Nucleoporin 107, 62 and 153 mediate Kcnq1ot1 imprinted domain regulation in extraembryonic endoderm stem cells

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Genomic imprinting is a phenomenon that restricts transcription to predominantly one parental allele. How this transcriptional duality is regulated is poorly understood. Here we perform an RNA interference screen for epigenetic factors involved in paternal allelic silencing at the Kcnq1ot1 imprinted domain in mouse extraembryonic endoderm stem cells. Multiple factors are identified, including nucleoporin 107 (NUP107). To determine NUP107’s role and specificity in Kcnq1ot1 imprinted domain regulation, we deplete Nup107, as well as Nup62, Nup98/96 and Nup153. Nup107, Nup62 and Nup153, but not Nup98/96 depletion, reduce Kcnq1ot1 noncoding RNA volume, displace the Kcnq1ot1 domain from the nuclear periphery, reactivate a subset of normally silent paternal alleles in the domain, alter histone modifications with concomitant changes in KMT2A, EZH2 and EHMT2 occupancy, as well as reduce cohesin interactions at the Kcnq1ot1 imprinting control region. Our results establish an important role for specific nucleoporins in mediating Kcnq1ot1 imprinted domain regulation.
Genomic imprinting is an epigenetic process that restricts expression of specific genes to predominantly the maternally- or paternally inherited allele. The biochemical mechanisms that generate this allele-specific asymmetry rely upon multiple protein families, broadly termed epigenetic factors. However, it is still not fully clear which specific epigenetic factors establish and maintain this duality. In this study, we investigated the Kcnq1ot1 domain to further understand the mechanisms involved in allele-specific asymmetry. The Kcnq1ot1 domain serves as an excellent model of imprinted domain regulation, since all known epigenetic regulatory mechanisms have some role at the Kcnq1ot1 imprinted domain, including differential DNA methylation and chromatin modifications, noncoding RNA expression, transcriptional interference, noncoding RNA-mediated silencing, CCCTC-binding factor (CTCF)/cohesin insulator activity and chromatin looping.\(^9\)

Within the Kcnq1ot1 domain resides the imprinting control region (ICR), the paternally expressed Cdkn1c gene, and several genes that escape transcript 1) noncoding RNA (ncRNA), 9 maternally expressed genes that contribute to the Kcnq1ot1 ICR deletion leads to paternal reactivation of imprinted genes in CAST7XB6 Cdkn1c\(^{\Delta neoR}\) embryonic, trophoblast and extraembryonic endoderm (XEN) stem cells. Reactivation of the silent Cdkn1c\(^{\Delta neoR}\) allele following depletion allowed for survival and selection of colonies in the presence of neomycin, and thus, identification of epigenetic factors crucial in maintaining its silent state. Albeit, only XEN cells displayed repression of the paternally inherited Cdkn1c\(^{\Delta neoR}\) allele to a level that would allow efficient screening (Supplementary Fig. 2b). Using this strategy, 696 colonies were picked for a second round of neomycin selection, following which 297 colonies were isolated. DNA was sequenced to identify shRNA-targeted factors controlling Cdkn1c\(^{\Delta neoR}\) repression (Supplementary Fig. 1). In total, 41 epigenetic modifiers were identified (Table 1), with stronger candidates having multiple independent colonies and hairpins recovered. Candidates included factors (RNF2, EZH2, EED, SUV420H1, and DMT1) previously shown to play a role in Kcnq1ot1 imprinted domain regulation, validating our screening strategy. However, they were recovered at a lower frequency than other candidates. Given the chance of off-target effects, validation of candidates will be required to delineate their role in paternal allelic silencing at the Kcnq1ot1 domain. Here, we focused on the candidate nucleoporin 107 (Nup107) (Table 1). Nucleoporins are proteins that constitute nuclear pore complexes that study the nuclear membrane, allowing nuclear-cytoplasmic transport. A more recent role identified for nucleoporins is gene regulation, although the mechanistic underpinnings of this regulation are not fully understood.

Nucleoporins regulate Kcnq1ot1 ncRNA expression and volume. To investigate the role and specificity of NUP107, we examined NUP107, as well as three additional nucleoporins with documented chomatin association (Nup98, Nup153, not included in our original screen) for their role in regulating the Kcnq1ot1 imprinted domain. B6XCAST XEN cells were transfected with two sets of Nup107, Nup62, Nup98/96 and Nup153 siRNAs to produce RNA and protein depletion (Supplementary Fig. 3a–d, antibody validation; Supplementary Fig. 4a, b). Cells were then assessed for total and allelic-specific Kcnq1ot1 ncRNA expression. Compared to controls, Nup107, Nup62 and Nup153 depletion significantly reduced Kcnq1ot1 ncRNA levels to 0.25, 0.45 and 0.76 of controls, respectively, while Nup98/96 depletion produced a 1.88 times increase in Kcnq1ot1 ncRNA levels (Fig. 1a). For all Nup depletions, the Kcnq1ot1 ncRNA maintained paternal expression (Fig. 1b), except for Nup153 depletion, where the normally silent maternal Kcnq1ot1 ncRNA was reactivated (24%). To determine absolute maternal and paternal expression levels instead of allelic ratios, we developed a highly sensitive, precision method for assessing absolute transcript abundance of each allele independently using droplet digital PCR with FAM and HEX strain-specific probes (Supplementary Fig. 5). Compared to control cells, there was a significant decrease in paternal Kcnq1ot1 transcripts by 9928-11,116 copies upon Nup107, Nup62, and Nup153 depletion, and a significant increase in paternal Kcnq1ot1 transcripts by 18,805 copies upon Nup98/96 depletion (Fig. 1c). While Nup107, Nup62, and Nup98/96 depletion did not change the low number of maternal Kcnq1ot1 transcripts, Nup153 depletion produced a significant increase in maternal Kcnq1ot1 transcripts by 4093 copies, compared to controls.

Paternal Kcnq1ot1 ncRNA transcripts coat the paternal Kcnq1ot1 domain in what is thought to be a repressive nuclear compartment. To determine whether reduced Kcnq1ot1 ncRNA abundance altered nuclear Kcnq1ot1 ncRNA volume, 3D Kcnq1ot1 RNA/DNA FISH was performed on G1-synchronized
control and Nup-depleted XEN cells, and Kcnq1ot1 ncRNA volume was calculated. Kcnq1ot1 ncRNA signal was restricted to the paternal Kcnq1ot1 domain in controls, with a reduction in cells with paternal Kcnq1ot1 ncRNA signal in Nup107−, Nup62−, and Nup153− but not Nup98/96-depleted XEN cells (Fig. 2a, b). Nup153-depleted XEN cells also displayed Kcnq1ot1 ncRNA signal at the maternal domain in 32% of cells. With respect to Kcnq1ot1 ncRNA volume, the majority of control cells possessed medium volumes (70–72%; low, 13–18%; high, 11–15%; very high, 0–1%) (Fig. 2d). Consistent with altered Kcnq1ot1 ncRNA levels, Nup107, Nup62, and Nup153 depletion generated a significant increase in cells with low Kcnq1ot1 ncRNA volumes (83, 76, 26%, respectively), while Nup98/96 depletion significantly increased the number of cells with high (35%) or very high (15%) volumes (Fig. 2d). In addition, upon Nup153 depletion, 76% of cells with maternal Kcnq1ot1 reactivation had low Kcnq1ot1 ncRNA volumes. Notably, changes in Kcnq1ot1 ncRNA abundance and volume were not due to altered Kcnq1ot1 transcript stability (Supplementary Fig. 6). Together, these results demonstrate that nucleoporins facilitate paternal Kcnq1ot1 ncRNA expression.

### Table 1 Candidate epigenetic factors of the Kcnq1ot1 imprinted domain

| Candidate | Gene name | Total colonies | Number of shRNAs |
|-----------|-----------|----------------|------------------|
| Ezh1      | Enhancer of zeste 1 | 21              | 1                |
| Smarca5   | Smarca subfamily a, member 5 | 16              | 1                |
| Trdmt1    | tRNA aspartic acid methyltransferase 1 (Dmnt2) | 15              | 1                |
| Taf6f     | TATA-box binding protein associated factor 6 like | 11              | 1                |
| Smarcc2   | Smarca subfamily c, member 2 | 9               | 4                |
| Smarcell  | Smarca, subfamily e, member 1 | 9               | 2                |
| Nup107    | Nucleoporin 107 | 9               | 1                |
| Atrx      | Alpha thalassemia/mental retardation syndrome X-linked | 9               | 1                |
| Smarcd1   | Smarca subfamily a, containing DEAD/H box 1 | 8               | 3                |
| Kat2a     | K(lysine) acetyltransferase 2A | 8               | 1                |
| Mbd4      | Methyl-CpG binding domain protein 4 | 7               | 1                |
| Kdm4a     | Lysine (K)-specific demethylase 4A | 7               | 1                |
| Smarce1-ps1 | Smarca, subfamily e, member 1 pseudogene 1 | 6               | 2                |
| Kat7      | K(lysine) acetyltransferase 7 | 6               | 2                |
| Suv420h1  | Suppressor of variegation 4-20 homolog 1 | 6               | 1                |
| Mbd3      | Methyl-CpG binding domain protein 3 | 6               | 1                |
| Hdac9     | Histone deacetylase 9 | 5               | 2                |
| Hdac1     | Histone deacetylase 1 | 5               | 1                |
| Smn3a     | Transcriptional regulator, SIN3A | 4               | 2                |
| Dmnt3a    | DNA methyltransferase 3A | 4               | 2                |
| Smyd3     | SET and MYND domain containing 3 | 4               | 1                |
| Sirt1     | Sirtuin 1 | 3               | 3                |
| Rnf2      | Ring finger protein 2 | 3               | 3                |
| Ezh2      | Enhancer of zeste 2 | 3               | 2                |
| Pcgf6     | Polycomb group ring finger 6 | 3               | 1                |
| Kat6b     | K(lysine) acetyltransferase 6B | 3               | 1                |
| Sns10     | Sorting nexin 10 | 3               | 1                |
| Dmnt1     | DNA methyltransferase 1 | 2               | 2                |
| Hdac2     | Histone deacetylase 2 | 2               | 2                |
| Smarcd2   | Smarca subfamily d, member 2 | 2               | 2                |
| Ep300     | E1A binding protein p300 | 2               | 2                |
| Hlf1      | Helicase-like transcription factor (Smarca3) | 2               | 1                |
| Hdac8     | Histone deacetylase 8 | 2               | 1                |
| Eed       | Embryonic ectoderm development | 2               | 1                |
| Taf5f     | TATA-box binding protein associated factor 5 like | 1               | 1                |
| Phc3      | Polyhomeotic-like 3 | 1               | 1                |
| Kat2b     | K(lysine) acetyltransferase 2B | 1               | 1                |
| Kmt2d     | Lysine (K)-specific methyltransferase 2D | 1               | 1                |
| Hdac5     | Histone deacetylase 5 | 1               | 1                |
| Hdac7     | Histone deacetylase 7 | 1               | 1                |
| Smarca1   | Smarca subfamily a, member 1 | 1               | 1                |

*Smars, SWI/SNF-related, matrix associated actin-dependent regulator of chromatin

NUPs position the Kcnq1ot1 domain to the nuclear periphery. Previous studies demonstrated that the Kcnq1ot1 ncRNA-coated domain can be situated at the nuclear periphery, and that the distance between distal probes within the Kcnq1ot1 domain differed for maternal and paternal alleles. To examine the position of the Kcnq1ot1 domain in XEN cells, distance of the Kcnq1ot1 DNA signal centroid from the nuclear rim was calculated. Here, size of the Kcnq1ot1 DNA volume identified the maternal (0.1–0.8 μm³) and paternal (0.9–1.3 μm³) domains (Supplementary Fig. 7). In control cells, the maternal Kcnq1ot1 domain was stationary at the nuclear periphery, subnuclear periphery and nuclear interior in 92–46, and 3–4% of cells, respectively (Fig. 2e). In Nup107−, Nup62− and Nup153-depleted XEN cells, nuclear periphery positioning of the paternal Kcnq1ot1 domain was significantly reduced to 54, 61, and 64% of the cells, shifting to the subnuclear periphery (26, 33, and 17%) and nuclear interior (20, 6, and 19%), respectively. Furthermore, maternal Kcnq1ot1 domain positioning was significantly increased at the nuclear periphery in Nup153-depleted XEN cells possessing a maternal Kcnq1ot1 ncRNA signal (+; 60%) compared to control cells (14–17%) and Nup153-depleted XEN cells without a maternal Kcnq1ot1 ncRNA signal (−; 9%).
We next investigated nucleoporin interactions with the Kcnq1ot1 imprinted domain. Quantitative chromatin immunoprecipitation (ChIP) was performed using mAb414 (primarily interacts with Nup62, Nup107, and Nup160 in XEN cells (Supplementary Fig. 3b)) and NUP153 antibodies; ChIP-grade NUP107 and NUP62 antibodies were not available. Antibodies were first validated at predictive positive and negative sites identified from mouse ES cells NUP153 DNA adenine methyltransferase identification sequencing (DamID-seq) data27 (Supplementary Fig. 9).

To investigate nucleoporin interactions at the Kcnq1ot1 domain, 21 sites across the Kcnq1ot1 ICR through to the reported H3K4me1-enriched enhancer element32,33,1–2 sites at imprinted gene promoters, and two control regions were assessed for total nucleoporin binding in WT XEN cells (Supplementary Fig. 10a, Fig. 3c, d). Significant mAb414 (NUP107/62) enrichment was observed at two sites within the Kcnq1ot1 ICR (IC3, 100 bp upstream; IC4, 1.7 kb downstream of the Kcnq1ot1 transcription start site), the Kcnq1ot1 enhancer element (E1 and E2) and the Osbp5 promoter (Os1 and Os2) on the paternal allele (Supplementary Fig. 10b, Fig. 3b, d). Significant NUP153 enrichment was observed at the Kcnq1ot1 ICR (IC3, IC4), where both parental alleles were equally enriched, and at the paternal Kcnq1 (Kc1, Kc2) and Cd81 (Cd1) promoters (Supplementary Fig. 10b, Fig. 3c, e). By comparison, no NUP98 enrichment was observed at the

Fig. 1 Nucleoporin depletion disrupted Kcnq1ot1 ncRNA expression. a Real-time Kcnq1ot1 ncRNA expression levels normalized to Gapdh (n = 3 biological samples with four technical replicates per sample). b Allelic Kcnq1ot1 ncRNA expression in control and Nup-depleted XEN cells (n = 3 biological samples; n = 4 technical replicates per sample). c Absolute allelic Kcnq1ot1 transcript abundance determined by droplet digital PCR in control and Nup-depleted XEN cells, as measured by RNA copies µg⁻¹ (n = 3 biological samples). Center lines, medians; box limits, 25th and 75th percentiles as determined by R software; whiskers, 1.5 times the interquartile range from 25th and 75th percentiles; B6/maternal, red; CAST/paternal, blue; error bars, s.e.m.; *, significance p < 0.05 compared to siNT control (t test); WT wild type, Veh vehicle, siNT nontargeting siRNA, si107 Nup107 siRNA, si62 Nup62 siRNA, si98/96 Nup98/96 siRNA, si153 Nup153 siRNA

depletion also significantly reduced paternal Kcnq1ot1 domain nuclear peripheral (84%) positioning, albeit to a lesser extent than other Nup depletions. Next, we examined the relationship between Kcnq1ot1 ncRNA volume and nuclear positioning. The majority of control cells had medium paternal Kcnq1ot1 ncRNA volume that resided at the nuclear periphery (Supplementary Fig. 8). By comparison, in Nup107- and Nup62-depleted cells with low paternal Kcnq1ot1 ncRNA volume, the Kcnq1ot1 domain was displaced from the nuclear periphery. In Nup153-depleted cells, paternal Kcnq1ot1 domains with low and medium Kcnq1ot1 ncRNA volumes shifted away from the nuclear periphery, while maternal domains with low and medium Kcnq1ot1 ncRNA volumes shifted toward the nuclear periphery. In contrast, Nup98/96-depleted cells with increased paternal Kcnq1ot1 ncRNA volumes primarily retained nuclear peripheral positioning of the Kcnq1ot1 domain. These results indicate that NUP107, NUP62, and NUP153 target the Kcnq1ot1 ncRNA-coated domain to the nuclear periphery.

NUPs bind the Kcnq1ot1 ICR and imprinted gene promoters.

We next investigated nucleoporin interactions with the Kcnq1ot1 imprinted domain. Quantitative chromatin immunoprecipitation (ChIP) was performed using mAb414 (primarily interacts with Nup62, Nup107, and Nup160 in XEN cells (Supplementary Fig. 3b)) and NUP153 antibodies; ChIP-grade NUP107 and NUP62 antibodies were not available. Antibodies were first validated at predictive positive and negative sites identified from mouse ES cells NUP153 DNA adenine methyltransferase identification sequencing (DamID-seq) data27 (Supplementary Fig. 9).

To investigate nucleoporin interactions at the Kcnq1ot1 domain, 21 sites across the Kcnq1ot1 ICR through to the reported H3K4me1-enriched enhancer element32,33,1–2 sites at imprinted gene promoters, and two control regions were assessed for total and allele-specific binding in WT XEN cells (Supplementary Fig. 10a, Fig. 3a). Significant mAb414 (NUP107/62) enrichment was observed at two sites within the Kcnq1ot1 ICR (IC3, 100 bp upstream; IC4, 1.7 kb downstream of the Kcnq1ot1 transcription start site), the Kcnq1ot1 enhancer element (E1 and E2) and the Osbp5 promoter (Os1 and Os2) on the paternal allele (Supplementary Fig. 10b, Fig. 3b, d). Significant NUP153 enrichment was observed at the Kcnq1ot1 ICR (IC3, IC4), where both parental alleles were equally enriched, and at the paternal Kcnq1 (Kc1, Kc2) and Cd81 (Cd1) promoters (Supplementary Fig. 10b, Fig. 3c, e). By comparison, no NUP98 enrichment was observed at the
Kcnq1ot1 ICR or enhancer element (Supplementary Fig. 10b). Next, we investigated whether nucleoporin—chromatin interactions were lost upon Nup depletion. Since the mAb414 antibody recognizes NUP62 and NUP107 (Supplementary Fig. 3b), double depletion was performed. We found a significant decrease in nucleoporin occupancy at the Kcnq1ot1 ICR and enhancer element in double-depleted cells at the paternal allele (Fig. 3d). Upon Nup153 depletion, NUP153 binding on the paternal and maternal Kcnq1ot1 ICR, and the paternal Kcnq1 and Cd81 promoters was significantly reduced (Fig. 3e). To determine whether there was interdependency between NUP107/62 and NUP153 binding, Nup153-depleted cells were examined for mAb414
enrichment, and Nup107- and Nup62-depleted cells were assessed for NUP153 enrichment. Nup153 depletion significantly decreased mAb414 (NUP107/62) binding at the Kcnq1ot1 ICR region but not at the Osbpl5 promoter and enhancer sites (Fig. 3f). Conversely, Nup107 and Nup62 depletion significantly decreased NUP153 occupancy at the Kcnq1ot1 ICR and Kcnq1 promoter, while no change in enrichment was observed at the Cdb1 promoter (Fig. 3g). These results indicate that nucleoporin interactions at the paternal Kcnq1ot1 ICR are cooperatively mediated through NUP107/NUP62 and NUP153.

We further investigated whether nucleoporins can interact with a Kcnq1ot1 ICR DNA fragment in nuclear lysates from control and Nup-depleted XEN cells. Electromobility shift assays were first performed using biotin-labeled Vin, Shank2 and Dchrl7 promoter fragments as controls. For the Kcnq1ot1, supershifts were observed for the biotin-labeled Kcnq1ot1 ICR fragment with mAb414 (NUP107/62), NUP107, and NUP153 antibodies but not for the biotin-labeled 2-kb upstream control fragment (Supplementary Fig. 11a, b), pointing to a direct interaction of these proteins with the Kcnq1ot1 ICR. No supershift was produced using the NUP98 antibody. Next, reverse ChIP was performed to isolate proteins bound to a biotin-labeled Kcnq1ot1 ICR and negative control fragments, followed by western blot analysis using NUP107, mAb414, NUP98, and NUP153 antibodies. NUP107 bound to the Kcnq1ot1 ICR fragment but not the 2-kb upstream control fragment (Supplementary Fig. 12). Nup107-depleted lysates abrogated NUP107 binding, as well as NUP62 and NUP153 binding, indicating that NUP62 and NUP153 binding at the Kcnq1ot1 ICR required NUP107 binding. NUP153 also bound directly to the Kcnq1ot1 ICR fragment but not the control fragment. Upon Nup153 depletion, binding to the Kcnq1ot1 ICR fragment was lost, although NUP107 and NUP62 binding was not altered, perhaps indicating sequential binding of NUP107, NUP62, and NUP153.

NUPs regulate paternal allelic silencing of imprinted genes. Since NUP107, NUP62, and NUP153 were bound within the Kcnq1ot1 domain, allelic expression of imprinted genes in the Kcnq1ot1 domain was assessed by a conventional method of allelic ratio quantification1. Nup107, Nup62, and Nup153 depletion resulted in paternal reactivation of the core group of genes, Slc22a18, Cdkn1c, and Kcnq1 (Supplementary Fig. 13), as well as gene-specific reactivation (paternal Osbpl5 and Phlda2 alleles upon Nup107 depletion, the paternal Osbpl5 allele upon Nup62 depletion, and the paternal Cdb1 allele upon Nup62 depletion). By comparison, Nup98/96 depletion had no effect on paternal allelic expression. To determine the absolute transcript abundance of maternal and paternal RNA copies, we developed allelic droplet digital PCR assays using FAM and HEX strain-specific probes for imprinted genes in the domains (Fig. 4, Supplementary Fig. 14). Compared to controls, we observed that Nup107 and Nup62 depletion increased paternal Osbpl5 transcripts by 13,472–13,662 copies, Nup107 depletion increased Phlda2 transcripts by 430 copies, Nup107, Nup62, and Nup153 depletion increased paternal transcripts of Slc22a18 by 29,547–32,214, Cdkn1c by 10,882–13,153, and Kcnq1 by 728–805 copies, and Nup153 depletion increased Cdb1 transcripts by 13,033 copies (Fig. 4, Supplementary Fig. 15). Notably, even though the normally silent maternal Kcnq1ot1 allele was reactivated in a proportion of Nup153-depleted XEN cells, no change was observed in maternal transcript copies of imprinted genes upon Nup153 depletion. Overall, our data indicate that paternal allelic reactivation was not domain-wide. Instead, NUP107, NUP62, and NUP153 regulate paternal allelic silencing of a core group of genes, Slc22a18, Cdkn1c, and Kcnq1, while paternal allele silencing of genes more distal to the Kcnq1ot1 ICR, Phlda2, Osbpl5, and Cdb1, was nucleoporin-specific.

Nuclear-cytoplasmic transport not impaired by Nup depletion. An alternate explanation for altered regulation of the Kcnq1ot1 domain upon nucleoporin depletion is impaired nuclear-cytoplasmic transport. The nuclear pore complex is composed of multiple copies of ~30 nucleoporins (~500 proteins in total)34. Impaired function may result from incorrect assembly of the nuclear pore complex when one component is deleted. Examining the levels of various nucleoporins within different structural components of the nuclear pore (Supplementary Fig. 16a), we found no change in nucleoporin levels in Nup-depleted XEN cells compared to controls (Supplementary Fig. 16b), suggesting that nuclear pore complex assembly was not affected upon Nup depletion. Previous studies have shown that import of cargo containing a classical bipartite nuclear localization signal (NLS) was impaired in Nup153-depleted HeLa cells35. We found no change in protein import in control and Nup-depleted XEN cells as measured by levels of the transcription factor 3 (E47)-red fluorescence protein (RFP)-NLS construct36 compared to invermectin-treated cells, where RFP import was inhibited (Supplementary Fig. 17a), or between endogenous inner centromeric protein (INCENP) levels (Supplementary Fig. 17b) or LAMINB1 protein localization (Supplementary Fig. 17c). With respect to export, previous studies found aberrant nuclear mRNA export in Nup107-depleted HeLa cells, with abnormal accumulation of polyA-mRNA in depleted nuclei37. Here, no significant difference in nuclear polyA-mRNA retention levels was observed between control and Nup-depleted XEN cells (Supplementary Fig. 17d). With regard to passive bidirectional diffusion, we found that Nup-depleted cells exhibited comparable levels of nuclear and cytoplasmic GFP mRNA as well as nuclear GFP protein38. With regard to nuclear transport, we found no significant difference in nuclear and cytoplasmic GFP mRNA or protein localization (Supplementary Fig. 17a), or between endogenous inner centromeric protein (INCENP) levels (Supplementary Fig. 17b) or LAMINB1 protein localization (Supplementary Fig. 17c). With respect to export, previous studies found aberrant nuclear mRNA export in Nup107-depleted HeLa cells, with abnormal accumulation of polyA-mRNA in depleted nuclei37. Here, no significant difference in nuclear polyA-mRNA retention levels was observed between control and Nup-depleted XEN cells (Supplementary Fig. 17d). With regard to passive bidirectional diffusion, we found that Nup-depleted cells exhibited comparable levels of nuclear and cytoplasmic GFP mRNA as well as nuclear GFP protein38. With regard to nuclear transport, we found no significant difference in nuclear and cytoplasmic GFP mRNA or protein localization (Supplementary Fig. 17a), or between endogenous inner centromeric protein (INCENP) levels (Supplementary Fig. 17b) or LAMINB1 protein localization (Supplementary Fig. 17c). With respect to export, previous studies found aberrant nuclear mRNA export in Nup107-depleted HeLa cells, with abnormal accumulation of polyA-mRNA in depleted nuclei37. Here, no significant difference in nuclear polyA-mRNA retention levels was observed between control and Nup-depleted XEN cells (Supplementary Fig. 17d). With regard to passive bidirectional diffusion, we found that Nup-depleted cells exhibited comparable levels of nuclear and cytoplasmic GFP mRNA as well as nuclear GFP protein38. With regard to nuclear transport, we found no significant difference in nuclear and cytoplasmic GFP mRNA or protein localization (Supplementary Fig. 17a), or between endogenous inner centromeric protein (INCENP) levels (Supplementary Fig. 17b) or LAMINB1 protein localization (Supplementary Fig. 17c).
Fig. 3 | NUP107/62 and NUP153 interaction with the Kcnq1ot1 ICR, and the Ospb15, Kcnq1, Cd81 promoters. | a | The Kcnq1ot1 domain with regions of analysis (arrowheads) at the Kcnq1ot1 ICR, IC3, IC4; enhancer element, E1, E2; imprinted gene promoters; Os1, Os2, Ph1, Ph2, Sl1, Sl2, Ck1, Ck2, Ts1, Ts2, Cd1, Th1; and negative control sites, Ctrl1, Ctrl2. Quantitative ChIP analysis using b mAb414 antibodies and c NUP153 antibodies in wild-type XEN cells at regions across the domain, respectively (n = 3 biological samples with four technical replicates per sample). Quantitative allelic analysis for d mAb414 and e NUP153 in siNT- and nucleoporin-depleted XEN cells. Allelic proportions are represented as percent of the total enrichment levels (n = 3 biological samples with four technical replicates per sample). f Quantitative ChIP analysis using mAb414 antibodies was performed in control and Nup153-depleted XEN cells at sites of NUP153 enrichment (n = 3 biological samples with three technical replicates per sample). g Quantitative ChIP analysis using NUP153 antibodies was performed in control and Nup107- and Nup62-depleted cells at sites of mAb414 (NUP107/62) enrichment (n = 3 biological samples with three technical replicates per sample). Error bars, s.e.m.; *, significance p < 0.05 compared to the IgG or siNT control (t test).
direct cell counting assay (Supplementary Fig. 18a, b), which was similar to NUP107-depleted HeLa cells. Overall, these results indicate that nuclear-cytoplasmic transport in XEN cells was not affected by nucleoporin depletion.

DNA methylation not altered by Nup depletion. Another explanation for a reduction in paternal Kcnq1ot1 ncRNA levels and subsequent paternal allelic reactivation upon Nup107, Nup62, and Nup153 depletion could be a gain of DNA methylation at the normal unmethylated paternal ICR. In addition, reactivation of maternal Kcnq1ot1 in Nup153-depleted XEN cells could be explained by a loss of DNA methylation at the methylated maternal ICR. Analysis of parental-specific methylation demonstrated that the maternal Kcnq1ot1 ICR was hypermethylated.
while the paternal ICR was hypomethylated in control and Nup-depleted XEN cells (Supplementary Fig. 19). Thus, changes in NUP107, NUP62, and NUP153 regulation at the Kcnq1ot1 imprinted domain were not through alterations in Kcnq1ot1 ICR methylation.

NUPs maintain chromatin at Kcnq1ot1 ICR and gene promoters. We next investigated whether altered Kcnq1ot1 nRNA expression and paternal allelic silencing at the Kcnq1ot1 imprinted domain is due to alterations in chromatin state4,9,15,40. ChIP assays were performed on control and depleted XEN cells using antibodies directed against RNAPII and histone 3 lysine 4 trimethylation (H3K4me3) as marks for active chromatin, and H3K9me2 and H3K27me3 for repressed chromatin. H3K4me3 and H3K27me3 peaks in XEN cells were used for primer placement41. Antibodies were first validated at the XEN cell expressed Foxa2 gene and XEN cell repressed MyoD gene42, where active and repressive modifications were observed, respectively (Supplementary Fig. 20). At the Kcnq1ot1 ICR domain, we first verified equal allelic detection in input chromatin (Supplementary Fig. 21) as well as equivalent enrichment for histone 3 (H3), a protein expected on both parental alleles (Supplementary Fig. 22). For the Kcnq1ot1 ICR, there was a significant decrease in RNAPII and H3K4me3 enrichment and a significant increase in H3K9me2 and/or H3K27me3 enrichment on the paternal Kcnq1ot1 ICR in Nup107-, Nup62-, and Nup153-depleted cells compared to control cells (Fig. 5a, b), which would account for reduced paternal Kcnq1ot1 nRNA levels. In addition, on the maternal Kcnq1ot1 ICR, we observed a significant increase in H3K4me3 and RNAPII enrichment along with decreased repressive histone modifications upon Nup153 depletion, which could account for reactivation of the maternal transcript. At the promoters of imprinted genes in the domain, there was significantly increased enrichment of RNAPII and/or H3K4me3 at the paternal Slc22a18, Cdkn1c, and Kcnq1 alleles upon Nup107, Nup62, and Nup153 depletion, at the paternal Ospbl5 and Phlda2 promoters upon Nup107 and Nup62 depletion, and at the paternal Cd81 promoter upon Nup153 depletion compared to control cells (Fig. 5a, b). This corresponded with significantly reduced levels of H3K9me2 and/or H3K27me3, thereby accounting for their paternal reactivation. No significant changes in histone modifications were observed upon Nup98/96 depletion (Fig. 5a, b). These results demonstrate that NUP107, NUP62, and NUP153 act to regulate histone modifications at the Kcnq1ot1 ICR and at specific imprinted gene promoters.

Changes in histone modifications could be a consequence of altered histone methyltransferases or RNAPII binding at the Kcnq1ot1 ICR and imprinted gene promoters upon nucleoparin depletion. First, we ruled out that nucleoparin depletion per se did not alter histone 3 lysine 4 methyltransferase 2a (KMT2A), EHMT2, EZH2, and RNAPII protein levels in control and Nup-depleted XEN cells (Supplementary Fig. 23a, b). We found increased KMT2A, and increased EHMT2 and EZH2 enrichment levels upon Nup107/62 and Nup153 depletion compared to control and Nup98/96-depleted cells (Fig. 6a–c). At imprinted gene promoters with altered histone modifications, KMT2A enrichment was increased on the paternal Ospbl5, Phlda2, Slc22a18, Cdkn1c, and Kcnq1 alleles upon Nup107/62 depletion, and on the paternal Slc22a18, Cdkn1c, Kcnq1, and Cd81 alleles upon Nup153 depletion, while a corresponding decrease in EHMT2 and EZH2 recruitment was found on the paternal Ospbl5, Cdkn1c, Kcnq1, and Cd81 alleles upon Nup107/62 and/or Nup153 depletion. Surprisingly, no change in EHMT2 and EZH2 levels were observed at the Slc22a18 promoter. Overall, these results suggest that changes in H3K4me3, H3K9me2, and H3K27me3 levels were conferred by alteration in KMT2A, EHMT2, and EZH2 binding, respectively, at the Kcnq1ot1 ICR and imprinted gene promoters upon Nup107/62 and/or Nup153 depletion.

NUPs promote cohesin interactions at paternal Kcnq1ot1 ICR. Another mechanism that may alter Kcnq1ot1 imprinted domain regulation is CTCF and/or cohesin complex binding. Previous studies identified two CTCF-binding sites within the Kcnq1ot1 ICR that were bound by CTCF and the cohesin complex in mouse embryonic fibroblasts (MEFs)43,44. Thus, we investigate the relationship between CTCF, cohesin proteins, and nucleoporins at the Kcnq1ot1 domain. Next, we ruled out that nucleoparin depletion per se altered CTCF, SMCA1, or SMC3 levels in control and Nup-depleted XEN cells (Supplementary Fig. 24a, b). As a positive control, CTCF, structural maintenance of chromosomes 1a (SMC1A), and 3 (SMC3) antibodies were validated in embryonic stem cells at the H19 ICR2, where strong CTCF, SMCA1, and SMC3 enrichment was observed on the maternal H19 ICR (Supplementary Fig. 25). To determine whether CTCF, SMCA1, and SMC3 localized at the Nup-positive enrichment sites at the Kcnq1ot1 domain, ChIP was performed using CTCF, SMCA1, and SMC3 antibodies in XEN cells. No CTCF occupancy was observed at the Kcnq1ot1 ICR and enhancer region in XEN cells (Supplementary Fig. 25b). By comparison, SMCA1 and SMC3 binding was significantly enriched at IC3 and IC4, but not at other tested sites, with preferentially cohesin binding at the paternal alleles (Fig. 7a, b). This binding was significantly decreased upon Nup107/Nup62 and Nup153 depletion, but not Nup98/96 depletion (Fig. 7a, b).

To determine whether nucleoparin depletion resulted in decreased occupancy at other sites outside the Kcnq1ot1 domain, we tested SMCA1 and SMC3 binding at positive and negative sites (ES cells ChIP sequencing data) within the promoters of Vim (NUP153 positive), Or2i2 (NUP153, mAb414 and NUP98 positive), Shank2 (NUP153, mAb414 and NUP98 positive), as well as the Dhc7 promoter (NUP153, mAb414, NUP98 negative site), in control and Nup-depleted cells. SMCA1 and SMC3 recruitment was not altered at these sites upon nucleoparin depletion (Supplementary Fig. 26), indicating that SMCA1 and SMC3 binding at Vim, Or2i2, Shank2, and Dhc7 were nucleoparin-independent, whereas binding at the Kcnq1ot1 ICR was Nup107-, Nup62-, and Nup153-dependent.

Finally, to gain an understanding of specific interactions between NUP107, NUP62, NUP153, and CTCF, SMCA1 and SMC3, we performed immunoprecipitation assays using control and Nup-depleted nuclear lysates and mAb414 and NUP153 antibodies, followed by western analysis with CTCF, SMCA1, and SMC3 antibodies. We first established that NUP107/62 and NUP153 interacted with CTCF, SMCA1, SMC3 in control cells, and that these interactions were reduced in Nup107/62- and Nup153-depleted cells (Supplementary Fig. 27). We next determined whether interaction with CTCF, SMCA1, and SMC3 occurred selectively through NUP107/62, and/or NUP153. In Nup107/62-depleted cells, interactions between NUP153 and CTCF, SMCA1, and SMC3 were reduced compared to control cells (Fig. 8a). By comparison, in Nup153-depleted cells, mAb414 (NUP107/62) maintained CTCF, SMCA1, SMC3 interactions similar to controls (Fig. 8a), indicating that NUP107/62 was required for NUP153 interactions with CTCF, SMCA1, and SMC3. To delineate whether these interactions were mediated via NUP107 or NUP62, NUP153 interactions were investigated in Nup107- and Nup62-depleted cells. Notably, NUP107 but not NUP62 reduced NUP153 interactions with
Fig. 5 Nup107, Nup62, and Nup153 depletion disrupts RNAPII enrichment and histone modifications at the Kcnq1ot1 ICR and imprinted gene promoters. 

a) RNAPII and H3K4me3 ChIP at the maternal and paternal Kcnq1ot1 ICR and imprinted gene promoters in control and Nup-depleted XEN cells (n = 3; n = 3 technical replicates).

b) H3K9me2 and H3K27me3 ChIP at the maternal and paternal Kcnq1ot1 ICR and imprinted gene promoters in control and Nup-depleted XEN cells (n = 3 biological samples with three technical replicates per sample). The y-axis indicates total and allelic ChIP enrichment levels represented as percent of input. Allelic proportions are represented as a percent of the total ChIP enrichment level. Error bars, s.e.m.; *, significance p < 0.05 of paternal/maternal levels compared to the siNT paternal/maternal control (t-test)
Fig. 6 Nup107, Nup62, and Nup153 depletion disrupts KMT2A, EHMT2, and EZH2 occupancy at the Kcnq1ot1 ICR and imprinted gene promoters. a KMT2A, b EHMT2 and c EZH2 ChIP at the maternal and paternal Kcnq1ot1 ICR and imprinted gene promoters in control and Nup-depleted XEN cells (n = 3 biological samples with three technical replicates per sample). The y-axis indicates total ChIP allelic enrichment levels represented as percent of input. Allelic proportions are represented as a percent of the total ChIP enrichment level. Error bars, s.e.m.; *, significance p < 0.05 compared to the siNT control (t test)
CTCF, SMC1A, and SMC3, indicating that CTCF and cohesin interactions are mediated through NUP107 (Fig. 8b).

**Discussion**

In recent years, studies in mammalian cells, yeast, and *Drosophila* have identified roles for nucleoporins in gene regulation, although the mechanistic action of nucleoporins in this regulation is not fully understood. Here, we identified a nucleoporin-dependent regulatory mechanism at the Kcnq1ot1 imprinted domain. More specifically, NUP107, NUP62, and NUP153 regulated paternal Kcnq1ot1 domain positioning at the nuclear periphery; Kcnq1ot1 ncRNA expression and volume; paternal allelic repression of multiple imprinted genes; maintenance of an active conformation at the paternal Kcnq1ot1 ICR and a repressed conformation at the paternal Osbp15, Phlda2, Slc22a18, Cdkn1c, Kcnq1, and Cd81 promoters; and cohesin complex interactions at the paternal Kcnq1ot1 ICR (Supplementary Fig. 28).

Interactions between NUP107, NUP62, and NUP153 have previously been documented in HeLa cells. Investigation of various nucleoporin interactions in U2OS cells found that three of the 11 tested had the capacity to recruit other nucleoporins, which included NUP153 and NUP107. The NUP153- and NUP107-induced structure also repositioned an integrated chromatin marker from the nuclear interior to the nuclear periphery. By comparison, NUP98 possessed very limited capacity to recruit nucleoporins or target chromatin to the nuclear periphery. In keeping with this, we found that Nup107, Nup62, and Nup153 act at the Kcnq1ot1 ICR to tether the Kcnq1ot1 domain to the nuclear periphery. Coordinate binding of NUP107 with NUP62 and NUP153 likely mediates their regulatory role in Kcnq1ot1 ICR. Having said this, other sites within the domain maintained their NUP107/62 and NUP153 enrichment upon Nup153 depletion or Nup107/Nup62 depletion, respectively. For example, we observed that NUP107/62 remained bound at the Osbp15 promoter and enhancer upon Nup153 depletion. However, even when these nucleoporin interactions were maintained, the Kcnq1ot1 domain shifted away from the nuclear periphery. The most direct explanation is that NUP107/62 and NUP153 coordinately tethered the Kcnq1ot1 ICR to the nuclear pore complex, while NUP107/62 and NUP153 binding at other regions of the domain...
were through an off-pore function. Recent studies have identified multiple nucleoporins (NUP62, NUP153, NUP98 and NUP50) that are present in the nucleoplasm as mobile fractions and regulate genes away from the nuclear periphery by binding their promoters\textsuperscript{7,51–53}.

Previous studies demonstrated that the Kcnq1ot1 ncRNA-coated domain is compartmentalized to the nuclear periphery or perinucleolar regions\textsuperscript{7,8,29}, which suggests a linkage between the Kcnq1ot1 ncRNA and nuclear positioning. Here, while Nup107, Nup62, and Nup153 depletion displaced nuclear peripheral positioning of the paternal Kcnq1ot1 domain, the Kcnq1ot1 ncRNA remained associated with the Kcnq1ot1 domain in Nup107-, Nup62-, and Nup153-depleted XEN cells, albeit with lower Kcnq1ot1 volumes. This suggests that the Kcnq1ot1 ncRNA per se does not regulate paternal Kcnq1ot1 domain positioning at the nuclear periphery. Alternatively, Kcnq1ot1 ncRNA coating must reach a specific threshold level to enable nuclear periphery positioning. However, the shift from random to nuclear periphery positioning of the maternal Kcnq1ot1 domain (low Kcnq1ot1 ncRNA volumes) in Nup153-deleted cells does not support the idea of threshold levels.

Previous studies found domain-wide loss of paternal allelic silencing in midgestation embryos harboring paternal deletion of the Kcnq1ot1 ICR or truncation of the paternal Kcnq1ot1 ncRNA\textsuperscript{5,6,12}. This contrasted with our findings in Nup107-, Nup62-, and Nup153-depleted XEN cells, where paternal alleles were reactivated at a core group of imprinted genes (Slc22a18, Cdkn1c, and Kcnq1), and at specific imprinted genes in a nucleoporin-specific manner (Osbp5 in Nup107 and Nup62, Phlda2 in Nup107, and Cd81 in Nup153-depleted cells). One possible explanation for lack of domain-wide loss of paternal allelic silencing is that the Kcnq1ot1 ncRNA acts to coordinately regulate paternal allelic silencing of the core group of genes, while genes more distal from the Kcnq1ot1 ICR were regulated by a Kcnq1ot1 ncRNA-independent mechanism. Alternatively, Kcnq1ot1 ncRNA may operate in a gene-specific capacity across the domain. In day 14.5 placenta, Kcnq1ot1 ncRNA interactions were found at genes within (Cdkn1c and Kcnq1) and outside (Osbp5 and Cd81) the core group\textsuperscript{8}. Interactions between the Kcnq1ot1 ncRNA and PRC2 or EHMT2 could direct repressive epigenetic marks, H3K27me3 and H3K9me2, respectively, to these paternally silent genes\textsuperscript{8}. This is supported by our data, since alterations in KMT2A, EHMT2, and EZH2 binding occurred concomitantly with changes in H3K4me3, H3K9me3, and H3K27me3 levels, respectively, at the Kcnq1ot1 ICR and paternal imprinted gene promoters upon Nup107/62 and/or Nup153 depletion. Recently, NUP153 was identified to play a role in PRC1 silencing of developmental genes in ES cells\textsuperscript{27}. This is consistent with reactivation of the normally silent maternal Kcnq1ot1 allele upon Nup153 depletion. Thus, nucleoporins may interact with different macromolecular epigenetic complexes to regulate gene expression. For example, NUP107, NUP62, and NUP153 may interact with a complex containing KMT2A to enable Kcnq1ot1 ncRNA expression, while NUP153 may engage with a repressive complex to regulate maternal Kcnq1ot1 ncRNA silencing.

A coordinate role for NUP107 and NUP62 has been identified at spindles and kinetochores\textsuperscript{54}, where they may function in chromatin reorganization upon nuclear membrane reformation\textsuperscript{23}. Our findings also point to a role for NUP107, NUP62, and NUP153 in conferring chromatin structure at the Kcnq1ot1 domain. Interactions between NUP107, NUP62, and NUP153 and the cohesin complex at the nuclear periphery on both sides of the Kcnq1ot1 ICR, enabling Kcnq1ot1 promoter could enable an open chromatin configuration at the paternal Kcnq1ot1 ICR, enabling Kcnq1ot1 transcription. This is supported by reactivation of the maternal Kcnq1ot1 ncRNA allele coincident with a shift in maternal Kcnq1ot1 domain positioning at the nuclear periphery upon Nup153 depletion. Demarcation of active chromatin by NUP107, NUP62, and NUP153 and cohesin may separate active chromatin at the paternal Kcnq1ot1 ICR from repressive chromatin at the paternal alleles of the core group of imprinted genes, blocking their expression. Upon Nup107/Nup62 and Nup153 depletion, cohesin binding was reduced at the paternal Kcnq1ot1 ICR,

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**Fig. 8 NUP107 is required for NUP153 and NUP62 interaction with CTCF, SMC1A, and SMC3.**

**a** NUP-immunoprecipitation with NUP153 and mAb414 antibodies was performed on control (siNT) and Nup107+62- and Nup153-depleted XEN nuclear lysates, following which western analysis was conducted using CTCF, SMC1A, and SMC3 antibodies (n = 3 biological replicates). **b** NUP-immunoprecipitation with NUP153 antibodies was performed on Nup107- and Nup62-depleted XEN lysates, followed by western analysis using CTCF, SMC1A, and SMC3 antibodies (n = 3 biological replicates).
possibly altering chromatin states at the paternal Kcnq1ot1 ICR and the core group of imprinted genes, leading to alteration in paternal allelic expression. In addition, NUP107/NUP62 binding and the core group of imprinted genes, leading to alteration in nucleoporins in imprinted domain regulation. Finally, identification of additional candidate epigenetic factors involved in paternal allele silencing at the Kcnq1ot1 domain opens a new dimension of investigations into understanding imprinted domain regulation.

RNA and DNA isolation, cDNA preparation and PCR amplification. DNA from surviving colonies from the lentiviral screen was isolated using Qiagen DNeasy Blood & Tissue Kit (Qiagen, 69054) and PCR amplification was performed for the hairpin region inside the shRNA construct with primers targeting the lentiviral vector sequence. cDNA was isolated using PureLink™ RNA Mini Kit (Life Technologies, 12183018A), QuickRT™ MicroPrep (Zymo Research, R1050) or RNeasy Plus Mini Kit (Qiagen, 74134) according to the manufacturers’ instructions. Before cDNA preparation, total RNA was subjected to DNase I (Life Technologies, 18068015) treatment as described1, cDNA was prepared using Transcript II cDNA Synthesis Kit (Roche, 12183018A), QuickRTM MicroPrep (Zymo Research, R1050) or RNeasy Plus Mini Kit (Life Technologies, 48190011). cDNA was treated with RNaseA (Roche, 1010912401) after preparation to remove residual RNA. PCR was performed on CFX1000 and MJ Research Thermocyclers (Bio-Rad).

Quantitative PCR analysis. Quantitative (q) PCR was performed as described1. Briefly, qPCR was performed using iQ SYBR Green Supermix (Bio-Rad, 1078880) or SensiFAST™ SYBR® No-ROX Kit (Bio-Rad, 980085) on an MJ Thermocycler (Bio-Rad). qPCR reaction systems were set up with 10 ng of total RNA and 250 different primers used in this experiment was previously described1, except for the DNA methylation analyses, where two biological replicates were prepared for each sample. Gene expression analysis, data were analyzed using the 2ΔΔCT method55. For ChIP, qPCR data analysis was performed as described56. Supplementary Table 1 lists primers, annealing temperatures, and amplicon sizes.

Droplet digital PCR allelic expression analysis. Droplet digital PCR (ddPCR) probes and primers were designed following Bio-Rad’s guidelines for ddPCR assays. Single nucleotide polymorphisms (SNPs) between C57BL/6 and CAST (CAST™) were targeted and tagged with OligoAnalyzer (Bio-Rad). SNPs that generated the greatest difference in melting temperature (Tm) between the alleles, in either forward and reverse complement strands, were selected as the probe target. Primers and probes were designed using Primer3plus18, RealTime Design™ software (BIOSEARCH Technologies)39, OligoAnalyzer 3.1 17, or advice from IDT. For primer sequences, a target Tm was set between 40 °C and 60 °C, where possible the SNP was centered within the probe. Caution was taken to ensure that a G nucleotide was not at the 5’ of target probe. A probe Tm of 3–10 °C higher than the primer pairs was required. Shorter probes incorporated locked-nucleic acids (LNA) to achieve stable Tm, which was confirmed on LNA oligo Tm Prediction20, was used to predict Tm changes from adding LNAs. Increased probe Tm was achieved with longer probes, in this case a ZEN-quencher (Integrated DNA Technologies; IDT) were added to the center of the probe to dual-quench the fluorophores. Following probe design, a Tm difference of ≥3 °C between the probes was confirmed using OligoAnalyzer 3.1 17. Probes for Cdh1, Cdkn1c, Th, and Tsc2 were designed by IDT. All probes were synthesized by IDT with FAM or HEX fluorophores quenched by Iowa Black FQ; Primer and probe sequences are listed in Supplementary Table 1.

For ddPCR sample preparation, RNA was extracted using the RNeasy Plus MinI Kit (Qiagen, 74134) according to the manufacturers’ instructions. RNA concentrations were determined using Qubit™ 3.0 Fluorometer (Life Technologies, Q32316), and RNA BR Assay Kit (ThermoFisher Scientific, Q32852). Before cDNA preparation, total RNA was subjected to DNase I (Life Technologies, 18068015) treatment. cDNA was prepared using Transcript II Reverse Transcriptase (NEB, M0388X), oligo(dT)23 (Sigma, O4387) and Random Primers (Life Technologies, 48190011). cDNA was treated with RNaseA (Roche, 1010912401) after incorporation to remove residual RNA. For each gene, ddPCR master mixes were made for a reaction final volume of 25 µL, containing 2–4 µL cDNA, 1 µL of primers each (250 nM), 1 µL of probes each (FAM, HEX 250 nM), 11 µL ddPCR Supermix for Probes (no dUTP) (Bio-Rad,1863024), and 3–5 µL DNA-RNAase free UltraPure dH2O (Invitrogen, 11877-015). Each reaction was loaded into a 96-well plate (Bio-Rad, 12001925), sealed with a foil seal (Bio-Rad, 1814080) and placed into a PX1 PCR Plate Sealer at 180 °C for 5 s. Sealed plates were loaded into the AutoDG (Bio-Rad,
cycle of 10 min at 98 °C. For
60 s, and a
the QX200 (Bio-Rad, 1864100), set for detecting absolute levels of FAM/HEX
molecule in the sample, allowing for absolute quanti
Cy5-UTP (GE Healthcare, 45001239) for DNA FISH as described1. For RNA FISH,
37 °C in a sealed formamide chamber (sealed with para
coverslips with 70% formamide in 2× SSC at 85 °C for 20 min. Coverslips were
IX81 motorized inverted system (Olympus). For DNA and RNA FISH, signal
primary antibody in 5% skim milk for 1 h at room temperature, washed three
times for 10 min, washed twice in 1× PBS and stored in 70% ethanol for up to a month.
Coverslips were then incubated with RNA FISH probe. RNA FISH was
overnight at 4 °C. For DNA/RNA FISH, DNA FISH was
frozen, dehydrated in a series of 25, 50, 75, and 100% ethanol followed by
Coverslips were mounted on glass slides with Vectashield DAPI (antifade
Statistical analysis
formed on mean values. Treatment samples were compared to controls. A

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Data availability. All data generated or analyzed during this study are included in this published article (and its supplementary information files). Data supporting the findings of this manuscript are available from the corresponding author upon reasonable request.

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Author contributions

S.S.S. performed experiments and wrote manuscript. L.S.L. performed shRNA screen. L.Z. performed Western analysis, immunoprecipitation, and provided technical assistance for ChIP analysis. C.R.W. performed bisulfite mutagenesis assay. W.A.M. designed droplet digital assays and provided bioinformatics analysis. M.C.G. designed and supervised the lentiviral screen. M.R.W.M. designed and supervised the project, helped with data interpretation and manuscript preparation. All authors have read and approved the final manuscript.

Additional information

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