EZH2-dependent chromatin looping controls \textit{INK4a} and \textit{INK4b}, but not \textit{ARF}, during human progenitor cell differentiation and cellular senescence

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Abstract

\textbf{Background:} The \textit{INK4b-ARF-INK4a} tumour suppressor locus controls the balance between progenitor cell renewal and cancer. In this study, we investigated how higher-order chromatin structure modulates differential expression of the human \textit{INK4b-ARF-INK4a} locus during progenitor cell differentiation, cellular ageing and senescence of cancer cells.

\textbf{Results:} We found that \textit{INK4b} and \textit{INK4a}, but not \textit{ARF}, are upregulated following the differentiation of haematopoietic progenitor cells, in ageing fibroblasts and in senescing malignant rhabdoid tumour cells. To investigate the underlying molecular mechanism we analysed binding of polycomb group (PcG) repressive complexes (PRCs) and the spatial organization of the \textit{INK4b-ARF-INK4a} locus. In agreement with differential derepression, PcG protein binding across the locus is discontinuous. As we described earlier, PcG repressors bind the \textit{INK4a} promoter, but not \textit{ARF}. Here, we identified a second peak of PcG binding that is located \textasciitilde 3 kb upstream of the \textit{INK4b} promoter. During progenitor cell differentiation and ageing, PcG silencer EZH2 attenuates, causing loss of PRC binding and transcriptional activation of \textit{INK4b} and \textit{INK4a}. The expression pattern of the locus is reflected by its organization in space. In the repressed state, the PRC-binding regions are in close proximity, while the intervening chromatin harbouring \textit{ARF} loops out. Down regulation of EZH2 causes release of the \textasciitilde 35 kb repressive chromatin loop and induction of both \textit{INK4a} and \textit{INK4b}, whereas \textit{ARF} expression remains unaltered.

\textbf{Conclusion:} PcG silencers bind and coordinately regulate \textit{INK4b} and \textit{INK4a}, but not \textit{ARF}, during a variety of physiological processes. Developmentally regulated EZH2 levels are one of the factors that can determine the higher order chromatin structure and expression pattern of the \textit{INK4b-ARF-INK4a} locus, coupling human progenitor cell differentiation to proliferation control. Our results revealed a chromatin looping mechanism of long-range control and argue against models involving homogeneous spreading of PcG silencers across the \textit{INK4b-ARF-INK4a} locus.
Background

Development and homeostasis require the coordinate regulation of cell proliferation and differentiation. The INK4b-ARF-INK4a tumor suppressor locus (Figure 1A) plays a central role in controlling the equilibrium between progenitor cell renewal and cancer risk [1-8]. This locus encodes three cell cycle inhibitory proteins: p15INK4b, p14ARF and p16INK4a [3,8]. p15INK4b and p16INK4a are closely related proteins and both act on the Rb-pathway through the inhibition of the proliferation-promoting cyclin-dependent kinases CDK4 and CDK6. p14ARF is structurally and functionally unrelated to p15INK4b or p16INK4a and works primarily through activation of the p53 pathway. A large number of studies have suggested a role for the INK4b-ARF-INK4a locus in cancer suppression and promotion of ageing. p16INK4a, in particular, has been implicated in balancing the need for tissue renewal and the risk of tumourigenesis [1-8]. Perhaps not surprisingly, the regulation of the INK4b-ARF-INK4a locus is highly complex. INK4b-ARF-INK4a expression is controlled by

![Figure 1](http://www.epigeneticsandchromatin.com/content/2/1/16)

**Figure 1**

Expression of the INK4b-ARF-INK4a locus during cellular ageing and differentiation. (A) Organization of the human INK4b-ARF-INK4a locus (not drawn to scale), encoding three distinct proteins, p15INK4b, p14ARF and p16INK4a. The untranslated regions (yellow boxes), the coding sequences of p15INK4b (green), p14ARF (blue) and p16INK4a (red) are indicated. (B) INK4b and INK4a, but not ARF, are upregulated in ageing human diploid fibroblasts (HDFs). RT-qPCR analysis of INK4b-ARF-INK4a expression in neonatal (yellow bar) versus adult (blue bar) HDFs. Bar graphs represent the mean of three independent biological replicate experiments, each analyzed in triplicate by RT-qPCR. mRNA levels are expressed relative to Gapdh. Error bars represent standard error of the mean. (C) INK4b and INK4a are selectively upregulated in ageing human embryonic fibroblasts (HEFs). Comparison of INK4b-ARF-INK4a expression in HEF cells (TIG3) at a low passage doubling (PDL 26, yellow) and high PDL (PDL 64, blue). (D) Flow cytometrical analysis of umbilical cord blood cells showing forward scatter (FSC) on the x-axes and CD34 staining on the y-axes. The immature CD34+ cells (blue) and mature CD34- cells (red) were sorted (left hand panel). Isolated CD34+ cells were reanalysed (middle panel; red dots represent 65% of the population). Following 6 weeks of culture these cells stained negative for CD34 (less than ~15%; right hand panel). (E) INK4b-ARF-INK4a expression in CD34+ (yellow) cells, CD34- (red) cells, and the progeny of seeded CD34+ cells after 6 weeks of culture (blue). (F) Erythroblasts (EB) kept under proliferation conditions for 2 days following induction of differentiation towards erythrocytes. Cells were cytocentrifuged onto glass slides and stained for haemoglobin (brown colour) and with standard cytologic dyes. (G) Expression of INK4b-ARF-INK4a in proliferating (yellow) erythroblast cells and cells differentiating towards erythrocytes (blue).
various signal transduction pathways and patterns of expression vary depending on physiological circumstances. Coordinated regulation of the whole locus, as well as differential gene expression, has been described [3]. Unfortunately, regulation of INK4b has received significantly less attention than that of ARF and INK4a.

The polycomb group (PcG) silencers form an important class of transcriptional corepressors that control the expression of the INK4b-ARF-INK4a locus [9]. This was first suggested by the finding that the Pcg protein, BMI1, promotes oncogenesis in mice through the silencing of INK4a [10]. Since then, other PcG proteins have also been implicated in the silencing of the INK4b-ARF-INK4a locus, including the histone H3 lysine 27 (H3K27) methyltransferase EZH2 [9-22]. However, in contrast to INK4a and ARF, the role of PcG proteins in INK4b expression control has not been extensively studied.

PcG proteins function as part of larger multi-protein assemblages, referred to as polycomb repressive complexes (PRCs) [23-25]. One major class is formed by PRC1-like complexes, which include assemblages harbouring BMI1. PRC1 class complexes have been implicated in chromatin compaction and histone H2A ubiquitylation. The second major class is formed by PRC2-like complexes, harbouring histone H3K27 methylases such as EZH2. However, it is important to stress that there is likely to be a great variety of PRCs and additional enzymatic activities. For example, PRC1 subunits are also part of alternate assemblages such as Drosophila dRAF [26] and its mammalian relatives [27,28]. The dRAF complex, harbouring the BMI1 homolog PSC, dRING and the demethylase dKDM2, ubiquitylates histone H2A and demethylates histone H3K36 during gene silencing [26]. Very recently, an essential role for N-acetylglucosaminilation in PcG repression was established [29]. Importantly, PRCs have also been implicated in the higher-order chromatin organization through looping [30,31].

Although the developmental roles of many PcG proteins await further analysis, recent research has emphasized their importance in dynamic gene control during the differentiation of precursor cells, in cancer and cellular senescence [9-22,32-36]. In particular, several of these studies have shown the importance of EZH2 for the dynamic regulation of gene silencing, orchestrating the differentiation of progenitor cells.

Here, we have addressed the role of PcG silencers in the regulation of the human INK4b-ARF-INK4a locus during the differentiation of progenitor cells, cellular ageing and cellular senescence of cancer cells. During these diverse physiological processes INK4b and INK4a were coordinately induced, whereas ARF remained unaltered. In order to investigate the underlying mechanisms, we analysed the spatial organization of the INK4b-ARF-INK4a locus. Our results revealed long-range control through chromatin looping rather than ‘blanket’ spreading of PcG proteins across the whole INK4b-ARF-INK4a locus. We conclude that PcG proteins control the higher-order chromatin conformation of the INK4b-ARF-INK4a locus, providing a molecular mechanism for coupling cell differentiation to proliferation control.

Results
Selective induction of INK4b and INK4a during cellular ageing, differentiation and senescence
We compared the expression of the human INK4b-ARF-INK4a locus in neonatal and adult human diploid fibroblasts (HDFs). The proliferation rate of adult HDFs is ~2-3 times slower than that of neonatal HDFs. In order to monitor INK4b-ARF-INK4a expression, we extracted RNA, which was followed by reverse transcription and real-time quantitative polymerase chain reaction (RT-qPCR) with gene-selective primers (Figure 1B). Compared to neonatal cells (yellow), the expression of INK4a and INK4b, but not of ARF, in adult HDFs (blue) is significantly higher. We also compared human embryonic fibroblasts (HEFs) with a low passage number (PDL 26) with old cells (PDL 64).

We observed a gradual, up to two- to threefold, increase in doubling time as the passage number became higher. INK4a and INK4b are clearly upregulated in HEFs with a high passage number, whereas ARF expression remained unchanged (Figure 1C). We conclude that, in these untransformed human diploid cells, INK4a and INK4b, but not ARF, are coordinately upregulated during ageing.

Next, we examined the expression of the INK4b-ARF-INK4a locus in human cells with a broad versus restricted potential for differentiation. We sorted CD34+ and CD34- cells isolated from human umbilical cord blood (UCB; Figure 1D). CD34+ UCB cells comprise quiescent stem cells, but they mainly represent the transiently amplifying compartment of multipotent and early myeloid progenitors [37]. In contrast, the CD34- fraction contains committed cells in late stages of differentiation. A fluorescence activated cell sorting analysis of the isolated CD34+ cells revealed a purity of about 65% (Figure 1D, middle panel). We cultured the purified CD34+ cells for 6 weeks, after which the majority of cells (~85%) matured to CD34- cells, representing mainly postreplicative erythroblasts and granulocytes. An expression analysis of CD34+ (yellow) and CD34- cells (red) immediately following isolation, and CD34+ derived cells cultured for 6 weeks (blue), demonstrated up-regulation of INK4b and very strong INK4a induction during differentiation (Figure 1E). In contrast, ARF expression remained unaltered.
As a model for senescence in cancer cells, we used MON human malignant rhabdoid tumour (MRT) cells. MRTs are caused by the loss of the hSNF5 subunit of the SWI/SNF chromatin remodelling complex [38]. Re-expression of hSNF5 in MRT cells restores SWI/SNF recruitment to INK4b and INK4a, causing the eviction of PRCs and a loss of silencing [20]. Consequently, these cells first undergo a G1/S cell cycle arrest and later become senescent [20,39]. hSNF5-induced cellular senescence of MRT cells is p16\(^{INK4a}\)-dependent [20,39]. We suspect that the hSNF5-mediated senescence of MRT cells might be due to the inability of oncogenic stress signalling to activate INK4a expression in the absence of hSNF5. In summary, we identified a number of diverse physiological processes in which human INK4a and INK4b are coordinately induced, while ARF expression remained unaltered.

**EZH2 is down-regulated during progenitor cell differentiation**

Because the PcG silencers EZH2 and BMI1 play important roles in the repression of the INK4b-ARF-INK4a locus, we investigated their expression in young versus adult HDFs and during cellular differentiation. RT-qPCR and Western immunoblotting revealed lower EZH2 levels in adult HDFs compared to the neonatal cells. In contrast, the BMI1 levels were comparable (Figure 2A). When we compared proliferating EBs with differentiating cells we again observed a reduction of EZH2 levels, but not of BMI1 (Figure 2B). Likewise, UCB CD34\(^+\) progenitor cells expressed much higher levels of EZH2 than mature CD34\(^+\) cells (Figure 2C). Upon culture, CD34\(^+\) cells matured and, concomitantly, EZH2 expression was strongly attenuated, whereas BMI1 levels remained stable (Figure 2C). However, re-expression of hSNF5 in MRT cells mediates INK4a and INK4b induction without affecting EZH2 or BMI1 levels [20]. We conclude that, in differentiating haematopoietic progenitor cells, the expression of the PRC2 subunit EZH2 wanes. In contrast, levels of the PRC1 subunit BMI1 remained constant.

In order to determine the effects of EZH2 down-regulation on chromatin occupancy at the INK4b-ARF-INK4a locus, we used chromatin immunoprecipitations (ChiPs) monitored by qPCR. Comparing neonatal and adult HDFs revealed an increased recruitment of RNA polymerase II (RNA POLII) to INK4a and INK4b in adult cells, consistent with their enhanced transcription (Figure 2E).

Next, we established the pattern of EZH2 and BMI1 binding, and the relative level of histone H3K27 me3 (Figure 2F-H). PRC binding at the INK4a promoter is already well established. We performed a detailed analysis of EZH2 and BMI1 binding to the INK4b upstream region and used a selection of primers targeting INK4a and ARF we published earlier [20] to serve as a reference. In addition to the INK4a promoter region (primer sets K and L), we identified a second peak of PRC binding to an area ~3 kb upstream of the INK4b promoter (primer sets C and D). Outside these two domains, binding of EZH2 and BMI1 across the INK4b-ARF-INK4a locus was low. H3K27 me3 levels follow EZH2 binding but are spread over a larger area (Figure 2H). Although only EZH2 was down-regulated in adult HDFs, both EZH2 and BMI1 occupancy at INK4a and INK4b was strongly reduced in these cells (Figure 2F and 2G). This observation agrees well with earlier studies showing that EZH2 can facilitate binding of other PcG proteins [23-25,40]. Like EZH2 binding, H3K27 me3 levels were strongly reduced in adult HDFs compared to neonatal cells (Figure 2H). In senescing MRT cells a distinct mechanism operates. hSNF5 re-expression does not affect EZH2 expression (Figure 2D), but enables SWI/SNF recruitment to INK4a and INK4b, leading to PRC eviction [20].

**EZH2 is required for coordinate silencing of INK4a and INK4b**

Our results showed that attenuation of EZH2 is accompanied by a loss of PRC1 and PRC2-binding, recruitment of RNA POL II and induction of INK4a and INK4b. ARF expression remained unaffected and is not co-regulated with INK4a and INK4b in the human cells studied here. These observations suggest a critical role for EZH2 in the selective regulation of INK4a and INK4b expression, during ageing and differentiation. To test whether EZH2 is indeed required for the silencing of INK4a and INK4b, we used a shRNA strategy to attenuate its levels in neonatal HDFs. Cells were transduced with lentiviruses expressing either shRNAs targeting EZH2 mRNA (EZH2 KD) or a scrambled control. Three days following the transduction, EZH2 levels were effectively reduced in cells treated with the appropriate shRNA (Figure 3A). In contrast, BMI1 levels were unaffected. Loss of EZH2 caused a strong induction of INK4a and INK4b but not of ARF (Figure 3B). RNA POL II was selectively recruited to the INK4a and INK4b loci, as revealed by ChIP-qPCR (Figure 3C). Depletion of EZH2 leads to its expected disappearance from INK4a and INK4b, but also causes loss of BMI1 binding (Figure 3D and 3E). Similar results were obtained in MRT cells (Additional file 1, Figure S2). Taken together, these results suggest that EZH2 attenuation is sufficient for the dissociation of PcG silencers and induction of INK4a and INK4b.
**EZH2 attenuation during progenitor cell differentiation.** (A-D) PRC2 subunit EZH2, but not the PRC1 subunit BMI1, is down-regulated during cellular ageing and differentiation. The expression of EZH2 was analysed by RT-qPCR and Western immunoblotting in: (A) neonatal and adult human diploid fibroblasts (HDFs); (B) proliferating and differentiating erythroblasts, (C) immature CD34+ progenitors and mature CD34- cells isolated directly from umbilical cord blood and CD34+ cells that differentiated and lost CD34 expression following 6 weeks of culture; (D) malignant rhabdoid tumour cells that either lack or express hSNF5. For characterization of these cells see Figure 1. In parallel, BMI1 and histone H3 levels were determined. (E) Selective RNA POL II recruitment to \( \text{INK4a} \) and \( \text{INK4b} \), but not \( \text{ARF} \), in ageing HDFs. Chromatin immunoprecipitation (ChIP)-quantitative polymerase chain reaction (qPCR) analysis of RNA POL II binding to the \( \text{INK4b-ARF-INK4a} \) locus in neonatal (light blue) and adult (dark blue) HDFs. The following primer sets were used: H (\( \text{INK4b} \)), I (\( \text{ARF} \)) and L (\( \text{INK4a} \)). All ChIP data presented in this study are the result of at least three biological replicates. Background levels were determined using antibodies directed against GST. The abundance of specific DNA sequences in the immunoprecipitates was analysed by qPCR and corrected for the independently determined amplification curves for each primer set. ChIP signal levels for each region are presented as percentage of input chromatin. Bar graphs represent the mean of three independent experiments, each analysed in triplicate by qPCR. Error bars represent standard error of the mean. (F) PRCs bind \( \text{INK4a} \) and \( \text{INK4b} \) in neonatal but not in adult HDFs. Chromatin immunoprecipitation (ChIP)-quantitative polymerase chain reaction (qPCR) analysis of RNA POL II binding to the \( \text{INK4b-ARF-INK4a} \) locus in neonatal HDFs (light blue) and adult HDFs (dark blue). The following primer sets were used: H (\( \text{INK4b} \)), I (\( \text{ARF} \)) and L (\( \text{INK4a} \)). All ChIP data presented in this study are the result of at least three biological replicates. Background levels were determined using antibodies directed against GST. The abundance of specific DNA sequences in the immunoprecipitates was analysed by qPCR and corrected for the independently determined amplification curves for each primer set. ChIP signal levels for each region are presented as percentage of input chromatin. Bar graphs represent the mean of three independent experiments, each analysed in triplicate by qPCR. Error bars represent standard error of the mean. (G) Following waning of EZH2, BMI1 binding to \( \text{INK4a} \) and \( \text{INK4b} \) is reduced significantly. ChIP-qPCR analysis of BMI1 binding to the \( \text{INK4b-ARF-INK4a} \) locus in neonatal and adult HDFs. The positions of the amplified regions (A-M) of the \( \text{INK4b-ARF-INK4a} \) locus are indicated at the bottom. (H) ChIPs using antibodies directed against histone H3K27 me3 revealed increased H3K27 methylation at- and around the PRC binding sequences upstream of \( \text{INK4b} \) and at the \( \text{INK4a} \) promoter. H3K27 me3 ChIPs were normalized against histone H3.
A chromatin loop, linking the repressed INK4a and INK4b, is released during induced expression

We wondered whether, because of their coordinate regulation by PcG silencers, INK4a and INK4b might be in close physical proximity, in spite of their being over 35 kb apart. In order to investigate the three-dimensional conformation of the INK4b-ARF-INK4a locus, we used chromatin conformation capture (3C) technology in combination with qPCR [41,42]. We first compared the INK4b-ARF-INK4a locus higher-order chromatin structure in neonatal and adult HDFs (Figure 4A). Cells were cross-linked with formaldehyde, followed by chromatin isolation and restriction digestion with EcoRI. Our preliminary analysis yielded 10 suitable EcoRI fragments across almost 70 kb of DNA encompassing the INK4b-ARF-INK4a locus (see Methods). Samples were ligated under conditions that favour the union of DNA fragments that are physically connected and qPCR across junctions was used to determine the relative cross-linking frequency between restriction fragments. All 3C data presented here are the result of three independent biological replicate experiments. The ‘constant’ primer and the TaqMan probe (grey bar) were designed in the EcoRI fragment ~4 kb to 2 kb upstream of INK4b, harbouring the PcG-binding sequences. Plotting of the ligation frequencies to this ‘bait’ fragment revealed a clear peak at fragment 9 overlapping the INK4a promoter proximal region. These experiments were complimented by 3C analysis using a bait fragment near the INK4a promoter. Now, we observed a peak at fragment 2, encompassing the PcG-binding domain upstream of the INK4b promoter (Figure 4B). We conclude that, in neonatal HDFs, the repressed INK4b-ARF-INK4a locus has a looped structure. The PcG-bound regions upstream of INK4b and proximal to the INK4a promoter are close in nuclear space, whereas the ~35 kb of intervening DNA, including the ARF promoter, loops out.

In adult HDFs, the higher-order chromatin conformation of the INK4b-ARF-INK4a locus is dramatically different. 3C-qPCR analyses revealed a loss of long-range interaction between INK4a and INK4b, suggesting the locus adopted a linear conformation (Figure 4A-B). In order to study the effects of progenitor cell differentiation, we undertook a similar 3C analysis of the chromatin structure in proliferating and differentiating ERs (Figure 4C and 4D). Our results revealed the presence of a chromatin loop between INK4a and INK4b in proliferating cells, which is similar to that in neonatal HDFs. When ERs were induced to differentiate towards erythrocytes, the silent chromatin loop dissolved and INK4a and INK4b became de-repressed. Finally, we compared proliferating MRT cells with senescing cells after hSNF5 expression (Figure 4E and 4F). Following hSNF5 expression and PcR removal, the repressive chromatin loop is released and the INK4b-ARF-INK4a locus assumes a linear conformation.

Together, our results showed that, in neonatal HDFs, haematopoietic progenitor cells and MRT cancer cells, the repressed INK4b-ARF-INK4a locus assumes a looped conformation. The ~35 kb chromatin loop links the PcG-binding regions of INK4a and INK4b, whilst excluding ARF. Concomitant with the increased transcription of INK4a and INK4b in adult HDFs, following differentiation or senescence, the chromatin loop dissolves. The loss of looping is concomitant with the loss of PcG-binding, which, in HDFs and differentiating EBs, is caused by attenuation of EZH2. In MRT cells driven towards senescence, EZH2 levels remain stable, but the PcGs are removed by SWI/SNF action. This dually leads to a release of the repressive loop and gene activation, again suggesting that PcG-binding is required for looping.

PcG-binding is required for looping between INK4a and INK4b

To test whether EZH2 is crucial for loop formation, we transduced neonatal HDFs with lentiviruses expressing either a shRNA targeting EZH2 mRNA or a scrambled control. As shown above, depletion of EZH2 leads to a loss of PcG binding to INK4a and INK4b upstream regions (Figure 3). 3C-qPCR analyses revealed that a loss of EZH2 also results in the release of the repressive chromatin loop that links INK4a and INK4b (Figure 5A and 5B). As shown above, the restoration of SWI/SNF targeting in MRT cells provides an alternate mechanism of PcG removal and loop release (Figure 4E and 4F; Additional file 1, Figure S1). However, EZH2 depletion in the absence of hSNF5 expression, also led to loss of EZH2 and BMI1 binding (Additional file 1, Figure S2) and loss of looping (Figure 5C and 5D). Taken together, these results show that PcG binding is critical for chromatin looping at the INK4b-ARF-INK4a locus. When PcG binding is lost, due either to diminished EZH2 levels or because of SWI/SNF action in MRT cells, the repressive chromatin loop is released concomitant with INK4a and INK4b induction (Figure 5E).

Discussion

PcG proteins control the balance between differentiation and proliferation

Our results and those of others [18,34,36,40,43], emphasize a crucial role for EZH2 in orchestrating progenitor cell differentiation and proliferation control. Ezhkova et al. [18] observed derepression of INK4a and INK4b due to EZH2 down-regulation, controlling the balance between the proliferative basal layer of progenitor cells and non-proliferating differentiated epidermal cells. These results in the mouse epidermal lineage are highly reminiscent of our findings in human haematopoietic progenitor cells. The histone H3K36 demethylase JHDM1b/KDM2b, a mammalian homolog of the dRAF signature subunit dKDM2, binds and regulates INK4b [19]. Reminiscent of the results described here, He et al. [19] observed that a
knockdown of JHDM1b/KDM2b or RING1b in primary mouse embryonic fibroblasts causes induction of INK4b and INK4a but not of ARF or the p53-pathway. Consequently, these cells arrest and undergo senescence, for which p15INK4b are critical. In response to oncogenic RAS-signalling in human fibroblasts, the H3K27 me3 demethylase JMDJ3/KDM6B activates INK4a, but not ARF [11,13]. In mouse embryonic fibroblasts, JMJD3/KDM6B activates both INK4a and ARF, possibly reflecting a difference in INK4/ARF regulation between mice and man [3,44]. However, as mentioned above, other studies have also presented induction of INK4a and INK4b, but not ARF in mouse cells (see, for example, [18] and [19]). Here, we identified EZH2- and BM11-binding sequences upstream of the INK4b promoter, providing additional evidence for the control of INK4b by PcG repression. Finally, we emphasize that, under different physiological conditions, different combinations of INK4b-ARF-INK4a expression will be relevant. See, for example, the many examples of co-induction of ARF and INK4a [3,7,8]. Related to this point, it is easy to imagine that, in those situations, the INK4b-ARF-INK4a locus might assume a more distinct conformation than that described here.

Regulation of INK4a and INK4b by EZH2 might be a conserved mechanism required to balance progenitor cell proliferation and differentiation. Unfortunately, this coupling might have sinister consequences when control of EZH2 expression is lost [40]. EZH2 is over-expressed in a variety of tumours, potentially blocking the tumour suppression function of INK4b-ARF-INK4a. In these cells, EZH2 seems to promote de-differentiation and uncontrolled proliferation. Notably, the expression level of EZH2 in early murine haematopoietic cells correlates with their expansion potential. EZH2 overexpression in early...
haematopoietic progenitors led to a loss of their repopulating ability [43]. Together with early studies in Droso-
phila, these observations emphasize the importance of PcG protein regulation and dosage.

Following the hSNF5 expression in MRT cells, which triggers the cellular senescence programme, EZH2 levels do not change. In these cells, the SWI/SNF chromatin remodeler is crippled due to a loss of hSNF5 and, therefore, unable to evict PRCs from INK4a and INK4b regulatory elements [20]. Following hSNF5 expression, SWI/SNF is able to remove PRCs from INK4a and INK4b, causing the release of the repressive chromatin loop and gene expression. Thus, a loss of function of a remodeler that counteracts PcG silencing may have similar consequences as EZH2 over-expression. Together with the earlier identification of SWI/SNF as a suppressor of PcG mutations in flies [23,45], these findings emphasize the evolutionary conservation of this regulatory antagonism.

**Higher-order chromatin structure and gene expression control**

Several studies have implicated higher-order chromatin structure in the regulation of complex multi-gene loci [30,31,46-51]. Typically, models explaining gene control at a distance invoke either spreading of factors along a
chromatin fibre or long-range protein-protein interactions that cause looping-out of the intervening chromatin.

Two earlier studies proposed a continuous spreading across the whole INK4b-ARF-INK4a locus of either heterochromatinization [52] or PcG proteins [15]. However, the ChIP data in the latter study actually showed a clear peak of PRC binding at the INK4a promoter, which tapers off and is near background at the ARF gene. This pattern of binding at INK4a is reminiscent of what we observed [20]. For the cell types and physiological processes studied here, we favour a discontinuous looping mechanism of INK4b-ARF-INK4a locus control over models invoking blanket spreading of silencers. First, there are two distinct peaks of PRC binding: first, ~3 kb upstream of the INK4b promoter and, second, at the INK4a promoter. Outside these domains, PRC binding is near background levels and we detected no significant PRC-binding at ARF. We note that the PRC-binding peak upstream of INK4b, albeit that it is nearby, does not coincide with the RD regulatory region identified by Gonzalez et al. [52]. In addition,
INK4a and INK4b are coordinately derepressed during cell differentiation, ageing and senescence, whereas ARF remained unaffected. Finally, the PRC-bound INK4a and INK4b regulatory regions are linked in nuclear space. Loss of PRC-binding causes the release of chromatin looping and induction of INK4a and INK4b.

Our findings for the INK4b-ARF-INK4a locus dove-tail well with other studies revealing PcG-mediated chromatin looping [30,31]. Chromatin looping is a consequence of the association of proteins bound to two or more distal regulatory elements (see, for example, [50,51,53-55] and references therein). When two genes are brought into the same microenvironment, such as the PRC bound INK4a and INK4b, it facilitates coordinated regulation. The looping-out of an intervening gene, such as ARF, provides a physical separation which allows independent regulation. Thus, in our view, the looped-out chromatin per se is neither active nor repressive. Rather, transcriptional consequences are determined locally, by repressors or activators that can alter the local chromatin status. Here, we presented an example where the higher-order chromatin conformation of a human multi-gene locus reflects its differential pattern of gene expression during diverse physiological processes.

Conclusion
Two classic problems in the study of transcription are the mechanism of long-range gene control and the regulation of multi-gene loci. Here, we studied how PcG silencers control expression of the human INK4b-ARF-INK4a tumour suppressor locus. We concentrated on a variety of physiological processes during which INK4a and INK4b are coordinately upregulated, whereas ARF expression remains unaltered. In agreement with differential regulation, our ChIP analysis revealed non-homogeneous PRC binding to the INK4b-ARF-INK4a locus. In addition to the INK4a promoter, we identified a PRC-binding sequence ~3 kb upstream of the INK4b promoter. PRC binding to these regions mediates the formation of a ~35 kb chromatin loop, linking INK4b and INK4a but excluding ARF. EZH2 attenuation causes the release of the repressive loop and upregulation of INK4a and INK4b. Thus, EZH2 levels determine the higher-order chromatin structure and expression pattern of the INK4b-ARF-INK4a locus, coupling human progenitor cell differentiation to proliferation control. Our findings revealed a looping mechanism of INK4b and INK4a control, but are difficult to reconcile with models invoking the continuous spreading of PcG silencers.

Methods
Cell isolation, cell culture and lentiviral procedures
UCB was collected in 10 ml Hanks+Hepes (H+H) with 1% heparin by nursing staff of the Department of Obstet-
infrared Imageing System according to the supplier's instructions. In Vivo detection kit (PIERCE) or visual antibodies. Primary antibodies: SUZ12 (Abcam, ab12073), BMI1 (Abcam; ab14389), EZH2 (Santa Cruz; Sc-25383), POL II (Santa Cruz; Sc-899), Histone H3 (Abcam; ab1791) and H3-K27 me3 (Upstate; 07-449). The abundance of specific DNA sequences in the immunoprecipitates was determined by qPCR and corrected for the independently determined amplification curves of each primer set. ChIPs using species and isotype-matched immunoglobulins directed against an unrelated protein (GST) were used to determine background levels analysed by qPCR as described above. Enrichment of specific DNA sequences was calculated using the comparative C\textsubscript{T} method [60]. ChIP levels for each region are presented as percentage of input chromatin. H3K27 me3 ChIPs were normalized against histone H3. All data presented are the result of at least three biological replicate experiments. PCR primer sequences are provided in Additional file 1, Table S1. Statistical analysis was performed using R software http://www.r-project.org/.

Cell extracts and western blotting

Cell extracts were prepared in RIPA buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% NaDOC, 0.1% SDS) and protein concentration determined. ~50 μg of extract was resolved in SDS-PAGE and transferred to 0.45 μm nitrocellulose membrane. Haematopoietic progenitor cells were lysed directly in an equal volume of 2× SDS loading buffer (0.1× Tris.Cl pH6.8, 20% glycerol, 4% SDS, 0.2 M DTT, 0.001% bromophenol blue). After transfer, membranes were blocked for 1 h in T-PBS with 5% milk and 0.1% Tween20 prior to incubation with primary (overnight) and secondary (1 h) antibodies. Primary antibodies: SUZ12 (Abcam, ab12073), BMI1 (Abcam; ab14389), EZH2 (Santa Cruz; Sc-25383) and Histone H3 (Abcam; ab1791). Western blots were developed with the ECL detection kit (PIERCE) or visualized with the IRDye 680/800 CW (LI-COR) and ODYSSEY Infrared Imaging System according to the supplier's instructions.

RT-qPCR and ChIP-qPCR assays

Total RNA was extracted from cells using the SV Total RNA Isolation System (Promega, WI, USA). cDNA was synthesized from 1 μg of total RNA using random hexamers and Superscript II RNase H-Reverse Transcriptase (Invitrogen). Quantitative real-time PCR (MyIQ, Bio Rad) was performed using SYBR Green I. PCR primers were designed using Beacon designer (Premier Biosoft). The qPCR Core Kit (Invitrogen) was used with 400 nM of each primer under the following cycling conditions: 3 min. at 95°C followed by 40 cycles of 10 s at 95°C and 45 s at 60°C. Gapdh was used as an endogenous reference for normalization. Enrichment of specific sequences was calculated using the comparative C\textsubscript{T} method [60]. ChIPs were performed essentially as described by the Upstate protocol http://www.upstate.com. Cross-linked chromatin was prepared from ~2 x 10\textsuperscript{7} cells. Cells were treated with 1% formaldehyde for 20 min at room temperature. Chromatin isolation, sonication yielding fragments of 300-600 bp and immunoprecipitations were performed according to protocol. The following antibodies were used: BM11 (Abcam; ab14389), EZH2 (Santa Cruz; Sc-25383), POL II (Santa Cruz; Sc-899), Histone H3 (Abcam; ab1791) and H3-K27 me3 (Upstate; 07-449). The abundance of specific DNA sequences in the immunoprecipitates was determined by qPCR and corrected for the independently determined amplification curves of each primer set. ChIPs using species and isotype-matched immunoglobulins directed against an unrelated protein (GST) were used to determine background levels analysed by qPCR as described above. Enrichment of specific DNA sequences was calculated using the comparative C\textsubscript{T} method [60]. ChIP levels for each region are presented as percentage of input chromatin. H3K27 me3 ChIPs were normalized against histone H3. All data presented are the result of at least three biological replicate experiments. PCR primer sequences are provided in Additional file 1, Table S1. Statistical analysis was performed using R software http://www.r-project.org/.

Chromatin conformation capture assay

The 3C-qPCR assay was performed as described [61]. Formaldehyde-fixed nuclei, prepared from ~10\textsuperscript{7} cells, were digested with EcoRI overnight, followed by ligation with T4 DNA ligase at 16°C for 4 h. Cross-links were reversed and DNA was purified. The PCR control template, primer efficiency and ligation efficiency were determined by digesting and ligating a BAC clone, which encompassed the entire \textit{INK4b-ARF-INK4a} locus, as previously described [61]. To correct for differences in the quality and quantity of templates, ligation frequencies between the fragments were normalized to a fragment in the human \textit{Ercc3} locus. Sample purity and digestion efficiency has been carefully assessed as described [61]. Quantification of ligated products was performed by real-time qPCR with a Taqman probe corresponding to a sequence within a DNA fragment overlapping the PRC-binding region of either \textit{INK4b} or \textit{INK4a}. Our analysis yielded 10 suitable EcoRI fragments covering the \textit{INK4b-ARF-INK4a} locus. The primers and probe sequences are listed in Additional file 1, Table S2. The amplification conditions used in 3C assays are available on request. Cross-linking frequencies were calculated as previously described [51,61].

Abbreviations

ChIP: chromatin immunoprecipitation; EB: erythroblast; HDF: human diploid fibroblast; HEF: human embryonic fibroblasts; MRT: malignant rhabdoid tumour; PcG: polycomb group; PCR: polymerase chain reaction; PRC: PcG repressive complex; RT-qPCR: real-time quantitative PCR; SCF: stem cell factor; UCB: umbilical cord blood; 3C: chromatin conformation capture; H3K27: H3 lysine 27.

Competing interests

The authors declare that they have no competing interests.
Authors' contributions
SKK and CPV conceived and designed the study and wrote the manuscript. SKK and PSK performed most of the experiments. EF, FP and MvL participated in the experiments involving haematopoietic cells and the writing of the parts describing them. All authors have read and approved the manuscript.

Additional material

Additional file 1
Supplementary tables and figures.
Click here for file [http://www.biomedcentral.com/content-supplementary/1756-8935-2-16-S1.PDF]

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