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Regional chromatin decompaction in Cornelia de Lange syndrome associated with NIPBL disruption can be uncoupled from cohesin and CTCF

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Cornelia de Lange syndrome (CdLS) is a developmental disorder caused by mutations in NIPBL, a protein which has functionally been associated with the cohesin complex. Mutations in core cohesin complex components have also been reported in individuals with CdLS-like phenotypes. In addition to its role in sister chromatid cohesion, cohesin is thought to play a role in regulating gene expression during development. The mechanism of this gene regulation remains unclear, but NIPBL and cohesin have been reported to affect long-range chromosomal interactions, both independently and through interactions with CTCF. We used fluorescence in situ hybridization to investigate whether the disruption of NIPBL affects chromosome architecture. We show that cells from CdLS patients exhibit visible chromatin decompaction, that is most pronounced across gene-rich regions of the genome. Cells carrying mutations predicted to have a more severe effect on NIPBL function show more extensive chromatin decompaction than those carrying milder mutations. This cellular phenotype was reproduced in normal cells depleted for NIPBL with siRNA, but was not seen following the knockdown of either the cohesin component SMC3, or CTCF. We conclude that NIPBL has a function in modulating chromatin architecture, particularly for gene-rich areas of the chromosome, that is not dependent on SMC3/cohesin or CTCF, raising the possibility that the aetiology of disorders associated with the mutation of core cohesin components is distinct from that associated with the disruption of NIPBL itself in classical CdLS.

INTRODUCTION

Cornelia de Lange syndrome (CdLS; OMIM 122470) is a genetic disorder characterized by characteristic facial features, abnormal upper limb development, delayed growth and cognitive retardation (1). These diverse clinical features are indicative of a developmental disorder affecting the expression of multiple genes. Interestingly, all causative mutations identified in cases defined as CdLS have been in genes encoding proteins in the cohesin complex or in proteins that interact with this complex. These include NIPBL (2,3), SMC1A and SMC3 (4–7), RAD21 (8) and HDAC8 (9). The cohesin complex was initially identified for its role in keeping sister chromatids together during cell division until anaphase; however, recent studies have expanded the role of this complex outside of mitosis and meiosis.

The core components of cohesin are SMC1/SMC3, Scc1/Mcd1/Rad21 and Scc3/stromalin/SA/stag. Together these proteins form a ring-like structure that is responsible for holding sister chromatids together (10). While mutations in genes encoding cohesin complex proteins have been identified in a small subset of patients diagnosed with CdLS, up to 60% of CdLS mutations, and 80% of mutations in the most severe forms of the disease, involve NIPBL (nipped-B-like) which is not a core component of cohesin (3,11–13). NIPBL (Scc2 in Saccharomyces cerevisiae) is important for loading the cohesin complex onto chromatin during S-phase (14). Interestingly, another clinical syndrome, Roberts-SC phocomelia, has been found to have mutations in a modulator of cohesin, ESCO2. While sharing some similar clinical characteristics, this syndrome has features that are distinct from CdLS (1), suggesting that subtle changes in the regulation of cohesin function can result in different phenotypes.

Individuals with CdLS are heterozygous for the mutant NIPBL allele. Most mutant alleles are predicted to result in
either a complete absence of protein or the production of a severely truncated one and thus are considered to be loss of function alleles, though missense mutations have also been reported (3,11,13). Mouse models that are heterozygous for alleles have some of the phenotypes characteristic of CdLS individuals, including growth retardation, craniofacial abnormalities, heart defects and behavioural changes (15). The type of mutation in NIPBL in the four CdLS LCL cell lines studied is indicated as is the predicted effect on NIPBL protein. Patient phenotypes were reported in the indicated references. Karyotypes for three of the four cell lines were normal. For CdL125P cell line, 50% of metaphases were normal 46XX but in 50% of cells there was a fusion chromosome. This did not appear to involve any of the regions analysed in this paper.

RESULTS

Nuclear size in CdLS cell lines

To determine whether there are global changes in chromatin compaction associated with the mutation of NIPBL, we first analysed nuclear size in fixed lymphoblastoid cell lines (LCLs) from individuals with a severe CdLS phenotype. Many factors affect nuclear size within a particular cell type, but chromatin decompaction is known to result in nuclear swelling (39–41).

Cell lines were chosen that carry different types of mutations in NIPBL with predicted differing effects on the resulting protein. CdL223P carries an NIPBL allele with a deletion of exons 2–17 that removes the start codon for NIPBL (Fig. 1A and Table 1) (42). CdL125P carries an allele with a very early frameshift (11) that is the most common mutation found in CdLS (43) and is expected to create no, or a severely truncated, Amino acid change in NIPBL Karyotype Reference

Table 1. CdLS cell lines

| Cell line | Mutation type | Location of mutation in NIPBL | Amino acid change in NIPBL | Karyotype | Reference |
|-----------|---------------|-------------------------------|---------------------------|-----------|-----------|
| CdL 223 P | Microdeletion  | Deletion of exons 2–17        | No start codon            | 46XX      | (42)      |
| CdL 125 P | Frameshift    | 2475, 2480delAG               | R827GfsX2                 | 50% 46XX, 50% 44XX + fusion (poss 1;16) | (11,43)   |
| AG0908    | Frameshift    | 7306, 7307 ins G              | S2435X                    | 46XX      | (2)       |
| AG0805    | Splice site   | 5575–2 A > G                  | No exon 30                | 46XX      | (2)       |

The type of mutation in NIPBL in the four CdLS LCL cell lines studied is indicated as is the predicted effect on NIPBL protein. Patient phenotypes were reported in the indicated references. Karyotypes for three of the four cell lines were normal. For CdL125P cell line, 50% of metaphases were normal 46XX but in 50% of cells there was a fusion chromosome. This did not appear to involve any of the regions analysed in this paper.
Figure 1. Characterization of NIPBL cell lines. (A) Representation of NIPBL protein with the main protein domains shown below and the location of CdLS mutations in LCLs indicated above. Orange represents a microdeletion, red a frameshift, blue a splice site mutation and green a nonsense mutation. (B) Box plots showing the distribution of nuclear sizes (μm²) in fixed nuclei from control and CdLS LCLs. Data from CdLS lines CdL223P and CdL125 are shown on the left together with a wild-type unaffected sibling (WT). AG0805 and AG0088 are shown on the right together with an independent WT control (575). Horizontal lines show the means and the boxed area is the interquartile range (IQR). Whiskers show 1.5 IQR of the upper and lower quartile. n > 100 loci each, P < 0.001. (C) Sample images of DAPI-stained nuclei from wild-type and patient cells demonstrating differences in nuclear size. Scale bar = 10 μm. (D) Fluorescence-activated cell sorting (FACS) analysis of DNA content from PI stained WT (575), AG0805 and AG0088 cells.
protein. Two other cell lines have point mutations within (AG0805), or just after (AG0088), the conserved C-terminal HEAT domains (2), which have been shown to be necessary to recruit NIPBL to sites of DNA damage (44) as well as mediating protein–protein interactions (45,46). By imaging 4′,6-diamidino-2-phenylindole (DAPI) stained fixed nuclei, we found that CdLS cell lines had a significant increase in nuclear size distribution relative to wild-type LCLs ($P < 0.001$; Fig. 1B and C).

Nuclear size increases during the cell cycle, so one possibility was that CdLS cell lines have an altered cell cycle profile, perhaps resulting from aberrant sister chromatid cohesion. However, fluorescence-activated cell sorting (FACS) analysis showed that the CdLS LCLs have a normal cell cycle distribution relative to control LCLs (Fig. 1D). Therefore, we conclude that the increased nuclear size we observe in CdLS cells is not the result of abnormal cell cycle dynamics and may instead reflect altered chromatin compaction.

### Regional chromatin decompaction in CdLS cell lines correlates with NIPBL mutation

To determine whether NIPBL mutations affect higher-order chromatin compaction at particular loci, we selected specific regions of the human genome to examine in more detail. The five regions were chosen to represent a variety of different genomic characteristics (Table 2). Because of the reported binding of NIPBL at gene promoters in association with Rpol II and Mediator (36), we selected two regions of low gene and CpG island ( CGI) density, 11p12 and 18q22.2 (Fig. 2A and B), one of moderate gene density, 18p11.3 (Fig. 2C), and two regions, 11q13.3 and 19q13.3, of high gene and CGI density (Fig. 2D and E). Moreover, 11q13.3 is also a region of the human genome (RIDGEs) where densely packed genes are also expressed to a high level (47).

Rpol II binding was also ascertained from the ChIP data for LCLs established by ENCODE (48). No Rpol II binding is detected at either of the low gene-density regions, while peaks of binding are found scattered throughout the other three regions. In agreement with this, gene expression data from human LCLs show transcripts originating from the high and moderate gene-density regions, while no transcripts are detected in the regions of low gene density (49).

The selected regions also have different CTCF binding profiles that largely follow the trends in gene density consistent with the location of many CTCF sites close to the transcription start sites of genes (50,51). Finally, and as expected given the correlation between CTCF and cohesin subunit ChIP signals (25–28), cohesin density (SMC3 ChIP peaks) also varies across the selected genomic regions in line with gene density (Fig. 2 and Table 2).

We assayed higher-order chromatin compaction in control and CdLS LCLs at these five selected genomic regions by FISH using probe pairs separated by 250 kb (Fig. 2 and Table 2). The mean-squared interprobe distance ($d^2$) between hybridization signals for such probe pairs is known to have a linear relationship with genomic separation (kb) over this size range and can be used to measure changes in chromatin compaction both between different regions of the same genome (52,53) and between different cell types—e.g. during differentiation and development (54,55). Moreover, such analysis can determine the role of specific histone modifications and proteins in chromatin compaction in wild-type and mutant cells (38). In order to detect effects that are locus-specific as opposed to those that are just a reflection of genome-wide chromatin decompaction and increased nuclear size, data were normalized to the nuclear radius ($r^2$), as described previously (38).

FISH analysis at regions of low to moderate gene density (Fig. 3) showed that in three of the four CdLS cell lines, there was no significant change ($P > 0.05$, Table 3) in regional chromatin compaction compared with wild-type cells. There was also no altered chromatin compaction detected at the 18q22 locus in CdLS lines AG0805 and AG0088 with a probe pair separated by a larger (400 kb) genomic distance (data not shown). The only cell line where there was a significant decrease in regional chromatin compaction (increase in $d^2/r^2$) in regions of low gene density, relative to control cells, was CdL223P, the cell line that is expected to have the most profound impairment of NIPBL function.

In contrast, at the two regions of high gene and high CTCF/SMC3 density, significant chromatin decompaction was seen across the 250 kb size range in both CdL223P and CdL125P cell lines (Fig. 4A, B and D). Moreover, for CdL223P cells, the extent of decompaction measured at these loci relative to

### Table 2. Characteristics of Fosmid probe locations

| Chromosome | Whitehead probe name | Other probe name | Start (bp) | End (bp) | Midpoint (bp) | Separation between probe pair midpoints (bp) | Genes/100 kb | CGI/100 kb | CTCF/100 kb | SMC3/100 kb |
|------------|----------------------|-----------------|-----------|----------|--------------|---------------------------------------------|-------------|-----------|-------------|-------------|
| 11p12      | WI2-1843D17          | G248PS6589B9    | 40 702    | 40 738   | 40 720       | 256 605.0                                   | <1          | 0         | 0           | 0           |
| 18q22.2    | WI2-1702P7           | G248PS7689H4    | 64 134    | 64 175   | 64 155       | 238 833.5                                   | 0           | 1         | 1.6         | 1           |
| 18p11.3    | WI2-0672M24          | G248PS8001G12   | 3 467     | 3 506    | 3 486        | 250 195.0                                   | 3           | 1.5       | 5.5         | 3.5         |
| 11q13.3    | WI2-1795M06          | G248PS6030G3    | 3 217     | 3 255    | 3 236        | 249 082.0                                   | 9           | 9         | 9           | 10          |
| 19q13.3    | WI2-1832N17          | G248PS6553G9    | 46 105    | 46 147   | 46 126       | 241 287.0                                   | 7           | 7         | 12          | 13          |
| 12       | WI2-1336P19          | G248PS4001H10   | 45 865    | 45 905   | 45 885       | 45 885 404                                  |             |           |             |             |

Probe names are from the Whitehead Fosmid database (http://bacpac.chori.org/library.php?id=275). Alternative probe names can be used to view fosmids on the UCSC genome browser. All genome locations are reported as hg19 coordinates. Gene, CGI, CTCF and SMC3 peak densities are estimates based on UCSC genome browser and Encode datasets and averaged for a 100 kb region.
Figure 2. Genomic regions used for FISH analyses. UCSC genome browser images showing the location of FISH probes in the five tested genomic regions. Map position (Mb) and RefSeq gene annotations are from the February 2009 (hg19) assembly of the human genome (http://genome.ucsc.edu). Positions of UCSC genes and CGIs are also indicated. ChIP peaks for CTCF, PolII and SMC3 are from ENCODE data for GM12878 LCLs. (A) Chromosome 11p12 region with low gene density. (B) Chromosome 18q22.2 region with low gene density. (C) Chromosome 18p11.3 region with moderate gene density. (D) Chromosome 11q13.3 region with high gene density. (E) Chromosome 19q13.3 region with high gene density.
wild-type cells was greater (2.4-fold for 11q13.3 and 3.7-fold for 19q13.3) than that at the regions of moderate to low gene density (2-fold; Table 3). The 11q13.3 region was also analysed in AG0805 and AG0088 CdLS cell lines (Fig. 4A). Significant chromatin decompaction was seen in AG0805, but not in AG0088 which potentially has the mildest mutation, a nonsense mutation at the extreme C-terminus of NIPBL (Fig. 1A). Significant chromatin decompaction was, however, seen in AG0088 cells when the probe pair separation was increased to 500 kb (Fig. 4C).

To exclude that the increased nuclear distances seen by FISH in CdLS patients could be due to a genomic alteration, e.g. copy number variation, in the regions being tested, we used array comparative genome hybridization (array CGH) on genomic DNAs prepared from AG0805 and AG0088 CdLS cell lines (Fig. 4A). Significant chromatin decompaction was seen in AG0805, but not in AG0088 which potentially has the mildest mutation, a nonsense mutation at the extreme C-terminus of NIPBL (Fig. 1A). Significant chromatin decompaction was, however, seen in AG0088 cells when the probe pair separation was increased to 500 kb (Fig. 4C).

To exclude that the increased nuclear distances seen by FISH in CdLS patients could be due to a genomic alteration, e.g. copy number variation, in the regions being tested, we used array comparative genome hybridization (array CGH) on genomic DNAs prepared from AG0805, AG0088 and CdL125P. The results showed no indication of gross genomic rearrangements at a genome-wide level in any of the three samples analysed. Detailed inspection of the regions examined by FISH revealed no significant changes in genomic copy number in those regions (Supplementary Material, Fig. S1).

From our data, we conclude that loss of NIPBL function causes a large-scale unfolding of higher-order chromatin structure that can be detected by FISH and that this occurs to the greatest extent in genomic regions with the highest gene density and the highest density of CTCF and cohesin binding sites. Our data also suggest that different mutations of NIPBL impact on the extent of chromatin decompaction, with the most severe cellular phenotype seen in the cases where the mutation is predicted to have the most severe effect on NIPBL protein and protein function.

**Chromatin decompaction after knockdown of NIPBL**

Because NIPBL expression levels are reported to be reduced to only 60–70% of wild-type levels in CdLS (15,20), we analysed the effects on chromatin compaction of more severely reduced levels of NIPBL, achieved by siRNA knockdown in HT1080 human fibrosarcoma cells. Knockdown of NIPBL at both the mRNA and the protein level was confirmed by quantitative reverse transcription PCR (qRT-PCR) and western blot (Fig. 5A.
DISCUSSION

The most common gene mutated in individuals diagnosed as CdLS is NIPBL. However, the link between the genotype of

### Table 3. Statistical results from FISH analysis of CdLS cell lines

|                | 11p12 | 18q22.2 | 18p11.3 | 11q1.3 | 19q13.3 |
|----------------|-------|---------|---------|--------|---------|
| **Normalized interprobe distance (d^2/r^2)** |       |         |         |        |         |
| Unaffected sibling | 0.00162 | 0.00161 | 0.00206 | 0.00295 | 0.00206 |
| CdLS 223P       | 0.00324 | 0.00222 | 0.00365 | 0.00713 | 0.00758 |
| P = 0.0054      | P = 0.0388 | P = 0.0031 | P = 0.0006 | P < 0.0001 |
| CdLS 125P      | 0.00155 | 0.00122 | 0.00278 | 0.00552 | 0.00575 |
| P = 0.8593      | P = 0.0822 | P = 0.1022 | P = 0.0085 | P < 0.0001 |
| WT (575)        | 0.002  | 0.002   | 0.0036  | 0.0036  |         |
| AG0085          | 0.0013 | 0.0043  | 0.003   | P = 0.007 |
| AG0088          | 0.002  | 0.002   | 0.0035  | 0.0035  | P = 0.178 |
| **Squared interprobe distance (d^2)** |       |         |         |        |         |
| Unaffected sibling | 0.1698 | 0.1782  | 0.1991  | 0.3305  | 0.1991  |
| CdLS 223P       | 0.3548 | 0.1726  | 0.4181  | 0.7442  | 0.7598  |
| P = 0.0014      | P = 0.7038 | P = 0.0001 | P = 0.0016 | P < 0.0001 |
| CdLS patient 125 | 0.2003 | 0.1251  | 0.3429  | 0.8425  | 0.8723  |
| P = 0.4797      | P = 0.1336 | P = 0.0035 | P < 0.0001 | P < 0.0001 |
| WT (575)        | 0.15   | 0.41    |         |         |         |
| AG0085          | 0.25   | 1.04    |         |         |         |
| AG0088          | 0.16   | 0.49    | 0.697   |         |         |
| **P-values**    |       |         |         |        |         |

Mean normalized distance (d^2/r^2) and squared interprobe distances (d^2) for FISH data from control and CdLS LCLs at five genomic loci, using probe pairs separated by 250 kb. P-values were generated using the Mann–Whitney U non-parametric test comparing CdLS to wild-type controls. Significant P-values (<0.05) are indicated by shaded boxes.
these individuals and their biological phenotypes remains unclear. It is accepted that the CdLS clinical phenotypes associated with NIPBL mutation are not the result of mitotic/sister chromatid cohesion defects, but instead result from the altered regulation of gene expression. This is consistent with the reported genomic localization of NIPBL with components of the transcriptional machinery (36). How perturbation of NIPBL affects gene expression has not been established. Here, we have demonstrated that there is a decompaction of higher-order chromatin structure at a subset of genomic regions in cells from CdLS individuals and that this is seen to the greatest degree at regions that are gene-rich and characterized by a high density of binding sites for cohesin and CTCF as assessed by ChIP (Figs 3 and 4). We provide evidence that the extent to which chromatin compaction is perturbed is linked to the likely severity of the NIPBL mutation, and this is further supported by the extensive chromatin decompaction we report after NIPBL knockdown by siRNA (Figs 5 and 6). These observations are consistent with the chromatin decondensation seen in budding yeast with mutations analogous to CdLS mutations, in Scc2—the yeast homologue of NIPBL (58).

Given that both NIPBL and cohesin affect long-range chromatin interactions in genetic assays, and cohesin affects chromatin conformation as measured in 3C cross-linking assays (29,30,32), we evaluated chromatin compaction in cells after the knockdown of the cohesin component SMC3. This did not phenocopy the effect seen either with NIPBL knockdown or in CdLS-associated NIPBL mutant cells.

Despite the fact that many NIPBL sites appear coincident with CTCF binding sites (25–28) and CTCF has been suggested to affect higher-order chromatin conformation (32,56,57), we also did not see chromatin decompaction after CTCF knockdown, even at genomic regions with the highest density of CTCF binding sites. We note that we were unable to decrease the levels of CTCF below 60% by siRNA treatment—CTCF is required for cell viability and division and is notoriously hard to knockdown (59). However, we did paradoxically observe an increase in chromatin compaction at the 11q13.3 locus after CTCF knockdown, indicating that knockdown was significant.

Figure 4. Chromatin compaction at gene-rich regions in CdLS. As in Figure 3 but for (A) 11q13.3 gene- and CTCF-rich region, n > 100 and (B) 19q13.3 gene- and CTCF-rich region, n > 95. All P-values are reported in relation to unaffected sibling in Table 3 and significant values are indicated below the graphs. (C) 11q13.3 gene- and CTCF-rich region, assayed with probes separated by 400 kb, n > 100. (D) Example FISH images from WT, CdL223P and CdL125P cells for 19q13.3. Bar represents 5 μm.
Figure 5. Knockdown of NIPBL, SMC3 and CTCF. (A) Real-time quantification of NIPBL mRNA levels relative to those for the mRNA of NADH dehydrogenase (ubiquinone) 1 alpha (NDUFA1) in untreated cells, in mock transfected cells and in cells transfected with random siRNAs or with NIPBL-specific siRNA. All values are normalized to levels in untreated cells (value of 1). All analysis performed in triplicate. (B) Western blot for NIPBL and actin in untreated cells and in cells after siRNA knockdown for CTCF, NIPBL and SMC3. Data for transfection with a random siRNA control are also shown. (C) Quantification of NIPBL protein levels based on western results. Samples normalized with actin levels and relative to the ratio in untreated cells. (D) As in (A) but for SMC3 knockdown. (E) As in (B) but for SMC3 and PCNA. (F) As in (C) but SMC3 protein levels normalized to those of PCNA. (G, H and I) As in (D), (E) and (F) but for CTCF knockdown.
Figure 6. Regional chromatin compaction after NIPBL, SMC3 and CTCF knockdown. Box plots show the distribution of interprobe distances $d^2$ normalized for nuclear radius $r^2$ in untreated cells and after knockdown with siRNAs against NIPBL, SMC3, CTCF and with random siRNAs. All $P$-values are reported in relation to untreated cells (Table 4) and are indicated here by $^*$ $P < 0.05$ and $>0.01$, $^{**} P < 0.01$ and $>0.001$ and $^{***} P < 0.001$ and $>0.0001$. Analysis at (A) 11p12 gene- and CTCF-poor region, $n > 105$; (B) 18q22.2 gene- and CTCF-poor region, $n > 100$; (C) 18p11.3 region with moderate gene and CTCF density, $n > 110$; (D) 11q13.3 gene- and CTCF-rich region, $n > 100$; (E) 19q13.3 gene- and CTCF-rich region, $n > 100$. 
enough to have an impact on chromatin structure, albeit not in the expected direction. Our data, therefore, suggest that NIPBL can affect higher-order chromatin folding, independent of cohesin and CTCF. This raises the possibility that the underlying aetiology of disorders associated with the mutation of core cohesin components is different from that associated with the disruption of NIPBL. Individuals identified with mutations of SMC1A and SMC3 have relatively mild phenotypes, often with atypical facial features and fewer limb and digit abnormalities than those which characterize classic CdLS with NIPBL mutation (6,13,43). This has been attributed to the fact that the SMC1A/SMC3 mutations have a predicted modest affect on protein structure—more severe mutations are assumed to be incompatible with birth—but it could also reflect a fundamental difference in the cellular functions of NIPBL and cohesin and a different aetiology for NIPBL-associated CdLS and the CdLS-like diseases associated with mutations in cohesin components. This would be consistent with the differences in dysregulated gene expression seen in zebrafish morphants for nipbl and those depleted in cohesin components (60).

Future work to dissect the regional differences in susceptibility to chromatin decompaction associated with NIPBL mutation, to establish how this level of chromatin structure is linked to the disruption of gene expression and to examine this in different cell types and at different developmental stages in animal models of CdLS has the potential to provide new insight into the mechanisms of CdLS-like diseases.

**MATERIALS AND METHODS**

**Cell culture**

LCLs were cultured in RPMI-1640 supplemented with 0.2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 20% fetal calf serum (FCS). HT1080 fibrosarcoma cells (61) were cultured in Dulbecco’s modified Eagle medium (Invitrogen) supplemented with 0.2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FCS. For cell cycle analysis, harvested cells were resuspended in 50% FCS/phosphate buffered saline (PBS), 3 x volume 70% ice-cold ethanol was added and cells were stored at 4°C. Cells were...
then washed with PBS and resuspended in propidium iodide solution (50 µg/ml PI + 100 µg/ml RNAse) for 1 h prior to FACS analysis on a BD FACSAria2 SORP. Cells were excited at 488 nm and measured at 562–588 nm. Data were analysed using BD FACSDiva Software version 6.1.3.

**FISH, image capture and analysis**

Nuclei were isolated in hypotonic buffer (0.25% KCl) and fixed with 3:1 v/v methanol/acetic acid. Fosmid clones (Table 2) were prepared and labelled with digoxigenin-11-dUTP or with biotin-16-dUTP as described previously (55). After hybridization, digoxigenin-labelled probes were detected using Rhodamine anti-digoxigenin and Texas Red anti-sheep IgG (Vector Laboratories). Biotin-labelled probes were detected using fluorescein–streptavidin and biotinylated anti-avidin (Vector Laboratories).

Slides were analysed as described previously (55) except that a Chroma #83000 triple band pass filter set (Chroma Technology Corporation, Rockingham, VT, USA) and a motorized filter wheel (Prior Scientific Instruments, Cambridge, UK) were used.

**Array CGH**

Genomic DNA was prepared from LCLs using a Nucleon DNA extraction kit (Tepnel Life Sciences, UK). DNA was quantified by NanoDrop spectrophotometry (Thermo Scientific).

Genome-wide analysis of DNA copy number aberrations was carried out using the Roche Nimblegen 135k whole-genome array (median probe spacing of 130 kb). The results were analysed by obtaining the log2 ratios of the case (labelled with Cy-5) compared with the control (labelled with Cy-3). A heterozygous deletion and a homozygous duplication are expected to result in log2 ratios of 1.0 and 0.58, respectively. Similarly, a homozygous deletion and a homozygous duplication are expected to result in log2 ratios of −2.0 and 1.0, respectively. Genomic coordinates were converted from hg19 to hg18 for analysis, using the UCSC Lift Genome Annotations utility.

**SiRNA-mediated knockdown**

SiRNA-mediated knockdown was performed using ON-Targetplus SMARTpool siRNA mixes (Thermo Scientific), designed with dual-strand modification that decreases off target effects and containing a mix of four individual siRNAs (Table 5). The randomer was the ON-TARGETplus Non-targeting Control Pool. siRNAs were diluted as directed by the manufacturer to a stock concentration of 100 µM in the supplied 1 × siRNA buffer. A 10 cm dish of HT1080 cells at 90% confluence was lipofected with 165 pmol siRNAs using Lipofectamine 2000. Cells were harvested for RNA and protein 50 h post-transfection.

**Real-time PCR**

RNA was isolated using Qiagen RNA Easy Kit. 500 ng of RNA were reverse transcribed in a 20 µl reaction using Superscript II. 1 µl of cDNA was amplified using specific primers (Table 6) and SYBER green real-time master mix (Life Technologies). Real-time analysis was performed using a Roche LightCycler 480. The programme for amplification was 95°C × 3 min, then 45 cycles of 95°C × 15 s, 62°C × 15 s and 72°C × 30 s. The melting curve programme was 95°C × 5 s and 65°C × 1 min, and then readings were acquired while increasing the temperature by 0.11°C/s to 97°C.

**Immunoblotting**

Protein was isolated from a 6-well plate of cells. 100 µl of 4× SDS SLB (2.0 ml 1 M Tris–HCl, pH 6.8, 0.8 g sodium dodecyl sulphate SDS), 4.0 ml of 100% glycerol, 0.4 ml of 14.7 M β-mercaptoethanol, 1.0 ml of 0.5 M ethylenediaminetetraacetic acid 8 mg bromophenol blue) and 300 µl PI were added to the cells. The cells and liquid were collected from the wells, boiled for 5 min and then sonicated for 20 s (Bioruptor NextGen Diagenode) prior to loading directly onto gels. For analysis of NIPBL, samples were run on a NuPage TrisAcetate 3–8% gel (Life Technologies) using the NuPage running buffer. For CTCF and SMC3, samples were run on a standard 10% SDS polyacrylamide gel. Gels were transferred onto polyvinylidene fluoride membranes by semidry transfer at 10 V for 1 h. The membrane was blocked for 1 h in 5% milk PBST (0.1% Tween-20 in Dulbecco A PBS; Oxoid). NIPBL was detected with the anti-IND3 rat monoclonal antibody (AbCAM #131913) diluted 1:500. For Smc3, anti-SMC3 rabbit polyclonal antibody (Bethyl A300-060A) was used at a 1:1000 dilution. For CTCF, anti-CTCF rabbit polyclonal antibody (Upstate #131913) diluted 1:500. For Smc3, anti-SMC3 rabbit polyclonal antibody (Bethyl A300-060A) was used at a 1:1000 dilution. For CTCF, anti-CTCF rabbit polyclonal antibody (Upstate 07-729) was used at a 1:2000 dilution. Membranes were incubated with antibodies overnight in 1.5% milk PBST at 4°C, washed 3 × with PBST and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody at a 1:10 000 dilution at room temperature for 1 h. Signal was detected by ChemiGlow West (Alpha Innotech) and imaged using Image Quant LAS4010 (Version 1, Build 1.0.0.52; GE Healthcare).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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