Developmentally regulated higher-order chromatin interactions orchestrate B cell fate commitment

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Received May 25, 2017; Revised July 21, 2017; Editorial Decision August 07, 2017; Accepted August 09, 2017

ABSTRACT

Genome organization in 3D nuclear-space is important for regulation of gene expression. However, the alterations of chromatin architecture that impinge on the B cell-fate choice of multi-potent progenitors are still unclear. By integrating in situ Hi-C analyses with epigenetic landscapes and genome-wide expression profiles, we tracked the changes in genome architecture as the cells transit from a progenitor to a committed state. We identified the genomic loci that undergo developmental switch between A and B compartments during B-cell fate determination. Furthermore, although, topologically associating domains (TADs) are stable, a significant number of TADs display structural alterations that are associated with changes in cis-regulatory interaction landscape. Finally, we demonstrate the potential roles for Ebf1 and its downstream factor, Pax5, in chromatin reorganization and transcription regulation. Collectively, our studies provide a general paradigm of the dynamic relationship between chromatin reorganization and lineage-specific gene expression pattern that dictates cell-fate determination.

INTRODUCTION

It is increasingly evident that the assembly of higher-order genome structures and their associated sub-nuclear compartments are intimately linked with transcriptional activity (1,2). Recent advances in high-throughput Chromosome Conformation Capture (3C)-derived methods have enabled quantitative measurement of physical interactions of chromatin in 3D nuclear space (2–6). These studies have demonstrated that chromatin is organized into transcriptionally permissive (A) and repressive (B) compartments indicating that chromatin positioning in 3D nuclear space may be associated with gene activity. For instance, B cell specification is associated with relocalization of Igh alleles from the nuclear periphery (a repressive compartment) towards center of the nucleus (an active compartment), where they undergo long-range interactions and subsequent rearrangements (7–9). These findings provide a functional link between sub-nuclear localization of the chromatin and gene activity. Recent studies indicate that chromatin compartments are further organized into varying sizes of dense and highly self-interacting regions, known as Topologically Associating Domains (TADs). These chromatin domains have been found to be stable and conserved across various cell types (10). In mammalian cells, insulator binding protein, CTCF, is found to be enriched in TAD boundaries (10). The deletion of boundary regions results in an increase in inter-domain interactions indicating the structural and functional role of insulators in maintenance of discrete, functional chromatin domains (11,12). Further it was demonstrated that loss of CTCF results in dose dependent insulation defects at most of the TAD boundaries (13). However, recent studies suggest that depletion of cohesin-loading factor Nipbl, but not CTCF, results in genome-wide disappearance of TADs, reinforcing the critical role of cohesin in the formation of TADs by loop extrusion mechanism (Schwarzer et al., 2016; Kubo et al., 2017, Unpublished). Although TADs are invariant, the intrinsic interactions within these TADs were found to be varying (10,14). Moreover, several studies show that the cell typespecific gene expression is regulated through interactions between promoters and distantly located cis-regulatory elements, particularly enhancers, by looping out of intervening DNA sequences (15–18). These long-range interactions were found to be associated with changes in histone modifications and DNA methylation (19–21). Furthermore, the transcriptional output is controlled by a combinatorial binding of transcription factors at cis-regulatory elements (22–25). Thus, a number of molecular mechanisms contribute to the precise regulation of gene expression pat-
tern that drives lineage differentiation to maintain cell identity.

Gene ablation studies demonstrate that the B cell developmental program is orchestrated by unique sets of transcription factors including PU.1 (Sfpi1), Ikaros (Ikzf1), E2A (Tcf3), Ebf1 (Ebf1) and Pax5 (Pax5) (26–31). PU.1, Ikaros and E2A promote the lymphoid developmental competence of multipotent progenitors (MPPs) (30,32,33). Loss of any of these factors results in a severe block to the development of B cells prior to IgH V to DJ recombination (26,28,34). These mutant progenitors fail to express both Ebf1 and Pax5. While PU.1, Ikaros and E2A are required for B cell development in vivo, their functions can be bypassed in vitro by complementing with Ebf1 but not with Pax5 (33,35,36). Thus, PU.1, Ikaros and E2A are necessary for the development of early lymphoid progenitors, whereas Ebf1 and Pax5 function as primary and secondary regulators of B cell fate determination (37–40). Correspondingly, Ebf1−/− hematopoietic progenitors display multilineage developmental potential, both in vitro and in vivo. Restoration of Ebf1 expression in Ebf1−/− progenitors inhibits their alternative lineage choice and induces B cell development, independently of Pax5 (41). Although much is known about the concerted interplay of transcription factors that are important for B cell determination, far less is known about the genome-wide composition of cis-regulatory interactions controlling B-lineage-specific gene expression program and relevance of these interactions on hierarchical organization of the chromatin during early B cell developmental transition.

To obtain a comprehensive view of the relationship between higher-order chromatin reorganization and induction of developmentally regulated B lineage-specific gene expression program, we carried out in situ Hi-C, in pre-pro-B cells (Ebf1−/− progenitors) and pro-B cells (Rag2−/−). Our comparative analysis of chromatin coexists between pre-pro-B and pro-B cells revealed a distinct set of genomic loci that switch between A and B compartments. In addition, we show that TADs serve as coordinated sub-units of chromatin and undergo structural alterations during developmental transition from pre-pro-B to pro-B stage. Correspondingly, we demonstrate that the cis-regulatory interaction landscape displays extensive rewiring, thereby, modulating the transcriptional activity during pre-pro-B to pro-B cell transition. Finally, we show that Ebf1 regulates differential gene expression pattern, at least in part, through chromatin relocalization and establishment of long-range promoter-cis-regulatory interactions. Collectively, our results demonstrate that, B lineage-specific gene expression pattern is closely associated with dynamic reorganization of chromatin in a developmental stage-specific manner.

MATERIALS AND METHODS

Cell culture

Pre-pro-B cells (Ebf1−/− progenitors) were maintained on stromal layer (OP9 cells) in the presence of Opti-MEM (Gibco) containing 4% (v/v) fetal calf serum, β-mercaptoethanol (50 μM), penicillin (10 U/ml) and streptomycin (10 μg/ml) and supplemented with SCF (10 ng/ml), Flt3L (10 ng/ml) and IL-7 (5 ng/ml). Pro-B cells (Rag2−/− cells) were maintained under similar conditions except that the media was supplemented with only IL-7 (5 ng/ml). Both pre-pro-B cells and pro-B cells were used for preparation of RNA for RT-PCR and chromatin for the 3C and Hi-C assays.

In situ Hi-C and 3C experiments

In situ Hi-C as well as 3C experiments were carried out using pre-pro-B and pro-B cells as described previously (2,3). During in situ Hi-C, chromatin cross-linking, restriction enzyme (HindIII) digestion, biotin fill-in and ligation reactions were performed in intact nuclei (42,43). In case of 3C experiments, chromatin ligation following restriction digestion were performed in intact nuclei and the interaction frequencies between pre-pro-B and pro-B cells were normalized using a control region in Ercc3 gene.

Identification of topologically associated domains

Iteratively corrected relative contact probability matrices at 40 kb resolution, generated by implementing HiResHiC module of hiclib were converted into the format specified by Domain Caller (10), where the first three columns represent the chromosome number followed by start and end of the bin. Domain Caller is a simple and straightforward approach with greater flexibility to identify biologically relevant domain structures.

Generation of 3D structures of TADs

We have generated 3D structures of TADs in both pre-pro-B and pro-B cells by implementing AutoChrom3D (44), which uses a novel sequencing-bias-relaxed parameter to normalize chromatin interactions.

Determination of statistically significant cis-regulatory interactions

To discriminate between random polymer loops and specific chromatin loops, we have used Fit-Hi-C (45), a tool for assigning statistical confidence estimates to mid-range contacts. We have prepared ‘FRAGSFILE’ containing midpoints (or start indices) of the fragments and ‘INTERSFILE’ containing interactions between fragment pairs from the dict-file obtained through fragment level filtering. The BIASEFILE is prepared by using the python code that implements the iterative correction in sparse mode by filtering out loci that are less mappable than the threshold (cut off ≥ 0.5). The significant interactions obtained by implementing Fit-Hi-C, were further integrated with various epigenetic modifications (H3K4me1, H3K4me3, H3K4me2 and H3K9/14ac) to identify potential cis-regulatory interactions.

Microarray analysis

Pre-pro-B cells were transduced by spin-infection with retrovirus encoding GFP or Ebf1-GFP or Pax5-GFP and maintained for 2 days in lymphoid culture conditions as
previously described (41). After two days, GFP+ transduc-
tants were FACS-sorted, total RNA was isolated with TRI-
zol reagent (Invitrogen) and further purified on RNAse-
free columns (Qiagen). RNA quality control analysis was
performed as previously described (41). Biotin-labeled cRNA
was generated and hybridized to the Mouse Genome 430
2.0 Array (Affymetrix, Santa Clara, USA) according to the
manufacturer’s instructions. Data were analyzed as previ-
ously described (41).

Statistical analysis

All statistical analyses except for identifying significant
promoter-Cis-interactions, were performed using R pack-
age. Statistical significance was evaluated by unpaired two
sample t-test. For all the tests performed, statistical signifi-
cance was assessed as *P < 0.05, **P < 0.01, ***P < 0.001.

RESULTS

Differential chromatin compartmentalization promotes the B
lineage gene expression program

To determine programmatic changes in chromatin or-
ganization during B cell development, we performed in situ
Hi-C (Supplementary materials and methods), a high-
throughput molecular approach (42,43) that captures
genome-wide chromatin interactions, using Eβfl−/− pro-
genitors that represent the pre-pro-B cell stage (41) and
Rag2−/− cells that represent the pro-B cell stage (Sup-
plementary Figures S1 and S2A-D). The in situ Hi-C ap-
proach is similar to the previously described dilution Hi-C
method (2), except that the reactions: chromatin crosslink-
ing, restriction enzyme digestion (HindIII), fill-in of 5’ over-
hangs and ligation of chromatins ends present in close prox-
imity, were performed in intact nuclei (42). The Hi-C li-
braries were generated from both pre-pro-B and pro-B cells
and then subjected to paired-end sequencing. Following
high-throughput sequencing, the uniquely aligned (refer-
ce to supplementary materials and methods). As expected, these analyses revealed that
chromatin is segregated into A or B compartments, which
are defined by enriched or minimal interactions respectively
(Figure 1A; Supplementary Figure S3). A compartments
were found to contain a higher number of genes (Figure
1B) with an increase (4 fold) in CpG islands than the B com-
partments. Accordingly, the A compartments were substi-
tially enriched for active histone modifications (H3K4me3,
H3K4me1 and H3K9/14ac) (47) (Figure 1C) and displayed
higher transcript levels when compared to the B compart-
ments (Figure 1D), indicating that chromatin compartment-
alization mirrors gene activity in both cell-types.

To investigate the possibility that selective changes
in chromatin compartmentalization provide a structural
framework for B-lineage gene expression (2,8), we per-
formed PCA analysis at a higher resolution (100 kb). From
these analyses, we were able to define the chromatin state
of a total number of 22,360 common genes that were cap-
tured by in situ Hi-C in both the cell types. Of these, 16,045
genes in pre-pro-B cells and 16,643 genes in pro-B cells
were found to be present in A compartments, whereas 6,315
genres in pre-pro-B cells and 5,717 genes in pro-B cells
were found to be present in B compartments. Further exami-
nation of these common genes between pre-pro-B cells and
pro-B cells revealed three distinct classes, including a com-
mon set of genes that are localized in either A (Group I:
68.44%) or B (Group II: 22.25%) compartments in both
cell types. Consistent with previous observations (47),
although a major fraction (90.69%) of genes remained in
the same compartment (Group I or II) in both cell types, a dis-
tinct set of genes (Group III; 9.31%) switched between A
and B compartments. Of these, 1,339 (5.98%) genes transi-
tioned from A to B compartment, while, 741 (3.31%) genes
relocalized from A to B compartment during differentiation
of pre-pro-B cells to pro-B cells (Supplementary Tables S1
and S2). These observations demonstrate that B cell devel-
opmental progression from a multipotential progenitor to
a specified state encompasses notable changes in chromatin
compartmentalization.

In order to test whether the differential chromatin com-
partmentalization is associated with B lineage-specific gene
expression pattern, we compared the abundance of nascent
transcript levels as determined by RNA-Seq (GSE52450)
of Group III genes in pre-pro-B cells and pro-B cells. We
observed that the genes, which switch from the B compart-
tment to the A compartment during differentiation, dis-
Figure 1. Chromatin compartmentalization is closely associated with gene activity. (A) Iteratively corrected intra-chromosomal contact count matrix of chromosome 2, representing the frequency of interactions at 1 Mb resolution. The first principal components (PC1) indicate the chromatin state on a linear genomic scale. (B) Distribution of genes in A and B compartments for both pre-pro-B and pro-B cell types (**p < 0.001). (C) A and B compartments that are defined by PC1 were integrated with active methylation marks (H3K4me1, H3K4me3 and H3K9/14ac). Normalized heat maps were generated by employing Matrix2png. Rows represent individual chromosomes, whereas the columns represent normalized count of respective methylation mark. (D) Comparative analysis of transcript levels of genes, based on RNA-Seq, present in A and B compartments (**p < 0.001) for both pre-pro-B and pro-B cells. (E) Comparative analysis of transcript abundance of genes that relocate from B to A compartment (left panel) and A to B compartment (right panel) during differentiation of pre-pro-B cells into pro-B cells (**p < 0.001). (F, G) Iteratively corrected contact count matrices derived from genomic regions comprising Satb2 (chr1) and Satb1 (chr17) for both pre-pro-B and pro-B cells. The PC1 values indicate the chromatin state of respective genomic loci. Dotted boxes represent genomic regions of Satb2 and Satb1.
played higher transcript levels in pro-B cells (Figure 1E, left panel). For instance, Satb2 (Figure 1F), Tead1, Pou2af1 and Tll4 that are essential for B cell development (51,52) are re-localized from the B compartment to the A compartment during pre-pro-B to pro-B cell transition (Supplementary Table S1). Likewise, genes that relocate from the A compartment to the B compartment displayed lower transcript levels in pro-B cells (Figure 1E, right panel). Notably, genes that are associated with multipotent progenitors such as Satb1 (Figure 1G), cKit and Cd43 as well as key alternate lineage determinants such as Gata3, Zbtb16, Klf4, Vav3 and Sox6 are found to be relocated to the B compartment in pro-B cells (Supplementary Table S2). In comparison with pre-pro-B cells, a significant number of genes within the chromosomes 10, 11 and 16 switch from the B compartment to the A compartment. Similarly, genes that are located in chromosomes 6 and 7, switch from the A compartment to the B compartment in pro-B cells (Supplementary Figure S5). Interestingly, our studies reveal that majority of functionally important B-lineage-specific genes (Ebf1, Pax5, Foxo1, Irf4, Irf8, Cd79a, Cd79b and Cd19) are localized in A compartments in both cell types. However, some of the key alternate lineage genes (Gata3, Zbtb16, Klf4, Vav3 and Sox6) switch to the B compartment in pro-B cells. Thus, these observations indicate that relocalization of alternate lineage genes into B compartments is closely associated with their transcriptional repression. Collectively, our studies demonstrate that switching of selective genomic loci between A and B compartments is closely associated with the B lineage-specific gene expression pattern. However, these studies cannot rule out the possibility that chromatin relocalization and its associated changes may be a result of alteration of transcription.

Global analysis of topologically associating domains (TADs) during B cell specification

At sub-megabase level, A and B compartments of chromatin are organized into dense and contiguous self-interacting regions termed topologically associating domains, TADs (10). These chromatin domains have been proposed to be stable and conserved across cell types, yet their intrinsic chromatin interactions were found to be varying (14). This raised a possibility that changes in the interaction patterns within TADs may serve as a framework for differential gene activity and contribute to the developmental progression of the cell. In order to capture the changes in chromatin structure within these domains in pro-pre-B and pro-B cells, we employed domain caller software (10) to identify TADs from iteratively corrected relative contact probability matrices generated at 40 kb resolution. Our analyses revealed that the genome of pro-pre-B cells is partitioned into a total of 2,008 TADs, whereas the genome of pro-B cells comprised of 1,810 TADs with a total genomic occupancy of 90.74% and 89.50%, respectively. Strikingly, we found that the median TAD size is higher in pro-B cells (920 kb) as compared to pro-pre-B cells (800 kb). Collectively, these studies provide the first indication that the structural organization of TADs may be subjected to alterations during developmental transition from pro-pre-B to pro-B cell stage.

To gain further insights into changes in structural organization of the chromatin at the sub-megabase level, we cross-compared the TADs between two cell types (pre-pro-B and pro-B cells) based on their linear genomic position. In line with the previous reports (10), a substantial number of TADs, 1,023 (pre-pre-B cells: 50.9%, pro-B cells: 56.5%) were found to be stable, in both cell types (Supplementary Figure S6). The remaining TADs (pre-pre-B: 985, pro-B: 787) exhibited re-organization in terms of their genomic positions and were categorized as ‘dynamic’. It is possible that the stable TADs may maintain persistent chromatin interactions and thus account for uniform gene activity between two cell types. Alternatively, chromatin regions within these stable TADs may be subjected to epigenetic modifications and concomitant changes in intra-molecular interactions, resulting in cell-type specific gene expression pattern. To investigate these possibilities, we have compared the transcript levels of genes present within the stable TADs between pre-pre-B and pro-B cells and observed significant differences in their activities. This differential gene expression pattern may possibly be due to alterations in intrinsic chromatin interaction landscape. To examine this, we have calculated Aggregation Preference (AP), a parameter that quantitatively measures interaction patterns of TADs (53). During this analysis, the local high-frequency chromatin interactions, violating the distance-dependence decay principle, were measured and segregated according to their spatial aggregation by employing DBSCAN. The weighted density of clustered groups, defined as Aggregation Preference (AP), was used to quantitatively measure interaction patterns within each TAD. As expected, we found that TADs with higher AP values were comprised of high density chromatin interaction blocks in both cell types. Accordingly, TADs with higher AP values were enriched with active methylation marks (H3K4me1, H3K4me2 and H3K4me3) and displayed higher nascent transcript levels. On the other hand, TADs with low AP values displayed sparse chromatin interactions and were found to be depleted with active epigenetic marks (Supplementary Figure S7A). Furthermore, permissive TADs displayed higher AP values as compared to repressive TADs (Supplementary Figure S7B). Thus, AP values define transcriptional status and may serve as an appropriate measure of functional activity of TADs.

Interestingly, of the 1,023 stable TADs, a majority (867, 85%) of them displayed similar AP values (<0.2) between pre-pre-B and pro-B cells, indicating that the cumulative number of chromatin interactions with these TADs are comparable. Thus the differential gene expression pattern observed within stable TADs between the two cell types may possibly be attributed to the combinatorial changes in their promoter and cis-regulatory interactions. To test this, we first identified statistically significant (P < 0.05) chromatin interactions in both cell types by implementing the spline-fit model (45) (Supplementary materials and methods). Next, these significant interactions were integrated with genome-wide epigenetic marks (H3K4me3, H3K4me1 and H3K4me2) to identify potential promoters and cis-regulatory interactions (32). Only those promoters located within close proximity (+2.5 kb) of transcription start sites (TSS) (Supplementary Figure S8) and the cis-regulatory elements located ≥1 kb away from putative promoters, were
considered for further analysis. In total, we have identified 30,150 and 46,263 potential interactions involving promoters and cis-regulatory elements in pre-pro-B and pro-B cells, respectively. We have mapped these promoter-cis-regulatory interactions to the stable TADs (867, 85%) with similar AP values. From these analyses, we found that 6,678 and 9,468 cis-regulatory interactions were associated with stable TADs in pre-pro-B and pro-B cells, respectively. Of these interactions, 1,715 promoters were found to be common in pre-pro-B and pro-B cells, whereas 1,147 (48.8%) and 952 (57.1%) promoters were found to be unique in pre-pro-B and pro-B cells, respectively. The majority of these promoters (common and unique) were found to interact with developmental stage-specific enhancers (Figure 2A). For instance, Polg2, which is highly expressed in pre-pro-B cells and Cd79b, which is induced at pro-B cell stage, are both located in a stable TAD with similar AP values. Interestingly, we observed that Polg2 interacts with multiple enhancers (3) in pre-pro-B cells, whereas no such promoter and cis-regulatory interactions were captured in pro-B cells. Conversely, Cd79b promoter interacts with multiple enhancers (4) in pro-B cells, while only one such interaction was observed in pre-pro-B cells (Figure 2B). These results suggest that although a substantial number of TADs are stable with respect to the genomic position, their intrinsic chromatin interactions involving cis-regulatory elements are dynamic. These intrinsic changes in chromatin interactions may have a limited effect on the structural maintenance of the TAD, but may be critical for sustaining the cell type-specific gene expression pattern.

Structural reorganization of TADs corresponds to changes in cis-regulatory interaction landscape

While a substantial number (1,023) of TADs are constant in both pre-pro-B and pro-B cell types, a considerable number of TADs (pre-pro-B:985, pro-B:787) were found to be altered as indicated by changes in their genomic positions. We classified these altered TADs into two groups: unique and merged. Unique TADs are defined as those present in pre-pro-B cells but not in pro-B cells and vice versa. We propose that unique TADs (pre-pro-B:100, pro-B:65) may have been generated as a result of increased local genomic interactions to facilitate cell type-specific gene expression pattern (Supplementary Tables S3 and S4). Consistent with this assumption, key alternate lineage genes like Ccs3, Serpini1 and Vav3 that are highly expressed in multipotent progenitors, were found to be associated with TADs in pre-pro-B cells. On the contrary, in pro-B cells, these genes are located in the boundary regions (Supplementary Figure S9A). Besides the unique TADs, we observed that few larger TADs in pre-pro-B cells (110) partitioned into two or more minor TADs in pro-B cells. Conversely, two or more minor TADs in pre-pro-B cells coalesce into a larger ‘merged’ TAD in pro-B cells (183). We propose that merged TADs may have been formed as a result of increased inter-TAD interactions in pre-B cells (Figure 2C). Accordingly, the normalized contact frequency of inter-TAD regions of minor TADs in pre-pro-B cells is significantly lower as compared to the counter regions of merged TADs in pro-B cells (Supplementary Figure S9B). These observations are in line with increased median TAD size (920 kb) of pro-B cells, as compared to the size of pre-pro-B cells (800 kb). Correspondingly, we observed a significant increase in the number (pre-pro-B cells: 30,150, pro-B cells: 46,263) as well as in the median distance (pre-pro-B cells: 298 kb, pro-B cells: 330 kb) between promoter-cis-regulatory interactions in pro-B cells. This raises the possibility that inter-TAD promoter-cis-regulatory interactions may contribute for re-organization of TADs. To test this, we have mapped promoter-cis-regulatory interactions of merged TADs in pro-B cells and compared to their counter TADs in pre-pro-B cells. We found a significant increase in inter-TAD promoter-cis-regulatory interactions (2,600) in pro-B cells as compared to those (1,570) in pre-pro-B cells (Figure 2C). Taken together, these analyses provide insight into the dynamic re-organization of TADs which is closely associated with changes in the cis-regulatory interaction landscape during developmental transition from pre-pro-B to pro-B cell stage.

To rigorously demonstrate the dynamic organization of TADs observed between pre-pro-B and pro-B cells, we employed two distinct strategies. First, we used Directionality Index (DI), which quantitatively measures the ‘interaction bias’ of a given genomic region (10), as a parameter to detect the structural variations of TADs between the two cell types. Comparative DI analyses revealed that stable TADs display significantly higher correlation as compared to the dynamic TADs (merged and unique) (Figure 3A). These observations suggest that, unlike stable TADs, dynamic TADs display dramatic structural alterations. Second, we built 3D models of merged and stable TADs to determine the changes in position order of chromatin using AutoChrom3D (44). Compared to conventional 3D modeling methods (54–57), AutoChrom3D employs a novel sequencing-bias-relaxed parameter to derive 3D chromatin models. Next, we compared the spatial distance between start and end regions of merged TADs in pro-B cells with their counter regions in pre-pro-B cells. We reason that, in pro-B cells, the ends of a merged TAD should be in close spatial proximity compared to their counter regions in pre-pro-B cells. Consistent with this supposition, we found that the spatial distance was significantly lower in pro-B cells as compared to pro-B cells (Figure 3B, left panel; Supplementary Figure S10A–D). In contrast, no significant difference in the spatial distance was observed for stable TADs (Figure 3B, right panel; Supplementary Figure S10E). Collectively, these analyses demonstrate that 3D models reflect the changes in 2D interaction maps. To validate these results, we performed 3C-qPCR (Chromosome Conformation Capture) for a merged TAD (Chr12:69720000–71160000) found in pro-B cells formed as a result of coalescence of three minor TADs in pre-pro-B cells. The spatial distance between start and end of this TAD is found to be lower in pro-B cells (2.41˚A) compared to pre-pro-B cells (8.00˚A) as shown by AutoChrom3D. Correspondingly, our 3C experiment using primers close to start (+1615 bp) and end regions (~7004 bp) of merged TAD revealed higher cross-linking frequency in pre-B cells than pre-pro-B cells (Figure 4) (Supplementary Table S5). These results support our hypothesis that TADs undergo dynamic structural alterations as a result of changes in chromatin in-
Figure 2. TADs are dynamic and undergo structural reorganization during early B cell development. (A) Venn diagram indicating the number of promoters interacting with cis-regulatory elements that are present in the stable TADs with similar AP values in both pre-pro-B and pro-B cells. The pie chart represents the number of common promoters tethered to same (grey) or cell type-specific (blue) enhancers. (B) Comparative analysis of promoter-cis-regulatory interactions between stable TADs with similar AP values spanning a genomic region (106.04–107 Mb) of chromosome 11. TADs are mapped with active epigenetic marks, H3K4me3 (enriched at promoter regions), H3K4me1 and H3K4me2 (enriched at enhancers) as determined by ChIP-Seq in both pre-pro-B and pro-B cells. TADs were defined by domain calling approach and are highlighted by dotted lines. The genomic positions of promoter-cis-regulatory interactions within the TADs are represented by arcs. Polg2 (black) and Cd79b (blue) interactions are highlighted. (C) Comparative analysis of promoter-cis-regulatory interactions between merged TAD (pro-B cells) and its counter TADs (pre-pro-B cells) spanning the genomic region (112.20–115.64 Mb) of chromosome 5. TADs are demarcated by domain calling approach and highlighted by dotted lines. TADs were mapped with active epigenetic marks: H3K4me3 (for promoters) and H3K4me1 and H3K4me2 (for enhancers) as determined by ChIP-Seq in both pre-pro-B and pro-B cells. Promoter-cis-regulatory interactions are represented by blue arcs.
Figure 3. Comparative analysis of structural organization of TADs between pre-pro-B and pro-B cells. (A) Pearson’s Correlation Coefficient for directionality index (DI) calculated for stable as well as dynamic TADs (merged and unique) between pre-pro-B and pro-B cells. (B) Genome-wide comparative analysis of 3D spatial distances between start and end regions of merged TADs in pro-B cells and their counter regions in pre-pro-B cells (**P < 0.01) (left panel). Similar analysis of 3D spatial distances for stable TADs in both pre-pro-B and pro-B cells (n.s. = not significant) (right panel).
Figure 4. Validation of TADs reorganization by 3C analysis. Comparative analysis of 3D spatial distances and promoter-cis-regulatory interactions between merged TAD (pro-B cells) and its counter TADs (pre-pro-B cells) spanning the genomic region (69.72–71.16 Mb) of chromosome 12. 3D models generated by AutoChrom3D were colored distinctly based on minor TADs in pre-pro-B cells and the same color code is given for corresponding genomic regions of merged TAD in pro-B cells. The start and end regions of merged TAD in pro-B cells and its counter regions in pre-pro-B cells are highlighted by green and red respectively in the backbone 3D structure and the spatial distance between these regions is indicated in Å units. 3D models were generated at 8 kb resolution (upper panel). TADs were demarcated by domain calling approach and highlighted by dotted lines. TADs were mapped with active epigenetic marks H3K4me3 (for promoters), and H3K4me1 and H3K4me2 (for enhancers) as determined by ChIP-Seq in both pre-pro-B and pro-B cells. Promoter-cis-regulatory interactions are represented by horizontal lines (middle panel). 3C analysis of interaction frequency between ends of merged TAD (Chr12: 69.72–71.16 Mb) in pro-B cells and its counter regions in pre-pro-B cells. HindIII restriction sites are shown above the 3C plots. The location of primers used for measuring cross-linking frequency is indicated by red arrows (lower panel).
teraction patterns that may be important for transcription regulation.

To investigate if our findings could be extrapolated to other cell types, we compared the structural organization of TADs in mESC and cortex cells using publicly available data (10). Similar to what we have observed with our cell types, the total number of TADs (mESC-2085, cortex-1519) as well as their median size (mESC-880 kb, cortex-1.3 Mb) differed between mESC and cortex cells. Moreover, the comparative analysis of DI and relative contact probabilities suggests that TADs undergo structural reorganization between mESC and cortex (Supplementary Figure S11A and B). In concordance with our data for pro-B cells, we noticed that cortex cells have more number of merged TADs (269) than the mESCs (95). In comparison with the pluripotent cells (pre-pro-B and mESC), the observed increase in number of merged TADs and the associated increase in the average size of TADs in differentiated cells (pro-B and cortex) can be attributed to their compact chromatin organization (58,59). These findings are further supported by an increase in long-range interactions in pro-B cells compared to those in pre-pro-B cells. Consistently, a recent study suggests that the increase in TAD size as well as long-range interactions in sperm cells may be due to the dense packaging of its genome (60). Collectively, these findings demonstrate that the differences in the TAD organization between various cell types are dependent on the differences in their long-range interactions and chromatin compaction.

**TADs constitute structural frameworks for coordinated gene expression**

Next, we sought to determine the relationship between the structural organization of TADs and the differential gene expression pattern in pre-pro-B and pro-B cells. For this, we integrated TAD regions with PC1 values at 100 kb resolution to assess their chromatin state. Interestingly, we found that majority of the TADs are either transcriptionally permissive or repressive. However, a small percentage of TADs are comprised of both permissive and repressive chromatin regions and are referred as uncharacterized (Figure 5A). As expected, genes present in permissive TADs, displayed higher nascent transcript levels compared to those in repressive TADs (Supplementary Figure S12). These observations suggest the possibility that TADs serve as structural frameworks for coordinated regulation of genes. To rigorously demonstrate this, we calculated Pearson’s correlation coefficient (PCC) for all possible gene pairs, using publicly available genome-wide expression data sets for pre-pro-B and pro-B cell types (61). PCC for pre-pro-B cells was calculated by comparing microarray measurements of hematopoietic stem cells (HSCs) and CLPs as they mimic pre-pro-B cells. Likewise, for pro-B cells, PCC was calculated using microarray measurements of CLPs and pro-B cells (pro-B.FrBC.BM). These analyses revealed that in both pre-pro-B and pro-B cells, genes within a given TAD exhibit significantly higher correlation values ($P < 0.001$) in relation to the genes that are present in other TADs, indicating that TADs represent co-regulated sub-units of the genome (Figure 5B–D). We note that such coordinated regulation of genes within the TADs facilitate activation/repression of gene clusters in a cell type-specific manner. For instance, the HOXA gene cluster (Chromosome 6), which is localized in a single stable TAD is transcriptionally active in pre-pro-B cells, whereas the same cluster is found to be transcriptionally inactive in pro-B cells; suggesting that TADs not only serve as fundamental sub-units for coordinate regulation of genes, but they also provide a framework to sustain lineage-specific gene expression pattern.

**The cis-regulatory interaction landscape undergoes rewiring during B cell fate commitment**

Although, it is well established that promoter-cis-regulatory interaction landscape determines a lineage-specific gene expression pattern (15,62), much less is known about the genome-wide composition of these interactions during B cell development. From *in situ* Hi-C analyses, we identified a total of 31,190 and 47,711 potential promoter-tethered interactions in pre-pro-B and pro-B cells, respectively. As expected, genes whose promoters are involved in cis-regulatory interactions showed significantly higher expression levels than the genes that are not involved in any such interactions (Supplementary Figure S13A). The majority of the promoter-cis-regulatory interactions (83.5% in pre-pro-B cells and 79% in pro-B cells) are within the range of 1 Mb with a median value of 298 kb and 330 kb in pre-pro-B and pro-B cells, respectively (Supplementary Figure S13B). We have classified these promoter-tethered interactions into three basic groups: intergenic (promoter–promoter), extragenic (promoter–enhancer) and intragenic (promoter–genebody). We observed 8,410 and 10,556 intergenic interactions in pre-pro-B and pro-B cells, respectively. Similarly, we have captured 1,040 and 1,448 intragenic interactions in pre-pro-B cells and pro-B cells, respectively. Strikingly, we observed a significant increase in promoter–enhancer interactions in pro-B cells compared pre-pro-B cells. We found a total number of 21,740 promoter–enhancer (extragenic) interactions involving 8,096 promoters and 10,637 enhancers in pre-pro-B cells, wherein each promoter on average interacts with 2.68 enhancers. In the case of pro-B cells, 35,707 promoter–enhancer (extragenic) interactions involve about 9,424 promoters and 14,904 enhancers, wherein each promoter on average interacts with 3.79 enhancers. Among 9,424 promoters captured in pro-B cells, 6,331 (67.2%) promoters were also captured in pre-pro-B cells. Interestingly, 5,101 (80.5%) of common promoters were found to interact with cell type-specific enhancers and only 1,230 (19.5%) promoters share common enhancers (Supplementary Figure S13C and D; Supplementary Table S6). These results reveal that during B lineage specification, the promoter–enhancer interaction landscape undergoes extensive rewiring. We also note that in pre-pro-B cells, nearly 13.3% of enhancers interact with more than three promoters. Likewise, in pro-B cells, ~19% of enhancers were found to interact with more than three promoters (Supplementary Figure S13E and F). These observations support the assertion that multiple genes interacting with the same enhancer may be co-expressed (63).

Furthermore, our data revealed that in pre-pro-B cells, nearly 25.4% of promoters interact with only one enhancer,
while remaining promoters interact with two or more enhancers. Likewise, in pro-B cells, the majority of the promoters (81.1%) were found to interact with two or more enhancers (Supplementary Figure S13G and H). To test whether transcriptional activity of a promoter depends on the number of its cis-regulatory interactions, we assessed the transcript levels of the corresponding genes. Interestingly, these studies depicted a positive correlation between gene expression and a number of cis-regulatory interactions in both cell types (Figure 6A). Next, we sought to determine if loss or gain of these interactions induce differential gene expression patterns. For this, we compared the cis-regulatory interaction landscape of genes that show +10-fold differential expression between pre-pro-B and pro-B cells. The analysis showed that the expression pattern is closely associated with an increase in the number of cis-regulatory
Figure 6. cis-regulatory interaction landscape determines differential gene expression pattern. (A) Box plots showing the relation between the number of cis-regulatory elements that are interacting with promoters and their expression levels, in pre-pro-B and pro-B cells. Transcript levels of genes were measured by RNA-Seq. (B) Box plots representing comparative analysis of promoter-cis-regulatory interactions for a set of genes with ≥10-fold differential expression in pre-pro-B cells (right panel) and in pro-B cells (left panel) (***P<0.001). (C, D) Circos plots showing promoter-cis-regulatory interactome of Cd24a (Chr11:43.3–44.1 Mb) and Flt3 (Chr5:14.68–14.89 Mb) in pre-pro-B (left panel) and pro-B cells (right panel). Black arcs represent promoter-cis-regulatory interactions.
interactions (Figure 6B; Supplementary Tables S7 and S8). For instance, Cd24a, which is highly induced in pro-B cells, interacts with 13 cis-regulatory elements, whereas, it is involved in only four such interactions at pre-pro-B cell stage, where its expression is considerably low (Figure 6C). Correspondingly, the genes: Flt3 and Ccl3, that are important for maintenance of MPPs and differentiation of T-cells, respectively, are transcriptionally active at the pre-pro-B stage. These genes were found to be involved in more number of cis-regulatory interactions (Flt3: 6, Ccl3: 8) in pre-pro-B cells as compared to pre-B cells (Flt3: 1, Ccl3: 0) (Figure 6D). The examples depicted here demonstrate the prevalence of dynamic promoter-cis-regulatory interactions across B cell developmental stages. To rigorously validate these findings, we carried out 3C analysis of promoter–enhancer interactions of Ccl3 locus in pre-pro-B and pro-B cells (Supplementary materials and methods) (Supplementary Table S5). We observed that the interaction frequency between the Ccl3 promoter and with its upstream enhancer (located 64 kb away) was higher in pre-pro-B cells compared to that in pro-B cells (Figure 7A). Correspondingly, the quantitative RT-PCR analysis revealed thirty-fold higher Ccl3 transcript levels in pre-pro-B cells as compared to the levels in pro-B cells (Figure 7B). These analyses confirm that reinforcement of lineage-specific gene expression is contingent upon specificity and frequency of interactions between promoters and their cis-regulatory elements.

Ebf1 coordinates B cell specific cis-regulatory interaction landscape

A major goal of this study was to understand the molecular relationship between chromatin architecture and differential transcriptional cascade. It has been shown that Ebf1 is essential for induction of early B lineage gene expression program and targeted inactivation of Ebf1 results in a complete block prior to B cell commitment (28, 41). This raises a possible role for Ebf1 in chromatin relocalization and establishment of B lineage-specific cis-regulatory interaction landscape. To test this, we scanned for highly specific and significant Ebf1 binding sites in cis-regulatory regions of genes that switched to A or B compartments in pro-B cells, using publicly available databases (Jasper, Homer and Uniprobe). From these analyses, we observed that Ebf1 and/or Pax5 bind to cis-regulatory sequences of differentially switched genes (65.3%). Although a subset of genes undergoes differential compartmentalization, this may not solely account for the induction of B lineage expression program. We propose that activation of B lineage-specific genes may be regulated at multiple levels including binding of lineage-specific transcription factors (E2A, Ebf1, Foxo1 and Pax5) to their target promoter-cis-regulatory interacting elements. To examine this, we integrated promoter-cis-regulatory interactions that are captured in pro-B cells with binding events of these factors. Importantly, we found that in pro-B cells, Ebf1 binds either alone or in combination with Pax5 to 5390 (57.2%) promoters and 7629 (51.2%) cis-regulatory elements that are involved in long-range interactions as defined by in situ Hi-C (Figure 8A). Consistent with these observations, binding of Ebf1/Pax5 at cis-regulatory elements of their target genes is positively correlated with increased expression levels (Figure 8B and C).

To rigorously demonstrate the induction of B lineage genes in response to Ebf1 and/or Pax5, we carried out genome-wide expression analysis following restoration of Ebf1 or Pax5 in Ebf1−/− progenitor cells (Supplementary materials and methods). As expected, Ebf1 and/or Pax5 induced a spectrum of genes associated with B cell identity, including those that are involved in pre-B and B cell receptor signaling, antigen presentation, DNA recombination, and repair. Conversely, Ebf1 and/or Pax5 repressed a subset of genes that are involved in the development and function of natural killer (NK), dendritic and T cells (Figure 8D and E). Integration of in situ Hi-C interactome (promoter-cis-regulatory interactions) with microarray data sets revealed that the genes that are upregulated (>2-fold) in response to Ebf1 (124, 39.7%) or Pax5 (231, 44%) or both (37, 72.5%) are involved in long-range interactions (Figure 8F). We note that activation of these genes could be due to direct binding of Ebf1 and/or Pax5 to their respective promoter or distant regulatory elements that are brought in close proximity by looping-out of intervening DNA sequences. To determine this, we scanned the promoter and their corresponding cis-regulatory elements that are upregulated in response to Ebf1 (161) and/or Pax5 (268) for their binding. De novo motif analyses revealed that Ebf1 binds to either promoter regions (17.6%) or distant cis-regulatory elements (45.3%) or both (36.9%). Similarly, Pax5 binds either at promoter regions (19.2%) or distant cis-regulatory elements (54.1%) or both (26%) (Figure 8G). We note that both Ebf1 and Pax5 co-bind to a number of key B lineage genes such as Cd19, Cd24a, Socs3 and Dtx1. Taken together, in situ Hi-C analyses in combination with genome-wide expression analysis and DNA occupancy studies, we demonstrate that activation of B lineage-specific genes is associated with changes in long-range interactions and many of these genes are potentially regulated by lineage-specific transcription factors, Ebf1 and Pax5.

DISCUSSION

Precise and coordinated control of gene expression is important for the cell fate determination of multipotent progenitors (1, 58, 64, 65). Recent studies indicate that structural organization of the genome in 3D nuclear space is closely associated with modulation of transcriptional activity and establishment of cell type-specific gene expression program, indicating a potential relationship between nuclear architecture and mechanistic control of transcription. 3C-based studies indicate that genome is organized in a hierarchical manner: folding of chromatin loops, TADs, and large-scale compartments (1, 2, 10, 14, 47). However, the comprehensive understanding of how multilayer organization of chromatin regulates cell-type-specific transcriptional activity remains unclear. Specifically, the following questions arise: Does chromatin relocalization precedes lineage commitment? Do chromatin domains undergo structural reorganization? What are the roles of lineage determinants during chromatin reorganization? We have attempted to address these questions by integrating genome-wide chromatin interaction data with epigenetic landscape and tran-
Figure 7. Validation of promoter–enhancer interactions by 3C analysis. (A) Ccl3 locus, overlaid with various epigenetic marks as determined by ChIP-Seq in both pre-pro-B and pro-B cells (upper panel). Interaction frequency between promoter region of Ccl3 and its distant putative enhancer located at 64 kb upstream of promoter in both pre-pro-B and pro-B cells (lower panel) was measured by 3C-qPCR analyses. Data are representative of two independent biological experiments (error bars, S.E.). (B) Relative transcript levels of Ccl3 as measured by quantitative RT-PCR in pre-pro-B cells and pro-B cells. Hprt was used as endogenous control and values were normalized against pro-B cells as a reference control. Data are representative of two independent biological experiments (error bars, S.E.).

scription profiling of cells that represent two distinct stages (pre-pro-B and pro-B) of B cell development. In line with previous reports, our in situ Hi-C analyses revealed that chromatin is non-randomly organized into A and B compartments (1,2). We identified that a distinct set of genes switch between A and B compartments during the developmental transition from pre-pro-B to pro-B cell stage. For instance, genes that are important for B cell development including Satb2, Tead1, Pou2af1 and Thr4 switch from the B to A compartment during the pre-pro-B to pro-B transition. Likewise, genes such as Gata3, Klf4, Satb1 and Zbtb16 that are important for disparate lineage differentiation programs localize to B compartment in pro-B cells, where they are silenced. In contrast, the majority of the downstream targets of these master regulators were found to be in A compartments in both cell types. These studies suggest that sequencing master regulators of alternate lineages into B compartments may ensure lineage specification. These observations are further supported by a previous study (66) wherein Th2-specific regulator, GATA3, was found to be localized in the nuclear periphery in a transcriptionally inactive state in Th1 cells. However, the downstream targets of GATA3 (IL-2, IL-3, and IL-4) were retained in the permissive compartment in both Th1 and Th2 cells. Thus selective relocalization of lineage determinants appears to play an important role during developmental transition from a progenitor to lineage-committed state. However, the mechanistic details of how the genomic loci switch between A/B compartments is unclear. We propose that binding of transcription factors along with chromatin activation complexes enable the genomic loci to relocate from B to A compartment, whereas binding of polycomb group (PcG) proteins enable the loci to relocate from A to B compartments.

Our findings show that, although, a substantial number of TADs are stable between pre-pro-B and pro-B cells, chromatin interaction patterns particularly promoter-cis regulatory interactions within these TADs remodel to facilitate cell type-specific gene expression pattern. Strikingly, in addition to the stable TADs, we found a number of TADs that are dynamic and display structural alterations during B cell developmental transition. Specifically, we uncovered a set of unique TADs that are exclusively present either in pre-pro-B or pro-B cells, which contain genes that are selectively expressed at these stages. Additionally, we found a distinct set of merged TADs in pro-B cells, which were generated by coalescence of contiguous TADs present in pre-pro-B cells. This may account for the presence of fewer TADs with an increase in average TAD size and the corresponding
Figure 8. Ebf1 regulates B lineage-specific gene expression pattern in part by binding at cis-regulatory interacting elements. (A) Venn diagram representing the motifs of the TFs (Ebf1 and Pax5) binding at the promoters and respective cis-regulatory elements. (B) Box plot representing genome-wide comparative analysis of transcript levels of genes with or without Ebf1/Pax5 binding sites in the promoter-cis-regulatory interacting elements (**P < 0.001). (C) Heat maps showing correlation between transcript levels of genes and Ebf1 and/or Pax5 binding events in the promoter-cis-regulatory interacting elements. (D) Heat maps showing the genome-wide expression patterns of B lineage-specific genes (fold change ≥2; P-value < 0.05) obtained by microarray analysis of pre-pro-B cells (Ebf1−/− progenitors) transduced with Ebf1 or Pax5. (E) Venn diagram indicating the number of genes that are regulated by Ebf1 and/or Pax5. Up headed arrow represents activated, down headed arrow represents repressed genes. (F) Venn diagram representing the percentage of upregulated targets of Ebf1 and/or Pax5 that are involved in cis-regulatory interactions in pre-B cells. (G) Venn diagrams representing the percentage of Ebf1 or Pax5 target genes containing Ebf1 and/or Pax5 binding sites within the cis-regulatory sequences that are involved in long-range interactions.
gain of promoter-cis-regulatory interactions in pro-B cells as compared to pre-pro-B cells. We propose that the merging of TADs is due to an increase of inter-TAD interactions that are associated with epigenetic modifications and regulation of lineage-specific transcription factors. However, the precise molecular mechanisms that regulate the merging of TADs remains to be elucidated. A recent study (67), in drosophila cells demonstrates a dramatic increase in inter-TAD promoter-cis-regulatory interactions upon heat shock treatment, which was shown to be associated with a corresponding redistribution of architectural proteins from borders to inside of TADs. Moreover, we show that genes that are present in a given TAD exhibit higher correlation of expression compared to genes present in other TADs, supporting the notion that TADs provide a structural framework for coordinated gene regulation (12). Thus the studies presented here, provide new insights into the structural organization of TADs and their propensity to undergo alterations during developmental progression.

In this study, we provide a comprehensive map of long-range interactions between promoters and their corresponding cis-regulatory elements in pre-pro-B and pro-B cells. We found a significant expansion in promoter-cis-regulatory interaction landscape during the developmental transition from pre-pro-B to pro-B stage is evidenced by not only an increase in a total number of promoter-enhancer interactions, but also by the average increase in the ratio of promoters to enhancers. Additionally, we show that the transcript levels are positively correlated with the number of cis-regulatory interactions in both cell types, indicating that enhancer usage dictates transcriptional output. These studies corroborate the observations that modulation of gene activity is regulated by the enhancer landscape (48,68). Furthermore, our studies reveal that a significant number of promoter and enhancer elements that are engaged in long-range interactions contain Ebf1 and/or Pax5 binding sites. Many of these cis-regulatory elements are important for expression of developmentally regulated genes during B cell fate commitment. Accordingly, Ebf1 targeted genes displayed high levels of gene expression in pro-B cells. These results are further strengthened by the fact that, a subset of Ebf1 targeted genes, activated upon complementation of Ebf1−/− progenitors with Ebf1 or Pax5 were found to be involved in long-range interactions. Our analyses revealed novel molecular functions of Ebf1 and its potential role in the establishment of cis-regulatory interactions and activation of B lineage-specific genes. Nevertheless, the molecular mechanisms by which Ebf1 regulates these interactions remains to be understood. Binding of Ebf1 has been shown to recruit chromatin remodeling complexes like SWI-SNF to the Cd79a promoter thereby increasing local chromatin accessibility for subsequent activation (69). Similar mechanisms may also operate for the establishment of long-range interactions, wherein binding of key lineage-determining transcription factors like Ebf1 to cell type-specific enhancers recruits a distinct combinatorial set of factors, thereby positioning the enhancers in close proximity to their target promoters. This raises the exciting possibility that Ebf1 may mediate lineage-specific long-range interactions crucial for B lineage gene expression program. Collectively, our studies demonstrate that dynamic alterations of chromatin organization associated with changes in cis-regulatory interactions that are regulated by lineage determinants impinge on the induction of lineage-specific gene expression. As our understanding of the detailed molecular mechanisms that govern the dynamics of higher-order chromatin organization continues to expand, the relationship between the 3D organization of the genome and lineage-specific gene expression will be better understood.

**ACCESSION NUMBERS**

*In situ* Hi-C and microarray data were deposited in the Gene Expression Omnibus database under accession number GSE85858.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

**ACKNOWLEDGEMENTS**

We thank C-CAMP, Bangalore, for providing technical assistance with Illumina sequencing. High-performance computational analyses were carried out at the Centre for Modelling Simulation and Design (CMSD), University of Hyderabad and Centre for DNA Fingerprinting and Diagnostics (CDFD), Hyderabad.

**FUNDING**

Department of Biotechnology (DBT) [BT/PR13507/BRB/10/763/2010, BT/PR1825/AGR/36/679/2011]; Department of Science and Technology (DST) [SR/SO/BB-0071/2013]. R.B., A.D.Y., and S.N. are recipients of CSIR/UGC fellowships; D.P. is a DBT India Alliance fellow. Funding for open access charge: University of Hyderabad.

Conflict of interest statement. None declared.

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