.**

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Cell culture:
Human diploid fibroblasts (HDFs), HEK293 and retrovirus producing Phoenix cells were grown in DMEM (Gibco), supplemented with 10% (v/v) FBS (Gibco) and 100 U/ml penicillin and 100 U/ml streptomycin (Gibco). For serum starvation experiments, serum was removed from HDFs at ~50% confluence for 96h. Media was changed at 24h intervals until 96h, at which point cells were re-stimulated to enter the cell cycle by addition of media containing 20% (v/v) FBS for 24h.

Cloning and plasmid generation:
The human PCL3 ORF was cloned previously (Brien et al., 2012). The PCL1 and PCL2 ORFs were PCR amplified from low-passage HMEC cDNA and inserted into the pCR8/GW/TOPO Gateway cloning entry vector (Invitrogen), following the protocol of the manufacturer. Sequence verified PCL1–3 ORFs were subcloned into Gateway cloning compatible expression vectors by recombination using the LR clonase enzyme (Invitrogen). The generation of the double point mutant PCL3 was described previously (Brien et al., 2012). The double point mutant PCL1 and PCL2 were generated using the GeneArt Site Directed Mutagenesis System (Invitrogen) in accordance with the manufacturers instruction.
**Retroviral transduction:**

Retroviral particles were produced in amphotrophic Phoenix cells. Cells were grown to ~60-70% confluence and transfected with a pBABE ORF expression vector. Media containing viral particles was collected at 48 and 72h post transfection, filtered through a 0.45µm filter, and used directly for transduction of target cells after addition of 5µg/ml Polybrene (Sigma). Target cells were transduced with viral particles for 4-8 hours on 2 consecutive days and subsequently selected in media containing 2µg/ml Puromycin (Sigma) for 48–72h immediately after transduction.

**Lentiviral transduction:**

Lentiviral particles were produced in HEK293 cells. Cells were grown to ~80% confluence and transfected with a pLKO.1 lentiviral shRNA expressing vector, and vectors encoding viral packaging (pPAX8) and envelope (pVSVG) proteins. Media containing viral particles was collected 24 and 48 hours after transfection and filtered through a 0.45µM syringe filter. Viral particles were purified from these supernatants by ultracentrifugation at 20,000 rpm for 2h at 4°C. Viral particles were resuspended in sterile PBS (Gibco) and added directly to target cell media supplemented with 10µg/ml Polybrene (Sigma). Target cells were transduced with virus for 16-20h, washed and selected in media containing 2µg/ml Puromycin (Sigma) for 48–72h immediately after transduction.
**RNA interference:**

Cells were seeded 24h prior to transfection, and transfected at 30–50% confluence with 20µM siRNA–Ctrl (SIC001), siRNA–TP53#1 (custom, target sequence –TGTTCCGAGAGCTGAATGA), siRNA–TP53#2 (custom, target sequence –GTGCAGCTGTGGGTTGATT), siRNA–PCL1#1 (custom, target sequence –CACACACCGGCACTTTACAT), siRNA–PCL1#2 (custom, target sequence – GCAACCGACAGCAGGTTA), siRNA–PCL3#1 (SASI_Hs01_00222697) or siRNA–PCL3#2 (SASI_Hs01_00222698). Cells were transfected using Lipofectamine RNAi MAX (Invitrogen) in accordance with the manufacturers in instructions. For experiments involving serum starvation, serum was removed from transfected cells 16h post transfection.

**3T3 growth assays:**

3T3 growth assays were conducted as follows, 7.5x105 cells were plated on 100mm plates, 3 days later, the total number of cells was counted and 7.5x105 cells were plated again. The cumulative increase in cell number was calculated according to the formula ‘Log(Nf/Ni)/Log2’ where Ni and Nf are the initial and final numbers of cells plated and counted after 3 days, respectively.

**FACS purification of haematopoietic lineages:**

Bone marrow was collected from tibias, femurs and humerus of 6–8weeks old C57/B6 mice (Taconic, NY). Red blood cells were removed using RBC lysis buffer (BD Biosciences) and the remaining cells were washed twice in PBS (Invitrogen). Labeling of cells for FACS analysis and cell sorting was performed on ice in PBS supplemented with 0.5% horse serum (Invitrogen).
using conjugated antibodies CD3-bio (17A2); CD4-bio (GK1.5); CD8a-bio (53-6.7); CD19-bio (1D3); B220-bio (RA3-6B2); Gr1-bio (RB-8C5); TER-119-bio (TER-119); CD117-APC (2B8); CD16/32-PE/Cy7 (93) Sca1-PacificBlue (E13-161.7); CD34-FITC (RAM34) eBiosciences (San Diego, CA) or Biolegend (San Diego, CA) and Streptavidine–Qdot605 Invitrogen (Carlsbad, CA). LT–HSC were isolated from mouse bone marrow as Lin– (CD3; CD4; CD8a; CD19; B220; Gr1; TER119; IL-7R) CD34–, CD117+, Sca1+ (Osawa et al., 1996) and ST–HSC/MPP as Lin– (CD3; CD4; CD8a; CD19; B220; Gr1; TER119; IL-7R) CD34+, CD117+, Sca1+. Myeloid progenitors were sorted as previously described (Akashi et al., 2000), CMP as Lin- Sca1- CD117 + CD16/32low CD34+Lin– Sca1– CD117+, CD16/32high CD34+; using FACS Aria high–speed cell sorter equipped with 407nm, 488nm, and 540nm lasers (BD Biosciences).

**FACS analyses of cell cycle profiles:**

For BrdU FACS analyses asynchronously growing HDFs were pulsed with 33µM BrdU for 45mins. Pulsed cells were harvested, washed twice in PBS and fixed in 70% ethanol. Staining was performed using a mouse anti–BrdUantibody (Sigma, SAB4700630) and an anti–mouse Alexa–Fluor 488 conjugated antibody (Abcam, ab150133) was used to detect BrdU incorporation. Stained cells were subsequently stained with 2.5µg ml⁻¹ Propidium Iodide (Sigma) and treated with 250µg ml⁻¹ RNase A (Thermoscientific). For G0/quiescence FACS, asynchronously growing or serum–starved HDFs were harvest, washed twice in PBS and resuspended in a Hank's balanced salt solution containing, 10% FBS and 10µM DRAQ5
(Biostatus) and incubated for 20 min at 20°C. After a single wash, cells were incubated in the same buffer containing Pyronin Y (5µg/ml) for an additional 15 min at 37°C. Stained cells were analysed on a BD AccuriTM C6 flow cytometer.

**Immunoprecipitations:**

Immunoprecipitation of endogenous proteins was performed essentially as previously described (Van Den Berg et al., 2010). Briefly, nuclear pellets prepared from HDFs cells were lysed in Buffer C containing protease inhibitors (20 mM HEPES (pH 7.6), 20% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, aprotinin 1µg ml⁻¹ and nuclear extracts were dialysed against Buffer C-100 (20 mM HEPES (pH 7.6), 20% (v/v) glycerol, 0.2 mM EDTA, 100 mM KCl, 1.5 mM MgCl2, 0.2 mM EDTA) for 5hrs. For each individual immunoprecipitation, a total of 5µgs of antibody was cross-linked to 20µls of protein A Sepharose (Sigma). Antibodies were subsequently covalently coupled to beads using dimethyl pimelimidate (DMP). Coupled beads were equilibrated in Buffer C-100, leupeptin 10µg ml⁻¹ containing 0.02% NP40 (C-100*), 0.1mg ml⁻¹ insulin (Sigma), 0.2mg ml⁻¹ chicken egg albumin (Sigma) and 1% fish skin gelatin (Sigma) and added to 1ml of dialysed nuclear extract containing 250 units of benzonase (Sigma) for 3 hours at 4°C in “No Stick” microcentrifuge tubes (Eppendorf). The beads were then washed 5 times with C-100* and elutions performed with 2XSDS loading dye. Eluted protein fractions were separated by SDS–PAGE and analysed by Western blot. For immunoprecipitation of exogenously expressed HA tagged proteins the procedure was carried out as above with slight modifications,
20µls of anti–HA (clone HA–7) agarose beads (Sigma) were used and immunoprecipitated proteins were eluted using 250µg ml⁻¹ HA peptide (Sigma).

**Chromatin immunoprecipitation:**
ChIP analyses were performed as described (Brien et al., 2012). Briefly, cells for ChIP assays were cross-linked using 1% formaldehyde. Fixed chromatin was sheared into fragments of approximately 200–1000bps by sonication. Chromatin samples were pre-cleared using 15µl of protein A or G sepharose beads (Sigma). Pre-cleared ChIP samples were incubated overnight at 4°C with 1–2.5µg of the relevant antibody. The next morning 25µL of protein A or G beads were added to ChIP samples and incubated for 4 hours at 4°C, the beads were extensively washed and immune complexes eluted in ChIP elution buffer (1% SDS, 0.1M NaHCO3) at 65°C for 2 hrs. The eluted material was purified by phenol/chloroform extraction and ethanol-precipitation and the DNA resuspended in nuclease–free water (Sigma) for downstream analysis.

**Quantitative Real–Time PCRs:**
Total RNA was extracted from cells using the RNeasy kit (Qiagen) according to the manufacturer's protocol. RNA was used to generate cDNA by reverse transcriptase PCR using the TaqMan Reverse Transcription kit (Applied Biosystems). Relative mRNA expression levels were determined using the SYBR Green I detection chemistry (Applied Biosystems) on the ABI Prism 7500 Fast Real-Time PCR System. The ribosomal constituent RPLPO was used as a control gene for normalization. All RT-PCR and ChIP experiments
are representative results from a single experiment, which were performed multiple times. Error bars indicate s.d. of triplicate qPCR data. Primer sequences used are available upon request.

**Western blotting:**
Whole-cell lysates were prepared in RIPA buffer containing protease inhibitors (25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% (v/v) NP–40, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 µg ml–1 aprotinin, 10 µg ml–1 leupeptin and 1 mM PMSF). Protein lysates were separated on SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were subsequently probed using the relevant primary and secondary antibodies, and relative protein levels were determined by chemiluminescence.

**Antibodies:**
The following antibodies were used for immunoblotting: mouse anti–PCL1 (Abgent, clone M04, AT3294a) (1:1000), rabbit anti–PCL2 (Genway Biotech, GWB-ML809H) (1:1000), rabbit anti–PCL3 (Brien et al., 2012) (1:1000), mouse anti–EZH2 (Pasini et al., 2004) (clone BD43) (1:8), mouse anti-EED (Bracken et al., 2003) (clone AA19) (1:10), mouse anti–p53 (clone DO1) (1:50), mouse anti–CCNA2 (BD Biosciences, 611268) (1:1000), mouse anti–HA (clone 12CA5) (1:10), mouse anti–ACTIN (MP Biomedicals, 08691001) (1:10,000), mouse anti–p21 (clone DCS–60) (1:3), mouse anti–p16 (clone JC8) (1:3), mouse anti–BMI1 (Bracken et al., 2007) (clone DC9) (1:30), mouse anti–GAPDH (Santa Cruz, sc–25778) (1:1000), rabbit anti–Histone H3 (Abcam, ab1791) (1:5000).
The following antibodies were used for immunoprecipitation and ChIP experiments: mouse anti–PCL1 (Abgent, clone M04, AT3294a) (2µg per ChIP, 5µg per IP), rabbit anti–PCL2 (Genway Biotech, GWB-ML809H) (2µg per ChIP, 5µg per IP), rabbit anti–PCL3 (Brien et al., 2012) (2µg per ChIP, 5µg per IP), mouse anti–EZH2 (clone AC22) (Bracken, 2006) (2µg per ChIP), mouse anti–p53 (clone DO1) (100µls of hybridoma supernatant per ChIP/IP), rabbit anti–E2F1 (Santa Cruz, C–20, sc–193) (2µg per ChIP), rabbit anti–E2F4 (Santa Cruz, C–20, sc–866) (2µg per ChIP) and rabbit anti-H3K27me3 (Millipore, 07-449) (1 µg per ChIP).

**Structural modeling of the PCL1 PHD1 domain**

The conformational propensity of the PCL1 PHD1 domain was studied by molecular dynamics (MD) simulations and clustering analysis. All these calculations were run with the GROMACS simulation package (Hess et al., 2008), version 4.6.3. The PHD1 domain was built by homology modeling (HM) with the MODELLER software package (Eswar et al., 2006). The structure of the PHD domain of TRIM24 (PDBid 1O37) was used as single template due to its optimal structure and sequence alignment to the target. Additionally, we were particularly interested in the comparison to the TRIM24 PHD domain as this was co-crystallized with its unmodified H3(1-10)K4 peptide substrate (Tsai et al., 2010), providing clues to the interaction between the PCL1 PHD1 domain and its p53 substrate. The HM procedure produced 5 structures and the 2 structures with the lowest discrete optimized protein energy (DOPE) score were chosen as starting structures for MD. The proteins termini were
capped with acetyl (ACE) and N-methylamide (NME) residues to avoid end-charge effects. The AMBER99SB-ILDN force field (Lindorff-Larsen et al., 2010) was chosen to represent the protein atoms and counterions in all MD simulations, while water molecules were represented by the TIP4P-Ew (Horn et al., 2004) potential. During the MD simulations the temperature was held constant at 300 K by a Langevin thermostat (Grest and Kremer, 1986) with coupling time constant of 0.1 ps. The Berendsen barostat (was used to hold the pressure constant at 1 bar, with a time constant of 0.5 ps. The equations of motion were integrated using a leap-frog stochastic dynamics integrator with a 2 fs timestep. The linear constraint solver (LINCS) was used to constrain all bonds with hydrogen atoms (Blomberg and Siegbahn, 2012). Long range electrostatics were treated with the Particle Mesh Ewald (PME) method (York et al., 1993; Essmann et al., 1995). The maximum spacing for the Fast Fourier Transform (FFT) grid was chosen as 1 Å. In all simulations cutoff values for Coulomb were set to 12 Å and van der Waals interactions were switched off also at 12 Å. The protein was initially centered in a cubic periodic box with minimum distances between the protein and the box edges equal to 1.3 Å. The total charge was neutralized by the addition of two Na\(^+\) counterions with the genion tool available in GROMACS. The positions of hydrogen atoms, counterions and water molecules was optimized through 500000 steps of steepest descent algorithm. Then the system was equilibrated for 500 ps in the NVT and in the NPT ensembles, respectively. During both of these equilibration steps only water molecules and counterions were left uncontstrained. The protein sidechains, except for the sidechains coordinating the two Zn\(^{2+}\) atoms, were equilibrated for 5 ns and subsequently
all backbone atoms were also equilibrated for 5 ns, except for the backbone of the residues coordinating the two Zn$^{2+}$ atoms and the residues immediately adjacent to these. The position of the Zn$^{2+}$ atoms have been constrained during all MD simulations and the coordination sphere was maintained through distance restraints, where the corresponding distances in the TRIM24 PHD were used as equilibrium values. Structure clustering was performed with the GROMACS tool g_cluster. Clusters were identified by means of the GROMOS algorithm (Daura et al., 1999) with a RMSD cut-off value of 1.5 Å.

**Evolutionary analysis**

A maximum likelihood phylogenetic tree of PCL proteins was generated using MEGA6 (Tamura et al., 2013) from a Clustal Omega alignment (Sievers et al., 2011) of drosophila and vertebrate sequences (Cunningham et al., 2014; Venkatesh et al., 2014).

**Purification of GST-fusion proteins**

PCL1/2/3-PHD1 wild-type and mutant fragments were cloned into the pGEX-6P1 expression vector. Sequence verified clones were transformed into protease deficient *E. coli* strain BL21–DE3. Colonies were picked and grown as started cultures overnight at 37°C in 10mls of TP media. The next day the 10ml started cultures were inoculated into 500mls of pre-warmed TP media. These cultures were grown for 3 hours at 37°C until OD600 was between 0.4-0.6. Protein expression was induced with 0.5 mM IPTG and cultures were grown overnight at 20°C. Cell pellets were harvested and lysed in PBS containing 0.25% Triton X-100 and protease inhibitors. Lysates were pre-cleared by centrifugation at 20000rpm for 45mins, and pre-cleared lysates
were incubated with Glutathione-agarose beads (Pierce) overnight at 4°C. Beads were washed extensively in wash buffer (PBS, 350 mM NaCl, 0.25% Triton X-100) and bound GST-fusion proteins were eluted using the same buffer following the addition of 20 mM Glutathione (Sigma).

**In vitro peptide binding assays**

A biotinylated p53 C-terminal domain peptide (residues 363-393, 1 µg), was incubated with bound to streptavidin-agarose beads (Invitrogen) for 2h at 4°C. GST-PCL1/2/3-PHD1 fragments (5µg) were subsequently incubated with peptide bound beads in binding buffer (50 mM Tris-HCl, pH 7.5, 650 mM NaCl, 0.5% (v/v) NP-40 and 1 mM EDTA) for 30 min at 4 °C. Beads were then washed extensively in binding buffer, and bound protein eluted using 2× Laemlili dye. Eluted protein was analyzed by western blotting.

**Surface Plasmon Resonance (SPR)**

SPR experiments were performed at 25 °C using a series S sensor chip SA with a BiaCore T200 SPR instrument (GE Healthcare). All experiments were performed in HBS-EP running buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3mM EDTA, 0.05% (v/v) surfactant P20). Biotinylated p53 C-terminal peptide was diluted in running buffer to 1 µM and immobilized at a flow rate of 10 µl min⁻¹ to a density of 1714 response units (RU). GST-PCL fusion-proteins or GST control protein (at concentrations 10–160 nM) were injected onto the chip surface for 180 s at a flow rate of 30 µl min⁻¹. The dissociation phase was monitored for 180 s, and the chip surface was then regenerated between each consecutive cycle with a 120 s pulse of regeneration buffer (0.25% [w/v]
SDS, 10 mM Glycine, pH 2). Individual sensorgrams were double-referenced against injection onto an empty flow cell, and GST-alone injections at equivalent concentrations. Data were fitted to a 1:1 Langmuir model using BIAevaluation analysis software (GE Healthcare). The observed results and apparent $K_D$ values were highly reproducible in replicate experiments.
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Venkatesh, B., Lee, A.P., Ravi, V., Maurya, A.K., Lian, M.M., Swann, J.B., Ohta, Y., Flajnik, M.F., Sutoh, Y., Kasahara, M., et al. (2014). Elephant shark genome provides unique insights into gnathostome evolution. Nature 505, 174–179.
Figure S1: PCL2 and PCL3, but not PCL1, are downstream of the E2F pathway
Quantitative ChIP analyses using the indicated antibody in HDFs following 96h of serum starvation (Q) or the same cells following treatment with 20% serum for 24hrs (C). Precipitated DNA was analyzed by qPCR using primers directed towards the promoters of the indicated genes. ChIP enrichments are presented as the percentage of protein bound normalised to input.
Figure S2: Ectopic expression of PCL2 and PCL3 leads to a proliferative advantage in HDFs, whereas PCL1 does not.

A. Representative Senescence-Associated β-Galactosidase stainings in HDFs infected with the indicated pBABE retrovirus at Population Doubling (PD)32. Staining was performed at PD48.

B. Quantification of senescence associated-β-Galactosidase stainings in 2 independent biological replicates.
Figure S3: PCL1 is the only PRC2 component immunoprecipitated with p53

A. PCL1 is the only PRC2 component immunoprecipitated with p53. Western blot analyses using the indicated antibodies of immunoprecipitations of the indicated endogenous proteins performed on nuclear protein lysates of HDFs at PD38.

B. MDM2 does not associate with PCL1. Western blot analyses using the indicated antibodies of immunoprecipitations of the indicated endogenous proteins performed on nuclear protein lysates of HDFs at PD38.

C. Quantitative–RT–PCR analyses of the indicated mRNA transcripts in HDF cells infected with the indicated pBABE retrovirus at PD32, where cells were previously infected with a control (shSCR) or TP53 targeting (shTP53) pLKO lentivirus at PD28. qPCRs were performed at PD48.
Figure S4: Expression of wildtype and mutant PCL proteins in HDFs.

A. Quantitative ChIP analyses using the indicated antibodies in HDFs infected with the indicated retrovirus at PD32. ChIP was performed at PD48. Precipitated DNA was analyzed by qPCR using primers directed towards the INK4A gene promoter. ChIP enrichments are presented as the percentage of protein bound normalized to input.

B. Quantitative–RT–PCR analyses of the indicated mRNA transcripts in human diploid fibroblasts infected with the indicated pBABE retroviral constructs. Cells were infected at PD32 and Q–RT–PCR was performed at PD48.
Figure S5: PHD1 of PCL1 binds the p53 C-terminal domain
A. Protein structure (disorder) plot of PCL1 generated using the IUPred program, a web based server for the prediction of protein disorder based on estimated energy content.
B. Representative SPR sensorgrams for the WT-PCL1–3 PHD1 domains binding to the p53 C-terminal domain peptide.
C. Table showing the binding affinities of WT-PCL1-3 PHD1 and point mutant PCL1-PHD1 fragments. Affinity rate constants were determined from a concentration series of each protein binding to the p53 C-terminal peptide.
Figure S6: MDM2 levels do not change between quiescent and growing HDFs.

A. Quantitative-PCR analysis of MDM2 mRNA levels in serum starved quiescent HDFs (-) or the same cells following serum stimulation for 24hrs (+).

B. Western blot analyses using the indicated antibodies on whole cell protein lysates from serum starved quiescent HDFs (-) or the same cells following serum stimulation (+).