Required enhancer–matrin-3 network interactions for a homeodomain transcription program

Dorota Skowronska-Krawczyk1, Qi Ma1, Michal Schwartz1,2, Kathleen Scully1, Wenbo Li1, Zhijie Liu1, Havilah Taylor1, Jessica Tollkuhn1, Kenneth A. Ogh1, Dimple Notani1, Yoshinori Kohwi1, Terumi Kohwi-Shigematsu1 & Michael G. Rosenfeld1

Homeodomain proteins, described 30 years ago1,2, exert essential roles in development as regulators of target gene expression3–5; however, the molecular mechanisms underlying transcriptional activity of homeodomain factors remain poorly understood. Here we investigate a developmentally required POU-homeodomain transcription factor, Pit1 (also known as Pou1f1), has revealed that, unexpectedly, binding of Pit1-occupied enhancers6 to a nuclear matrix–rich network/architecture6,7 is a key event in effective activation of the Pit1-regulated enhancer/coding gene transcriptional program. Pit1 association with Satb1 (ref. 8) and b-catenin is required for this tethering event. A naturally occurring, dominant negative, point mutation in human Pit1 (R271W), causing combined pituitary hormone deficiency9, results in loss of Pit1 association with b-catenin and Satb1 and therefore the matrix–rich network, blocking Pit1-dependent enhancer/coding target gene activation. This defective activation can be rescued by artificial tethering of the mutant R271W Pit1 protein to the matrix–3 network, bypassing the pre-requisite association with b-catenin and Satb1 otherwise required. The matrix–3 network-tethered R271W Pit1 mutant, but not the untethered protein, restores Pit1-dependent activation of the enhancers and recruitment of co-activators, exemplified by p300, causing both enhancer RNA transcription and target gene activation. These studies have thus revealed an unanticipated homeodomain factor/b-catenin/Satb1-dependent localization of target gene regulatory enhancer regions to a subnuclear architectural structure that serves as an underlying mechanism by which an enhancer-bound homeodomain factor effectively activates developmental gene transcriptional programs.

During pituitary development, the POU-homeodomain transcription factor, Pit1, is necessary for differentiation of thyrotrope, lactotrope and somatotrope cell types in both mice and humans10,11. To further understand the molecular basis for Pit1-mediated gene activation, we mapped the genomic localization of Pit1 by chromatin immunoprecipitation followed by sequencing (ChIP-seq) using antibody specific to matrin-3 (Extended Data Fig. 1d). Both matrin-3 and HNRNPU have been previously found in a salt extraction-resistant nuclear fraction, as co-precipitating factors identified with mass spectrometry. These proteins have thus revealed an unanticipated homeodomain factor/b-catenin/Satb1-dependent localization of target gene regulatory enhancer regions to a subnuclear architectural structure that serves as an underlying mechanism by which an enhancer-bound homeodomain factor effectively activates developmental gene transcriptional programs.

ChIP-seq using a specific anti-matrin-3 antibody revealed that >50% of matrin-3 binding sites co-localized with H3K4me2 peaks (Extended Data Fig. 1f), showing significant enrichment (~16-fold, P value < 10–100) over the ~3% predicted random co-localization with H3K4me2 peaks, covering ~5% of the rat genome. Of these matrin-3/H3K4me2

Figure 1 | Co-localization of Pit1 and matrin-3 on enhancers. a, ChIP-seq analysis of Pit1 and H3K4me2 genome-wide co-localization in GC rat pituitary somatotrope cell line. b, Most DNA sites co-bound by Pit1 and H3K4me2 lie outside gene proximal promoters. c, HA-tagged Pit1 immunoprecipitated from 293-T cells. Co-purifying factors identified with mass spectrometry. d, Co-immunoprecipitation of Pit1 followed by western blot analysis confirmed Pit1-endogenous matrix-3 and HNRNPUs interactions in GC cells. e, Most matrin-3/H3K4me2 sites in GC cells lie outside gene proximal promoters. f, Heat map of ChIP-seq data on non-promoter genome-wide association of Pit1, H3K4me2, H3K4me1, H3K27Ac, matrin-3 in GC cells centred on Pit1 sites and categorized as transcription termination sites (TTS), intergenic, intronic sites. g, Meta-analysis of matrin-3 ChIP-seq data. h, Example of immuno-FISH experiments showing matrin-3 spots (red) colocalized with GH locus spots (green) in GH-expressing GC cells. Chart represents count of percentage of signals exhibiting co-localization, n ≥ 200, ± s.d.
sites, ~80% were elements distal to transcription start sites (Fig. 1e). This finding provides an initial demonstration of matrin-3 association with DNA regulatory sequences in the genome. We noticed that Pit1-associated H3K4me2, H3K4me1 and H3K27Ac-marked enhancers, co-localized with matrin-3 (Fig. 1f, g, Extended Data Fig. 1g), and confirmed matrin-3 antibody specificity by knockdown of endogenous matrin-3 protein (Extended Data Fig. 1h, i). To investigate whether regulatory elements bound by Pit1 occupy the same nuclear compartment as matrin-3, we performed immuno-fluorescence in situ (FISH) experiments with DNA-FISH probes specific to the growth hormone (GH) locus and anti-matrin-3 antibodies, finding that >65% of the FISH signals co-localized with matrin-3 (Fig. 1h, Extended Data Fig. 1i).

Because the pituitary specific homeodomain protein, Prop1, interacts with β-catenin to activate target gene expression, we investigated β-catenin interacting partners in differentiated pituitary cells by expressing β-catenin fused to the biotin ligase recognition peptide (BLRP), along with biotinylating enzyme BirA, in GC cells. Biotinylated β-catenin was present in both the cytoplasmic and the nuclear compartments (Fig. 2a, Extended Data Fig. 2a). Streptavidin pull-down, followed by mass spectrometry, revealed that whereas cytoplasmic β-catenin-interacting factors included α-catenin, as previously described, Pit1 peptides predominated in the nuclear β-catenin fraction (Fig. 2a, Supplementary Tables 4–7). This putative Pit1–β-catenin interaction was confirmed between endogenous proteins by co-immunoprecipitation/western blot experiments using nuclear extracts from GC pituitary cells (Fig. 2b). Using glutathione-S-transferase (GST) pull-down assays, armadillo repeats 8–12 of β-catenin proved sufficient to mediate this interaction, exactly within a region that has been previously described to interact with Prop1 (ref. 14) as well as Lef/Tcf (Fig. 2c, Extended Data Fig. 2b).

We then examined whether β-catenin plays a role in regulating expression of Pit1 target genes, such as GH. Effective knockdown of β-catenin in GC cells (Extended Data Fig. 2c) markedly decreased the level of nascent GH transcripts (Fig. 2d), analogous to what was observed when Pit1 was knocked down (Fig. 2d, Extended Data Fig. 2c). β-catenin also forms a complex with Satb1 (ref. 17), a well-established genome organizer essential for multiple biological processes, including T-cell activation and cancer progression18,19. Reverse transcription coupled with quantitative PCR (RT-qPCR) analysis revealed that the upregulation of Satb1 expression in the mouse pituitary gland coincides with differentiation of Pit1 lineages during pituitary development (Extended Data Fig. 2d), and immunohistochemical analysis confirmed that SATB1 protein is expressed in somatotropes and other cells of the mouse pituitary gland (Extended Data Fig. 2e). Co-immunoprecipitation of Pit1 from GC cells revealed an interaction with Satb1 (Fig. 2b) which was reproduced by co-immunoprecipitation of HA-tagged Pit1 in 293T cells (Extended Data Fig. 2f). Indeed, conditional knockout of Satb1 in the mouse anterior pituitary significantly decreased expression of GH (Extended Data Fig. 2g). We therefore investigated whether interaction between Pit1 and Satb1 was dependent on β-catenin, and vice-versa. Exogenously expressed, HA-tagged Pit1 protein was pulled down after knocking down either β-catenin or Satb1 in 293T cells. In the absence of β-catenin, the

---

**Figure 2** Pit1 association with LIS resistant nuclear component is β-catenin- and SATB1-dependent. a, BLRP-β-catenin immunoprecipitated from cytoplasmic or nuclear fractions of GC cells. Co-purifying factors identified by mass spectrometry. b, Co-immunoprecipitation-Western analysis confirmed Pit1-β-catenin interaction and revealed interaction with SATB1. c, GST-pulldown showing β-catenin armadillo repeat 8 (within region previously shown to interact with Prop1 and Lef1) is required for interaction with Pit1. d, Nascent GH transcripts levels after siRNA knockdown of β-catenin and Pit1 analysed by RT-qPCR. Experiments were repeated 2 times, and P values calculated using Student’s two tailed t-test (± s.d.; **P < 0.01). e, Co-immunoprecipitation of HA-tagged Pit1 protein in 293T cells before and after β-catenin and Satb1 knockdown showing Pit1 interacts simultaneously with both proteins. f, LIS nuclear extraction before and after β-catenin or and Satb1 knockdown shows that both proteins are needed for Pit1 retention in LIS resistant fraction. g, Co-immunoprecipitation of Pit1 protein in GC cells before and after simultaneous knockdown of β-catenin and Satb1 shows interaction of Pit1 with matrin-3 is dependent on the presence of both proteins. h, Examples of immuno-FISH experiments showing GH locus (green) colocalizing with matrin-3 (red) with higher frequency in control culture conditions than after siRNA β-catenin and siRNA Satb1 treatment in GC cells. In Pit1 positive, non-GH-expressing MMQ cells, significantly fewer GH loci are associated with matrin-3. The chart represents the count of percentage of signals exhibiting co-localization in control vs. siβ-catenin and siSATB1 conditions compared to MMQ cells; n ≥ 200, ± s.d., ***P < 0.001.
interaction between Pit1 with Satb1 was diminished, indicating a role for β-catenin in mediating Pit1–Satb1 interaction. Reciprocally, the interaction of Pit1 with β-catenin was partially abolished by Satb1 knockdown (Fig. 2e).

Pit1 has been shown previously to co-fractionate with the insoluble, matrix-3 rich, nuclear fraction in in-vitro biochemical studies. We therefore used lithium 3,5-diadosalicylate (LIS), a chaotropic reagent that extracts a majority of nuclear proteins but preserves the nuclear matrix-3 rich network, to investigate Pit1 subnuclear locations (Extended Data Fig. 2f). When both β-catenin and Satb1 proteins were simultaneously depleted (Extended Data Fig. 2g), the presence of Pit1 in the LIS resistant insoluble fraction was significantly reduced (Fig. 2f). In the double knockdown, total Pit1 protein levels were unchanged and Pit1 protein remained localized to the nucleus (Extended Data Fig. 2k); however, it was now detectable in the looped-out DNA fraction obtained after DNAse I digestion of nuclear halos (Extended Data Fig. 2l). Together, these data indicate that localization of Pit1 protein to the insoluble fraction is dependent on interaction with β-catenin and Satb1. Additionally, when endogenous Pit1 protein was pulled down before and after knocking down both β-catenin and Satb1 in GC cells, the absence of these proteins highly affected interaction between Pit1 and matrix-3 (Fig. 2g).

To further investigate whether association of Pit1-bound enhancers with the matrix-3 fraction is indeed dependent on β-catenin and SATB1, we performed immuno-FISH experiments in GC cells with anti-matrix-3 antibodies and DNA-FISH probes specific to the growth hormone (GH) locus. In GH expressing GC cells, ~65% of the FISH signals co-localized with matrix-3. When both β-catenin and Satb1 were depleted, only ~35% of FISH signals loci co-localized with matrix-3, similar to the overlap of a GH locus in a Pit-1 positive, GH non-expressing cell line (MMQ) (Fig. 2h, Extended Data Fig. 2m). We thus conclude that association of Pit1 enhancers with the nuclear matrix-3 rich network/structure observed by immuno-FISH (Fig. 1h, Fig. 2h) and biochemical extractions (Fig. 2f, Extended Data Fig. 2h) is dependent on intact interactions of Pit1 with both β-catenin and Satb1.

Global run-on (GRO-seq) analysis using GC cells before and after simultaneous knockdown of both β-catenin and Satb1 revealed that in total, 1,350 gene nascent transcripts were downregulated and 916 genes were upregulated under these double knockdown conditions (Extended Data Fig. 3a). Out of the 1,350 genes positively regulated by both β-catenin and SATB1 (Supplementary Table 8), 991 had Pit1-bound enhancer elements within 200 kb of their start sites (Extended Data Fig. 3a), and all 991 were significantly downregulated (Fig. 3a). Consistent with their functional importance, the expression of the eRNAs associated with these 991 Pit1-bound enhancers was significantly affected in the simultaneous knockdown of β-catenin and Satb1 (Fig. 3b). ChIP-seq analysis revealed that Satb1 and β-catenin proteins were located at the centre of Pit1 positive enhancers (Extended Data Fig. 3b–d), strongly supporting the co-binding model of these three proteins. Based on this analysis, we selected several highly downregulated genes that contain Pit1 enhancers (Extended Data Fig. 3c) and observed that knockdown of either of the two proteins significantly diminished target gene expression (Extended Data Fig. 3g). Additionally, knockdown of either Pit1 or β-catenin affected Satb1 association with enhancer elements, as assessed by ChIP-qPCR (Fig. 3c, Extended Data Fig. 3e). Association of matrix-3 with Pit1-dependent enhancers was significantly reduced when either β-catenin or SATB1 were downregulated (Extended Data Fig. 3h). Finally, we confirmed that lack of Pit1 protein inhibited association of selected Pit1-dependent enhancers with matrix-3 (Fig. 3d, Extended Data Fig. 3f). Together, these data suggest that functional interaction between Pit1-dependent regulatory elements and matrix-3 requires association of the DNA-bound Pit1 with β-catenin and SATB1. Consistent with this suggestion, lack of matrix-3 had a negative effect on the expression of target genes (Extended Data Fig. 3i).

Naturally occurring mutations in Pit1 underlie number of combined pituitary hormone deficiency (CPHD) cases in humans, with the R271 residue being the most frequently mutated. Amino acid 271 maps to the last turn of helix 3 in the homeodomain and is located outside the DNA-binding surface. Although the R271W mutation in Pit1 confers dominant-negative properties (Extended Data Fig. 4a), it does not affect protein stability or binding to its cognate DNA sites (Extended Data Fig. 4b–d). We therefore investigated whether the R271W mutation affects the composition of the Pit1 complex. Co-immunoprecipitation of recombinant HA-tagged Pit1 proteins followed by western blot analysis revealed that the R271W mutation disrupted the ability of Pit1 to interact with matrix-3 and HNRNP-U, apparently owing to its inability to bind β-catenin and Satb1 (Fig. 4a). Additionally, biochemical fractionation indicated that the R271W mutant is largely lost from the insoluble fraction (Fig. 4b), although both wild-type and R271W proteins were expressed at similar levels and found entirely localized to the nucleus (Extended Data Fig. 4e). Instead, R271W was detected in the...
'looped-out DNA' fraction (Fig. 4b), suggesting that it had lost its ability to interact with the LIS-resistant fraction.

Together, these data indicated that a key function of homeodomain transcription factors such as Pit1 might be to join bound regulatory regions to the matrin-3-rich network, an association required to activate target gene transcription. Because the transcriptionally inactive R271W mutation interferes with this association, we experimentally tested this hypothesis by artificially tethering the R271W mutant to the matrin-3 network to try to restore target gene transcription. Several protein domains that are responsible for association with the matrin-3-rich 'matrix attachment regions' have been previously described and include the SAF/SAP domain (for example, in HNRNPU21, SAF-B, PARP proteins22; AT-hooks (HMG proteins23); and the atypical homeodomain, SATB118. We therefore grafted the SAF/SAP domain from rat HNRNU (amino acids 4–45), onto HA-tagged wild-type and R271W Pit1 (Fig. 4c). As predicted, R271W-SAF hybrid protein localized efficiently in the insoluble nuclear fraction (Extended Data Fig. 4f). Hybrid wild-type (WT)-SAF and R271W-SAF proteins were expressed in GC treated with short interfering RNAs to deplete endogenous Pit1 (Extended Data Fig. 4g).

As expected, matrin-3 association with Pit1-dependent enhancers and expression of Pit1-dependent coding genes and eRNAs were lost upon Pit1 knockdown, but were restored in the presence of either wild-type or WT-SAF Pit1 (Fig. 4d, Extended Data Fig. 4i). In contrast, neither the association of Pit1 enhancers with matrin-3 nor target gene and eRNA expression were rescued in R271W-expressing cells. However, the R271W-SAF hybrid rescued matrin-3 interaction and Pit1 transcriptional activation to levels close to those achieved by wild-type Pit1. These experiments indicate that specific subnuclear localization of Pit1 is a bona fide requirement for its full activity, and that this is the only functional defect in the R271W mutant Pit1 protein.

To investigate which step of enhancer activation is affected by R271W, we tested recruitment of p300, functional homologue of CREB binding protein (CBP), a known co-factor of Pit126, finding that p300 association with Pit1 enhancers was significantly reduced (Extended Data Fig. 4h). Overexpression of R271W was not able to rescue this recruitment whereas hybrid R271W-SAF did restore p300 association with Pit1 bound enhancers (Extended Data Fig. 4h). These data suggest that eRNA transcription and binding of critical co-activators, such as p300, require recruitment of Pit1 to the matrin-3-rich network.

To further substantiate these observations in intact pituitary, ex vivo rescue approach took advantage of the “Snell” mouse model that harbours a mutation (W261C) in Pit1 that renders it incapable of binding to hormone defficiency pituitary glands.

**Figure 4** Naturally occurring R271W mutation in human Pit1 affects ability of Pit1 to interact with matrin-3 rich network. a, Western blot analysis showing defective interaction of Pit1 R271W mutant with components of nuclear matrin-3 enriched network as well as β-catenin and Satb1. b, Biochemical extraction shows that the amount of R271W mutant protein decreased in the LIS-resistant fraction and associates instead with the looped-out DNA fraction. c, Schematic representation of Pit1 protein with added SAF domain. Red “x” - location of amino-acid 271. d, Analysis of effect of overexpression of different forms of Pit1 protein in absence of endogenous Pit1; top panels: ChIP-qPCR analysis of matrin-3 association; bottom panels: RT-qPCR analysis of transcriptional effect on selected targets. Experiments were repeated 3 or 4 times, and P values calculated using Student’s two tailed t-test (± s.d.; **p < 0.01, ***p < 0.001). e, Analysis of effect of ex vivo overexpression of R271W mutant with and without SAF domain in P2 snell mutant pituitary glands. GH expression is rescued only when the R271W-SAF protein is overexpressed. Bottom: quantification of percentage of HA-positive GH expressing cells. Two pituitaries were used per condition, P value calculated using χ2 test. f, Model: association of Pit1-bound regulatory element with matrin-3 rich network and loss of this interaction based on failure of R271W mutant to interact with β-catenin and SATB1 causing loss of gene activation as in CPHD.
to cognate DNA sites. Overexpressing HA-tagged Pit1-R271W-SAF hybrid, but not Pit1-R271W protein, rescued expression of endogenous GH as visualized by immunohistochemistry (Fig. 4e). On the basis of these data, we propose a model in which Pit1 association with the matrin-3-rich network is dispensable for its biological activity, as demonstrated by the lack of transcriotional activity of the naturally occurring mutant R271W Pit1 (Fig. 4f).

Nuclear organization and genome three-dimensional structure are postulated to have a prominent role in regulation of gene expression. Here, we have presented evidence that a homeodomain transcription factor regulates gene transcriptional programs through interaction with components of subnuclear structure (for example, matrin-3-rich structure). Other homeodomain proteins (for example, Lhx3, Prop1, Oct1), as well as different classes of transcription factors, have been shown to associate with the high-salt extraction-resistant fraction, corresponding to the matrin-3-rich network. This suggests a model in which this interaction may be required for transcriptional activation of enhancers regulated by many homeodomain proteins, and perhaps several other types of DNA binding transcription factors.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 2 December 2013; accepted 10 June 2014.

Published online 3 August 2014.

1. McGinnis, W., Levine, M. S., Hafen, E., Kuroiwa, A. & Gehring, W. J. A conserved DNA sequence in homoeotic genes of the Drosophila Antennapedia and bithorax complexes. Nature 308, 428–433 (1984).

2. Scott, M. P. & Weiner, A. J. Structural relationships among genes that control development: sequence homology between the Antennapedia, Ultrabithorax, and fushi tarazu loci of Drosophila. Proc. Natl. Acad. Sci. USA 81, 4115–4119 (1984).

3. Affolter, M., Schier, A. & Gehring, W. J. Homeodomain proteins and the regulation of gene expression. Curr. Opin. Cell Biol. 2, 485–495 (1990).

4. Prince, K. L., Walvoord, E. C. & Rhodes, S. J. The role of homeodomain transcription factors in heritable putative disease. Nature Rev. Endocrinol. 7, 727–737 (2011).

5. Heintzman, N. D. & Ren, B. Finding distal regulatory elements in the human genome. Curr. Opin. Genet. Dev. 19, 541–549 (2009).

6. Zeitz, M. J., Malyavantkar, K. S., Seifert, B. & Berzney, R. Matrin 3: chromosomal distribution and protein interactions. J. Cell. Biochem. 108, 125–133 (2009).

7. Nickerson, J. Experimental observations of a nuclear matrix. J. Cell Sci. 114, 463–474 (2001).

8. Kohwi-Shigematsu, T. et al. Genome organizing function of SATB1 in tumor progression. Semin. Cancer Biol. 23, 72–79 (2013).

9. Radovick, S. et al. A mutation in the POU-homeodomain of Pit-1 responsible for combined pituitary hormone deficiency. Science 257, 1115–1118 (1992).

10. Zhu, X., Wang, J., Ju, B. G. & Rosenfeld, M. G. Signaling and epigenetic regulation of pituitary development. Curr. Opin. Cell Biol. 19, 605–611 (2007).

11. Ingraham, H. A. et al. A tissue-specific transcription factor containing a homeodomain specifies a pituitary phenotype. Cell 55, 519–529 (1988).

12. Nakayasu, H. & Berezney, R. Nuclear matrices: identification of the major nuclear matrix proteins. Proc. Natl. Acad. Sci. USA 88, 10312–10316 (1991).

13. Romig, H., Fackelmayer, F. O., Renz, A., Ramsperger, U. & Richter, A. Characterization of SAF-A, a novel nuclear DNA binding protein from HeLa cells with high affinity for nuclear matrix/scaffold attachment DNA elements. EMBO J. 11, 3431–3440 (1992).

14. Olson, L. E. et al. Homeodomain-mediated beta-catenin-dependent switching events dictate cell-lineage determination. Cell 125, 593–605 (2006).

15. Su, L. K., Vogelstein, B. & Kinzler, K. W. Association of the APC tumor suppressor protein with catenins. Science 262, 1734–1737 (1993).

16. Graham, T. A., Weaver, C., Mao, F., Kimmelman, D. & Xu, W. Crystal structure of a beta-catenin/Tcf complex. Cell 103, 885–896 (2000).

17. Notani, D. et al. Global regulator SATB1 recruits beta-catenin and regulates T(H)2 differentiation in Wnt-dependent manner. PLoS Biol. 8, e1000296 (2010).

18. Dickson, L. A., Dickson, C. D. & Kohwi-Shigematsu, T. An atypical homeodomain in SATB1 promotes specific recognition of the key structural element in a matrix attachment region. J. Biol. Chem. 272, 11463–11470 (1997).

19. Cai, S., Han, H. J. & Kohwi-Shigematsu, T. Tissue–specific nuclear architecture and gene expression regulated by SATB1. Nature Genet. 34, 42–51 (2003).

20. Mancini, M. G., Liu, B., Sharp, Z. D. & Mancini, M. A. Subnuclear partitioning and functional regulation of the Pit-1 transcription factor. J. Cell. Biochem. 72, 322–338 (1999).

21. Mirkovitch, J., Mirault, M. E. & Laemmli, U. K. Organization of the higher-order chromatin loop: specific DNA attachment sites on nuclear scaffold. Cell 39, 223–232 (1984).

22. Li, W. et al. Functional roles of enhancer RNAs for oestrogen-dependent transcriptional activation. Nature 498, 516–520 (2013).

23. Kipp, M. et al. SAF-Box, a conserved protein domain that specifically recognizes scaffold attachment region DNA. Mol. Cell. Biol. 20, 7480–7489 (2000).

24. Aravind, L. & Koonin, E. V. SAF – a putative DNA-binding motif involved in chromosomal organization. Trends Biochem. Sci. 25, 112–114 (2000).

25. Reeves, R. Molecular biology of HMGA proteins: hubs of nuclear function. Gene 277, 63–81 (2001).

26. Xu, L. et al. Signal-specific co-activator domain requirements for Pit-1 activation. Nature 395, 301–306 (1998).

27. Liu, S. et al. Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene pit-1. Nature 347, 528–533 (1990).

28. Gibcus, J. H. & Dekker, J. The hierarchy of the 3D genome. Mol. Cell 49, 773–782 (2013).

29. Bickmore, W. A. & van Steensel, B. Genome architecture: domain organization of interphase chromosomes. Cell 152, 1270–1284 (2013).

30. S’iakste N. I. & S’iakste T. G. Transcription factors and the nuclear matrix [in Russian]. Mol. Biol. (Mosk.) 35, 739–749 (2001).

Supplementary Information is available in the online version of the paper.

Acknowledgements We thank M. Ghassaeinia for assistance with mass spectrometry; C. Nelson for cell culture assistance; J. Hightower for assistance with figures and manuscript preparation; T. Suter for help with images analysis. We acknowledge J. Santini and the UCSD Neuroscience Microscopy Shared Facility (Grant P30 NS047101) for imaging. These studies were supported by grants NS034934, DK039949, DK018477, HL065445, CA173903 to M.G.R. from NIH. D.S.-K. was supported by EMBO Long Term Fellowship, The Swiss National Science Foundation and The San Diego Foundation. M.G.R. is an Investigator with HHMI.

Author Contributions D.S.-K. and M.G.R. conceived the project. D.S.-K. performed the majority of the experiments; Q.M. performed the bioinformatic analyses; M.S. contributed three dimensional immuno-FISH W.L. contributed GRO-Seqs; K.A.O. assisted in deep-sequencing library preparations and sequencing; additional experiments/methods were contributed by K.S., J.T., Z.L. and D.D.N.; H.T. and K.S. assisted in animal-based experiments; Y.K. and T.K.-S. contributed critical insights and reagents. D.S.-K., K.S. and M.G.R. wrote the manuscript. All authors discussed the results and commented on or edited the manuscript.

Author Information Microarray data are deposited in GEO under accession number GSE58009. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.G.R. (mrosenfeld@ucsd.edu) or D.S.-K. (dkrawczyk@ucsd.edu).
METHODS
Antibodies. The antibodies used in this study are listed in Supplementary Table 9. siRNA and expression vector electroporations. siRNA and overexpression experiments in GC cells and in P2 pituitary were performed using Neon system (Invitrogen). Conditions of 2 pulses of 1,150 V with 30 ms spacing were used. 2.5 μM siRNA and 30 μg of DNA were used per 3 × 10^6 cells. Knockdown was repeated after 48 h to obtain optimal depletion of the protein, verified by western blotting. Overexpression experiments were harvested after 48 h. siRNAs used in the study are listed in Supplementary Table 10. siRNA and overexpression experiments in 293T cells were performed using Lipofectamine 2000 (Invitrogen) using manufacturer’s protocol. RT-qPCR. RNA was isolated using TRIzol (Invitrogen) and total RNA was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen). Quantitative PCRs were performed mostly with QIAexpressionist SYBR Green Supermix (BIO-RAD). For normalization, ΔCq values were calculated relative to the levels of GAPDH transcripts. The experiments were repeated at least three times as a biological replicates. P values were obtained using a two-tailed Student’s t-test; *p < 0.05, **p < 0.01, ***p < 0.001; primers are listed in Supplementary Table 10.
ChIP-seq. ChIP was performed as previously described1. Briefly, for ChIP-seq, approximately 10^7 cells were cross-linked with 1% formaldehyde at room temperature for 10 min and neutralized with 0.125 M glycine. After sonication, 20–30 μg of soluble chromatin was incubated with 1–5 μg of antibody at 4 °C overnight. Immunoprecipitated complexes were collected using G-protein Dynabeads (Invitrogen). Subsequently, immuno-complexes were washed, and DNA was extracted and purified by QiAquick Spin columns (Qiagen). For ChIP-seq, the extracted DNA was ligated to specific adaptors followed by deep sequencing by the Illumina’s HiSeq 2000 system according to the manufacturer’s instructions. Usually, the first 48 bp for each sequence tag returned by the Illumina Pipeline was aligned to the nr assembly using BFAST or Bowtie2. Only uniquely mapped tags were selected for further analysis. The data was visualized by preparing custom tracks on the UCSC genome browser using HOMER (http://homer.salk.edu/homer). The total number of mapable reads was normalized to 10^7 for each experiment presented in this study.
Identification of ChIP-seq peaks. The ChIP-seq peaks were identified by HOMER. Given different binding patterns of transcription factors and histones, parameters were optimized for the narrow tag distribution characteristic of transcription factors by searching for high read-enrichment regions within a 200-bp sliding window. Regions of maximal density exceeding a given threshold were called as peaks, and adjacent peaks were set to be >500 bp away to avoid redundant detection. The common artefacts from clonal amplification were circumvented by considering only one tag from each unique genomic position. The threshold was set at a false discovery rate (FDR) of 0.001 determined by peak finding using randomized tag positions in a genome with an effective size of 2 × 10^9 bp. For ChIP-seq of histone marks, seed regions were initially found using a peak size of 500 bp (FDR < 0.001) to identify enriched loci. Enriched regions separated by <1 kb were merged and considered as blocks of variable lengths. All called peaks were then associated with genes by cross-referencing with the ReSeq TSS database. Peaks from individual experiments were considered overlapping if their peak centres were located within 200 bp (for some analyses the distance between them could extend to 1 kb). The peaks within ±1 kb apart from the ReSeq TSS site were considered to be promoter bound.
GRO-seq. GRO-seq experiments were performed as previously reported.1, 3 Briefly, GC cells were swelled in swelling buffer (10 mM Tris pH 7.5, 2 mM MgCl2, 3 mM CaCl2) for 5 min on ice and then lysed in lysis buffer (swelling buffer with 0.5% IGEPAL and 10% glycerol) before being finally re-suspended in 100 μl of freezing buffer (50 mM Tris–HCl pH 8.3, 40% glycerol, 5 mM MgCl2, 0.1 mM EDTA). For the run-on assay, re-suspended nuclei were mixed with an equal volume of reaction buffer (10 mM Tris–HCl pH 8.0, 5 mM MgCl2, 1 mM dithiothreitol (DTT), 300 mM KCl, 20 units of Superase–In, 1% sarkosyl, 500 μM ATP, GTP, Br–UTP and 2 μM CTP) and incubated for 5 min at 30 °C. The nuclear–run on RNA (NRO–RNA) was then extracted with TRIzol LS reagent (Invitrogen) following the manufacturer’s instructions. After base hydrolysis on ice for 40 min and followed by treatment with DNase I and Antarctic phosphatase, the Br–UTP–labelled NRO–RNA was purified by anti-BrdU agarose beads (Santa Cruz Biotech) in binding buffer (0.5 × SSPE, 1 mM EDTA, 0.05% Tween) for 3 h at 4 °C while rotating. Then T4 PNK (NEB) was used to repair the end of NRO-RNA. Subsequently, complementary DNA synthesis was performed with addition of dCTP and dUTP (NEB) and in a reaction mixture containing 1 mM each of dNTPs, 50 units of T4 DNA polymerase (NEB), 1 mM dNTPs and 5 μl of T4 PNK (NEB) was added to the mix to a final concentration of 0.1% and samples were boiled for 5 min. TCEP (Tris (2-carboxyethyl) phosphine) was added to 1 mM (final concentration) and the samples were incubated at 37 °C for 30 min. Subsequently, the samples were carboxymethylated with 0.5 mg ml−1 of iodoacetamide for 30 min at 37 °C followed by neutralization with 2 mM TCEP (final concentration). Proteins samples prepared as above were digested with trypsin (trypsin:protein ratio 1:50) overnight at 37 °C. RapiGest was degraded and removed by treating the samples with 250 mM HCl at 37 °C for 1 h followed by centrifugation at 14,000 r.p.m. for 30 min at 4 °C. The soluble fraction was then added to a new tube and the peptides were extracted and desalted using C18 desalting columns (Thermo Scientific).
LC-MS/MS analysis. Trypsin-digested peptides were analysed by ultrahigh pressure liquid chromatography (UPLC) coupled with tandem mass spectrometry (LC-MS/MS) using nano-spray ionization. The nano-spray ionization experiments were performed using a TripleTOF 5600 hybrid mass spectrometer (ABSciex) interfaced with nano-scale reverse-phase-hypersil UPLC (Waters corporation nano ACQUITY) using a 20 cm-75 micron ID glass capillary packed with 2.5 μm C18 (130) C18HTM beads (Waters corporation). Peptides were eluted from the C18 column into the mass spectrometer using a linear gradient (5–80%) of ACN (acetonitrile) at a flow rate of 250 nl min−1 for 1 h. The buffers used to create the ACN gradient were: buffer A (98% H2O, 2% ACN, 0.1% formic acid, and 0.005% trifluoroacetic acid (TFA)) and buffer B (100% ACN, 0.1% formic acid, and 0.005% TFA). MS/MS data were acquired in a data-dependent manner in which the MS1 data was acquired for 250 ms at m/z of 400 to 1,250 Da and the MS/MS data was acquired from m/z of 50 to 2,000 Da. The independent data acquisition (IDA) parameters were as follows; MS1-TOF acquisition time of 250 ms, followed by 50 MS2 events of 48 ms acquisition time for each event. The threshold to trigger MS2 event was set to 150 counts for at least 2 days when the ion had the charge state +2, +3 and +4. The ion exclusion time was set to 4 s. Finally, the collected data were analysed using Protein Pilot 4.5 (ABSCIEX) for protein identification.
Three-dimensional-immuno-FISH. Cells were grown on poly-lysine coated cover slips. For immuno-FISH cells were fixed with 4% formaldehyde and permeabilized with 0.5% Triton X-100 followed by glycerol wash and freezing in liquid nitrogen. Cells were then treated with 0.1 M HCl and with pepsin. Cells were then washed, blocked in 4% BSA and incubated with the primary anti-matrix 3 antibody (ab70336). The cover slips were incubated with a biotinylated secondary antibody (Vector Labs BA-1000) followed by fluorescent streptavidin and post-fixed cells at 4% paraformaldehyde. The cover slips were then immersed in 50% formamide/2×SSC and in 0.5×SSC. Afterwards the cover slips were incubated with the appropriate fluorescent secondary antibodies, washed and mounted on slides with ProLong gold containing DAPI. The images were taken using an Olympus FV1000 confocal microscope.
Three-dimensional-immuno-FISH data analysis. Quantification of co-localization was determined using the Volocity Software (Perkin Elmer) as a double blind experiment. The FISH signals and Matrin-3 spots were identified automatically based upon intensity and object size. The number of overlapping signals was automatically determined. The data are based on at least 200 FISH signal per condition from two independent experiments. Up to 10 pictures per conditions were analysed, P value was calculated using Student’s two tailed t-test.

LIS extraction. Biochemical extraction of LIS resistant fraction was performed according to the published detailed protocol33 with the following modifications: LIS extraction was optimized to be the most successful at 7.5 mM, DNase I digestion (5 U per 50 μl of nuclei) was performed on ice 15 min.

Mice. Prop1-Cre transgenic mice were generated by using the 2.2 kb promoter and enhancer region of the Prop1 gene to drive the Cre recombinase expression in mixed background (X. Zhu, M.G.R. et al., submitted). Satb1flg/flq mice, which have LoxP recombination sites flanking Satb1 exons 3 and 5 has been generated in the T. Kohwi-Shigematsu laboratory (M.A. Balamotis, T.K.-S., K.Y. et al., submitted). Mice were collected at embryonic day 16.5, WT = 4, cKO = 12 embryos.

31. Wang, D. et al. Reprogramming transcription by distinct classes of enhancers functionally defined by eRNA. Nature 474, 390–394 (2011).
32. Core, L. J., Waterfall, J. J. & Lis, J. T. Nascent RNA sequencing reveals widespread pausing and divergent initiation at human promoters. Science 322, 1845–1848 (2008).
33. Gasser, S. M. & Vassetzky, Y. S. in Chromatin: A Practical Approach (ed. Gould, H.) pp. 111–124 (Oxford Univ. Press, 1998).
Extended Data Figure 1 | Matrin-3 co-localizes with enhancers in GC cells.  

**a**, Validation of Pit1 antibody specificity by qPCR analysis of ChIP signals on two known targets; the GH and NeuroD4 enhancers shows lack of Pit1 signal after Pit1 knockdown. Experiment repeated 2 times, *P* values were calculated using Student’s two tailed *t*-test (± s.d.; ***P < 0.001).  

**b**, Western-blot confirming Pit1 knockdown efficiency in samples used to assess Pit1 antibody specificity.  

**c**, Co-immunoprecipitation of HA-tagged, overexpressed Pit1 protein from 293T cells confirms interaction of Pit1 protein with endogenous matrin-3 and hnRNP-U proteins (nt, non-transfected).  

**d**, Reciprocal co-immunoprecipitation of matrin-3 followed by western blot analysis confirmed interaction of matrin-3 with Pit1.  

**e**, Super-resolution image (x100, OMX DeltaVision) of immunostaining with anti-matrin-3 antibody in GC cells reveals “structure-like” matrin-3 network.  

**f**, ChIP-seq analysis reveals that ~50% of matrin-3 DNA binding sites co-localize with the enhancer mark, H3K4me2, in GC cells.  

**g**, Meta-analysis plot of ChIP-seq data shows that Pit1 bound enhancers co-localize with matrin-3, H3K4me1, H3K4me2 and H3K27Ac, but not with a mark of silent chromatin, H3K27me3.  

**h**, Western blot confirming matrin-3 knockdown efficiency in GC cells.  

**i**, Validation of matrin-3 antibody specificity by qPCR analysis of ChIP signal on known Pit1 targets shows lack of matrin-3 signal after matrin-3 knockdown. Experiment repeated 2 times, *P* values were calculated using Student’s two tailed *t*-test (± s.d.; **P < 0.01, ***P < 0.001).  

**j**, Single optical sections of immuno-FISH experiment in GC cells. From left: matrin-3, GH-FISH, DAPI, merge; each staining shown separately in black and white, and merged as matrin-3 antibody staining in red and the GH locus labelled with a DNA probe in green.
Extended Data Figure 2 | Co-localization and interaction of Pit-1 with β-catenin and Satb1. 

**a**, Western blot showing that biotinylated BLRP-β-catenin is present in the nuclear and cytoplasmic fractions of GC cells. Biotinylated BLRP-β-catenin is visualized with streptavidin-horse radish peroxidase. 

**b**, Bacterially expressed GST-β-catenin fusion proteins used to map interaction with Pit-1 in GC cell extracts. 

**c**, Western blot validation of siRNA knockdown of β-catenin and Pit-1 proteins in GC cell samples. 

**d**, RT-qPCR analysis of Satb1 mRNA expression in embryonic and adult mouse pituitary glands showing increased expression that parallels differentiation of Pit1-dependent cell types, n = 3, ± s.d. 

**e**, Immunohistochemical staining of SATB1 and GH in the anterior lobe of an adult mouse pituitary showing co-localization of signals at the cellular level. 

**f**, Western blot showing co-immunoprecipitation of SATB1 with HA-tagged Pit1 in 293T cells. 

**g**, RT-qPCR analysis of GH nascent transcript levels in E16.5 mouse pituitaries harbouring a Prop1-CRE conditional deletion of Satb1, ± s.d., *P < 0.05, WT = 4, cKO = 12. 

**h**, Schematic representation of the LIS-extraction procedure used to isolate matrin-3-rich network. 

**i**, Pit-1 co-localizes with Satb1 and matrin-3 in the LIS-resistant insoluble fraction whereas β-catenin is present in both LIS-extracted and LIS-resistant insoluble fractions in GC cells. 

**j**, Western blot confirming β-catenin and Satb1 knockdown efficiency in GC cells. 

**k**, siRNA knockdown of β-catenin and Satb1 in GC cells does not affect either the subcellular distribution or the level of Pit1 protein. 

**l**, After siRNA knockdown of β-catenin and Satb1 followed by LIS extraction, Pit-1 is detectable in the “looped-out DNA” fraction. 

**m**, ImmunofISH in GC cells before and after siRNA knockdown of β-catenin and Satb1 in MMQ cells. Single optical section of each experiment is presented. In merge: Matrin3 antibody immunostaining in red and GH loci labelled with a fluorescent green DNA probe.
Extended Data Figure 3 | β-catenin and SATB1 have transcriptional effects on Pit1 targets. a, Most β-catenin/Satb1-activated target genes are associated with Pit-1-bound enhancers in GC cells. b, Example picture of ChIP-seq and GRO-seq analysis of a Pit-1/Satb1 target gene, the NeuroD4 locus. c, Location of Pit1-dependent enhancers (in yellow) relative to the transcription start sites of selected target genes. d, Heat-map plot of ChIP-seq tag distribution on Pit1 enhancers showing enrichment of Satb1 and β-catenin signal in centre of enhancer. e, ChIP-qPCR analysis of Satb1 association with Pit1 enhancers upon either Pit1 or β-catenin knockdown. Experiments repeated 3 times, P values calculated using Student’s two tailed t-test (± s.d.; *P < 0.05, **P < 0.01, ***P < 0.001). f, ChIP-qPCR analysis of matrin-3 association with Pit1 enhancers upon Pit1 knockdown. Experiments repeated 2–4 times, P values calculated using Student’s two tailed t-test (± s.d.; *P < 0.05, **P < 0.01, ***P < 0.001). g, qPCR analysis showing significant change in expression of selected Pit1 target genes upon either Pit1 or Satb1 knockdown (± s.d.; ***P < 0.001). h, ChIP-qPCR analysis of matrin-3 association with Pit1-dependent enhancers following siRNA knockdown of β-catenin or Satb1. Experiment repeated 2–3 times, P values were calculated using Student’s two tailed t-test (± s.d.; *P < 0.05, **P < 0.01, ***P < 0.001). i, RT-qPCR analysis of relative expression of target gene mRNA following siRNA knockdown of matrin-3.
Extended Data Figure 4 | Pit-1 R271W mutant protein binds cognate DNA sites but does not associate with the nuclear LIS-resistant insoluble fraction.

**a**, RT-qPCR analysis of mRNA expression of Pit1-dependent target genes in GC cells. Both overexpressed, wild-type and R271W, Pit1 proteins compete for binding to recognition sites with endogenous Pit1. Experiment repeated 2 times, $P$ values were calculated using Student’s two tailed t-test ($\pm$ s.d.; *$P < 0.05$).

**b**, Heat map of ChIP-seq data showing that overexpressed HA-Pit1 WT and HA-Pit1 R271W bind to the same enhancer DNA recognition sites as endogenous Pit1 protein.

**c**, Scattered dot plot of genome-wide peaks consistency analysis between the wild-type and R271W Pit1 to Pit1 enhancers with Pearson correlation coefficient shows a strong resemblance of the data.

**d**, ChIP-seq analysis comparing normalized tag density on GC samples containing overexpressed wild-type and R271W Pit1 protein. x-axis represents the number of base pairs from the centre of the Pit1 peak. 

**e**, Both wild-type and R271W Pit1 protein express at similar levels and partition in the nuclear fraction.

**f**, Fusion of the SAF matrix-association domain results in fractionation of R271W-SAF Pit1 protein in LIS-resistant insoluble fraction of GC cells.

**g**, Western blot showing equivalent expression of all HA-tagged Pit1 constructs in GC cells. Asterisk, product of degradation of overexpressed Pit1 protein.

**h, i**, ChIP-qPCR (**h**) and RT-qPCR (**i**) analysis of effect of overexpression of different forms of Pit1 protein on p300 association and enhancer RNA (eRNA) expression, respectively, in the absence of endogenous Pit1. Experiments have been repeated 2–4 times, $P$ values were calculated using Student’s two tailed t-test ($\pm$ s.d.; **$P < 0.01$, ***$P < 0.001$).