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Role for the MOV10 RNA helicase in Polycomb-mediated repression of the *INK4a* tumor suppressor

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Abstract

Several lines of evidence point to a role for non-coding RNA in transcriptional repression by Polycomb group (PcG) proteins but the precise mechanism remains unclear. Here we show that MOV10, a putative RNA helicase previously implicated in post-transcriptional gene silencing, co-purifies and interacts with components of Polycomb-repressive complex 1 (PRC1) from human cells. Endogenous MOV10 is mostly nuclear and a proportion associates with chromatin in an RNA-dependent manner. ShRNA-mediated knockdown of MOV10 in human fibroblasts leads to up-regulation of the *INK4a* tumor suppressor, a known target of PcG-mediated repression, accompanied by dissociation of PRC1 proteins from the locus and a reduction in trimethylation of histone H3 on lysine 27 (H3K27me3). As well as prompting reassessment of MOV10's role in other settings, our findings suggest that it is directly involved in transcriptional silencing by PcG complexes.

The Polycomb group (PcG) genes were initially identified in *Drosophila* as regulators of gene expression patterns during development [1]. The encoded proteins participate in two types of multimeric complex, termed Polycomb repressive complexes 1 (PRC1) and 2 (PRC2), that inhibit transcription by specifically modifying nucleosomal histones [2],[3]. The PRC2 complex, which comprises E(z), Su(z)12 and Esc, is responsible for trimethylation of histone H3 on lysine 27 (H3K27me3). This mark is recognized by the PRC1 complex which catalyzes monoubiquitination of histone H2A on lysine 119 (H2AK119ub). As the four proteins that form the core of the *Drosophila* PRC1 complex, Polycomb (Pc), Posterior sex combs (Psc), Polyhomeotic (Ph) and Sex combs extra (Sce) each have several orthologs in mammals [4],[5], there can be multiple permutations of the PRC1 complex within a single cell [6]. The reasons for this expansion remain unclear, as does the mechanism for establishing locus specificity. In *Drosophila*, PcG proteins are recruited to target loci by specific DNA

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AUTHOR CONTRIBUTIONS

S.elM.-A. performed most of the experiments described in the manuscript and drafted the figures and text. J.N. characterized the mCbx7 complex and together with E.B. provided the initial evidence for the interaction with MOV10. G.N.M. assisted with the gel filtration analyses and S.B. performed the immunofluorescence. G.P. directed the project and prepared the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

PREPARATION OF FIGURES

ECL images were scanned using Adobe Photoshop and imported into Microsoft Powerpoint for construction of figures. PCR data were compiled in Microsoft Excel, imported into Powerpoint and ungrouped for editing purposes. The Powerpoint figures were then saved as TIFF files. All the manipulations comply with NPG policy concerning image integrity.

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sequence elements, termed PREs [2,7] but it has proved difficult to find equivalent elements in mammalian cells [8].

One possibility, for which there is considerable support, is that PcG complexes are recruited to specific regions of chromatin by non-coding RNAs (ncRNAs) [9,10]. Examples include the silencing of imprinted genes by ncRNAs, such as Xist/RPA and Kncnq, which are responsible for recruitment of PcG complexes and their trademark modifications to the silenced allele [11,12]. On a more global scale, it was recently shown that at least 20% of the several thousand "long" ncRNAs expressed in mammalian cells may be bound by PRC2 proteins [13,14]. Moreover, down-regulation of specific ncRNAs can affect the expression of PRC2 target genes.

An implication of these recent findings is that long ncRNAs, such as HOTAIR and TUG1, can act in *trans* to control distant genes [13,14]. This contrasts with the situation in the *Drosophila* bithorax complex where intergenic transcription through PRE/TRE elements is viewed as having *cis*-acting effects on adjacent genes or as contributing to long range effects on higher order chromatin structure [15-18]. There are also a number of experimental systems in which siRNAs directed against promoters or transcriptional start sites result in transcriptional repression linked to recruitment of PRC2 proteins and H3K27 methylation but the physiological relevance of these systems remains uncertain [19-21].

Several large scale studies aimed at identifying the physiological targets of PcG complexes and H3K27 methylation have pointed to a key role in the maintenance of pluripotency [22-25]. In addition to HOX genes and other transcription factors, one of the critical targets of PcG-mediated repression is the *CDKN2A* tumor suppressor locus. The locus, commonly referred to as *INK4a/ARF*, encodes two unrelated proteins, $p16^{INK4a}$ and $p14^{ARF}$, that can block cell proliferation by activating the retinoblastoma protein (pRb) and p53, respectively [5,26]. These proteins contribute to cell intrinsic defences against oncogenic insults or cellular stress that depending on context, engage either apoptosis or the state of permanent growth arrest referred to as senescence [27].

Compelling evidence for the importance of PcG proteins in the regulation of *INK4a/ARF* and stem cell self-renewal came from studies on *Bmi1* null mice [28]. These mice sustain hematological and neurological defects that are rescued by concomitant ablation of *Ink4a/Arf* (reviewed in refs. 5, 26). Knockouts of several other PcG genes also result in premature senescence due to derepression of *Ink4a/Arf,* while over-expression of Cbx7, Cbx8 and Bmi1 can extend cellular lifespan by repressing *Ink4a/Arf* [29-32].

In our efforts to delineate the PRC1 complexes that regulate *INK4a* in human cells, we affinity purified the Pc homolog Cbx7 and identified the co-purifying proteins by mass spectrometry. In addition to known PcG proteins, which have been described elsewhere [6], we found a number of non-PcG proteins, including the putative RNA helicase MOV10 (ref 33). MOV10 is the mammalian homolog of Armitage in *Drosophila* and SDE1 in *Arabidopsis* and has been implicated in post-transcriptional gene silencing in these systems [34-36]. It was also shown to co-purify with components of the mammalian RISC complex and to localize in P-bodies when over-expressed [37-39]. Here we show that in human cells, most of the endogenous MOV10 is present in the nucleus, with a substantial proportion bound to chromatin. It associates with CBX7 and other PRC1 components, but not with PRC2 proteins, and can be detected on the *INK4a* locus by chromatin immunoprecipitation (ChIP). Furthermore, shRNA-mediated knockdown of MOV10 results in up-regulation of $p16^{INK4a}$ accompanied by displacement of PcG proteins and the H3K27me3 mark. To our knowledge, this is the first example of an RNA helicase implicated in PcG-mediated gene silencing.

RESULTS

Association of MOV10 with CBX7

To identify proteins that associate with the Pc homolog CBX7, we constructed an expression vector for mouse Cbx7 carrying the classical TAP tag at its carboxy terminus [40]. When expressed in primary human fibroblasts, this construct was able to suppress endogenous $p16^{INK4a}$ expression and extend the replicative lifespan of the cells, confirming that the tagged protein is capable of functional interactions (Supplementary Fig. 1a,b). We then generated transiently and stably transfected populations of HEK293T cells expressing mCbx7-TAP. After tandem affinity purification, the complexes were fractionated by SDS-PAGE, stained with colloidal Coomassie and selected bands were identified by mass spectrometry (Supplementary Fig. 1c).

In addition to known PcG proteins, which we described elsewhere [6], the complexes contained several other proteins that consistently co-purify with PRC1 complexes, such as casein kinase 2 (CK2), WDR68 and others [30],[41]. Interestingly, a prominent band of around 110 kDa contained peptides from the putative RNA helicase MOV10 (ref. 33). DE(X)D box helicases are considered to be common contaminants of affinity purified complexes, in the so-called Sepharose bead proteome [42]. However, there are situations where RNA binding proteins and helicases are expected to be present in the purified complexes, as in previous sightings of MOV10 (refs 37, 38, 43). In the event, MOV10 was the only helicase that we detected in the mCbx7 complex and it was identified in independent preparations from transiently and stably transfected cells.

To assess whether MOV10 is a bona fide interacting protein, we performed a pull down assay using bacterially expressed GST-mCbx7 and in vitro translated MOV10. MOV10 bound efficiently to GST-mCbx7 but not to GST alone (Supplementary Fig. 1d). We also generated rabbit antisera against a MOV10-specific peptide and commissioned a phage display screen (HuCAL®, AbD Serotec) to isolate a recombinant human antibody against the same immunogenic peptide. The bivalent antibody incorporates Flag and His_6 epitopes that can be used in conjunction with Flag antibody-coated or nickel beads, respectively. Validation of the antibodies is described in Supplementary Figure 2. Importantly, these reagents enabled us to detect endogenous MOV10 in a wide variety of human cells (Supplementary Fig. 2d) and to confirm that endogenous MOV10 co-precipitated with ectopically expressed Flag-tagged CBX7 in 293T cells (Fig. 1a). Note that the rabbit antiserum detects a doublet, the upper band of which represents authentic MOV10 as judged by shRNA-mediated knockdown (Supplementary Fig. 2b) and peptide competition (Supplementary Fig. 2c). Using the recombinant antibody, we were further able to demonstrate co-precipitation of endogenous MOV10 and endogenous CBX7 in 293T cells (Fig. 1b).

As CBX7 has been shown to bind RNA [44] and MOV10 is a putative RNA helicase, we considered the possibility that the interaction between the two proteins might be RNA-dependent. Intrigued by finding a peptide signature of the RNA binding protein IMP1 (also known as CRD-BP or ZBP1, ref 45) in one of the preparations of the mCbx7 complex (Supplementary Fig. 1c), we included this protein as a control. As shown in Figure 1c, the association between Flag-tagged mCbx7 and endogenous MOV10 was unaffected by treatment of the lysate with RNAse A. In contrast, the association between Flag-tagged mCbx7 and endogenous IMP1 was completely ablated by treatment with RNase. Similarly, although endogenous MOV10 could be co-precipitated with Flag-tagged IMP1, this interaction was dependent on RNA.

Association of MOV10 with chromatin

Much of the existing literature on MOV10 views it as a cytoplasmic protein that associates with the RISC complex [37-39,46]. When ectopically expressed, epitope tagged MOV10 accumulates in P-bodies [38,39]. An interaction with PRC1 complexes would therefore seem incongruous unless a significant fraction of the cellular MOV10 is present in the nucleus or a significant fraction of CBX7 localized in the cytoplasm. A simple cell fractionation experiment revealed that in 293T cells the endogenous MOV10 is predominantly nuclear (Fig. 2a). TFIID and GAPDH were used as controls for nuclear and cytoplasmic proteins respectively. A similar fractionation experiment with ectopically expressed Flag-MOV10 showed a higher proportion in the cytoplasm (Fig. 2b) suggesting that over-expression might distort the intracellular localization.

As a more stringent test, we applied a commonly used fractionation protocol [47] to prepare cytoplasmic (S1), nucleoplasmic (S2 and S3) and chromatin bound (P3) proteins from either 293T cells or primary human fibroblasts. Under these conditions, most of the endogenous MOV10 was again detected in the nuclear fractions, including a substantial amount in the chromatin pellet (Fig. 2c and Supplementary Fig. 3a). In this regard, it showed a similar distribution to CBX7 and TFIID, albeit with a somewhat lower proportion in chromatin. Transducing the cells with either of two shRNAs against MOV10 reduced the total amounts of the protein and depleted the chromatin bound pool, confirming that the band detected on the immunoblots was authentic MOV10. These findings suggested that while MOV10 is indeed present in chromatin, it might be less tightly associated than, for example, CBX7 and TFIID. Extracting the purified nuclei with increasing salt concentrations confirmed this suspicion in that MOV10 was released from the chromatin pellet by 200 mM NaCl whereas CBX7 and TFIID were only released at higher salt concentrations (Fig. 2d). All three proteins were released by DNAse (not shown). Interestingly, treatment of chromatin with RNAse A released most of the MOV10 and a substantial proportion of the CBX7 into the soluble fraction whereas histone H3 was unaffected (Fig. 2e). In contrast, MOV10 was not released by treating the chromatin with RNase H whereas CBX7 was clearly mobilized. This suggests that the association of MOV10 with chromatin is more likely to involve single- or double-stranded RNA rather than the formation of an RNA-DNA hybrid.

We also identified a commercially available antibody (validated in Supplementary Fig. 2c) which was able to detect endogenous MOV10 by immunofluorescence. As illustrated in Figure 2f, the staining is predominantly but not exclusively nuclear, in line with the idea that MOV10 might have multiple functions within the cell. A similar distribution was observed in two independent strains of human fibroblasts, FDF and the $p16^{INK4a}$-deficient Leiden strain [48]. Thus far, we have been unable to visualize the low levels of endogenous CBX7 in these cells.

Interaction between MOV10 and other PRC1 components

We next asked whether MOV10 can associate with other Pc homologs or components of the PRC1 complex, focusing initially on proteins that have been shown to regulate the human *INK4a* locus [6,30]. For example, when Flag-tagged versions of human CBX7 and CBX8 were transiently expressed in 293T cells, endogenous MOV10 clearly co-precipitated with both proteins, using a Flag antibody (Fig. 3a). Similarly, HA-tagged MOV10 was shown to co-precipitate with Flag-tagged BMI1 and MEL18 when the proteins were transiently co-expressed (Fig. 3b). Importantly, using the recombinant antibody to precipitate the endogenous MOV10 in 293T cells, it was possible to demonstrate co-precipitation of endogenous MEL18, BMI1, CBX8, CBX7 and RING2, but there was no discernible interaction with TFIID (Fig. 3c). These interactions appeared to be insensitive to treatment with RNAse A (Supplementary Fig. 3b). In a side-by-side comparison of the five Pc

homologs, ectopically expressed using the same vector and epitope tag, it appeared that MOV10 bound preferentially to CBX7 and to a lesser extent to CBX6 and CBX8 (Supplementary Fig. 4).

The association of MOV10 with multiple PRC1 components suggested that it might represent an ancillary protein for the core PRC1 complex. To assess this possibility, nuclear extracts from either 293T cells or primary fibroblasts were subjected to size exclusion chromatography on a Superose 6 column. Individual fractions were analyzed by SDS-PAGE and immunoblotted with antibodies against MOV10 and a panel of PcG proteins. Authentic MOV10, represented by the upper band of the doublet, eluted in a wide range of size fractions including a significant proportion in the void volume (Supplementary Fig. 5a). The apparent "peak" at approximately 600 kDa corresponds to the background band and the distribution was not affected by pre-treatment with RNase (not shown). Surprisingly, the two Pc homologs, CBX8 and CBX7, showed distinctive elution profiles. We have recently shown that these proteins participate in at least four permutations of the PRC1 complex and that their association with the *INK4a* locus is interdependent [6].

Although there was no obvious concordance between the elution of MOV10 and its proposed partners, immunoprecipitation of selected fractions with the recombinant MOV10 antibody or beads alone confirmed an association with CBX7 and CBX8 (Supplementary Fig. 5b). CBX8 co-precipitated with MOV10 in a relatively broad size range (fractions 21 through 30) with a peak at around 800 kDa. In contrast, CBX7 co-precipitated with MOV10 from fractions 27-32, with a peak at approximately 500 kDa. A more detailed analysis of endogenous and purified complexes will be required to determine whether MOV10 associates with typical PRC1 core complexes or is part of a specialized sub-complex.

Involvement of MOV10 in the regulation of *INK4a*

As CBX7, CBX8, BMI1 and MEL18 are all implicated in the transcriptional repression of *INK4a* [6], it was important to determine whether MOV10 is also involved. Infection of primary HDFs with lentiviral vectors encoding MOV10 shRNAs caused a substantial reduction in MOV10 expression as demonstrated by immunoblotting for the endogenous protein. Importantly, both shRNAs also caused up-regulation of $p16^{INK4a}$, at the protein and RNA levels (Fig. 4a, b). The effects were comparable to those achieved by knockdown of CBX7 and equivalent results were obtained in different strains of HDF (Supplementary Fig. 6). There were no appreciable effects on *ARF* expression, in line with previous indications that PRC1 complexes have little if any influence on human *ARF* [6,49]. As noted for the knockdown of PRC1 components, the cells transduced with MOV10 shRNA acquired the characteristics of senescence (Fig. 4c). In contrast, when a similar experiment was performed in the Leiden strain of $p16^{INK4a}$-deficient fibroblasts [48], their proliferation was unaffected by knockdown of MOV10, using either sh2 or an independent shRNA (sh4) cloned in the pRetroSuper vector (Supplementary Fig. 7). These results suggest that the arrest is at least partly dependent on $p16^{INK4a}$. Conversely, as there was no discernible increase in either p53 or $p21^{CIP1}$ levels in the cells transduced with MOV10 shRNA (Fig. 4d) it seems unlikely that the arrest reflects the reported interplay between *Drosophila armitage* and the ATR/CHK2 DNA damage pathway [50].

To investigate whether the effects of MOV10 are direct or indirect, we determined conditions in which the recombinant antibody could be used for chromatin immunoprecipitation using the Flag epitope engineered into the antibody backbone. The precipitated DNA was interrogated with primer pairs specific for the *INK4a/ARF* locus, as previously described [6,49]. The signal obtained with the MOV10 antibody was significantly higher than the Flag only control, and specificity was confirmed by the fact that knockdown of MOV10 with shRNA reduced the ChIP signal with each primer set (Fig. 5a). Curiously,

the enrichment extended to primer set PS2 which represents the first exon of *ARF*. Most studies conclude that *ARF* is not subject to PcG-mediated repression in human fibroblasts and knockdown of MOV10 has no discernible effect on *ARF* expression (Fig. 4).

Importantly, knockdown of MOV10 reduced the recruitment of CBX7 and CBX8 to the endogenous *INK4a* locus. As shown in Figure 5b and c, both of the shRNAs against MOV10 caused a reduction in the ChIP signal for CBX7 and CBX8 on the *INK4a* locus, particularly with the primer sets PS7 and PS8. The effects were less dramatic with PS6 and negligible on the *ARF* promoter (PS2). Loss of Pc binding was accompanied by a marked reduction in H3K27me3 at the *INK4a* locus (Fig. 5d). Note, however, that MOV10 knockdown had no effect on the interaction between PRC1 components, as judged by the co-immunoprecipitation of RING2, CBX7 and CBX8 with BMI1 (Supplementary Fig. 8).

We next asked whether knockdown of MOV10 affected the ability of mCbx7 to repress *INK4a*. Primary fibroblasts (FDF cells) expressing a control shRNA or an shRNA against MOV10 were super-infected with retroviruses expressing either GFP or mCbx7. The presence of MOV10 shRNA resulted in significantly higher levels of *INK4a RNA*, as assessed by qRT-PCR (Fig. 5e). Notably, mCbx7 caused a reduction in *INK4a* RNA levels in the cells expressing the control shRNA but not in cells containing MOV10 shRNA.

Lack of interaction between MOV10 and PRC2 complexes

Most previous reports on the role of ncRNA in PcG-mediated repression focus on PRC2 complexes and the notion that the RNA contributes to the recruitment of PRC2 and the establishment of the H3K27me3 repressive mark. It was therefore pertinent to ask whether MOV10 was likely to contribute to these events by determining whether it associates with ectopically expressed PRC2 components. Adopting the same strategy as used for the PRC1 proteins, we transiently transfected 293T cells with Flag-tagged versions of EZH2, SUZ12 and EED, the core components of the human PRC2 complex, and asked whether endogenous MOV10 could be co-precipitated with the Flag antibody. The OCT3/4 transcription factor was used as a control for an unrelated, chromatin associated protein. Under conditions in which the association between mCbx7 and endogenous MOV10 was readily detected, we saw no evidence for co-precipitation of MOV10 with any of the PRC2 proteins (Fig. 6a). Immunoblotting for SUZ12 confirmed that the PRC2 proteins co-precipitate in this setting.

We also asked whether MOV10 might be functionally involved in PRC2-related events. It was recently reported that in primary human fibroblasts, a long ncRNA transcribed from the *HOXC* locus, designated HOTAIR, can act in *trans* to repress transcription of the *HOXD* locus, by recruitment of PRC2 complexes [13]. However, in cells in which shRNA-mediated knockdown of MOV10 caused up-regulation of *INK4a*, there was no discernible effect on three of the known HOTAIR target genes within the *HOXD* cluster (Fig. 6b).

DISCUSSION

As virtually all facets of RNA biology require the action of a helicase, the identification of MOV10 within a human Polycomb complex has important implications for the proposed role of RNA in PcG-mediated gene silencing. Importantly, we find that MOV10, a helicase previously implicated in post-transcriptional gene silencing, interacts with components of PRC1 but not PRC2 complexes. Among the Pc family, the strongest interaction was with CBX7 and CBX8. These are the Pc proteins that show the greatest affinity for RNA [44] but at least in the case of CBX7, the interaction with MOV10 appeared to be direct and resistant to RNAse treatment. We have not evaluated direct binding to other PRC1 components. Co-precipitation of MOV10 with MEL18, BMI1, and RING2 may therefore reflect participation

in high molecular weight complexes and the gel filtration analyses would be consistent with this idea. However, the broad elution profile of MOV10 suggests that it might participate in complexes other than PRC1 and at this point we do not know the stoichiometry of the association between MOV10 and PcG proteins.

We recently reported that multiple permutations of PRC1 complex purified from human cells each contain single representatives of the Pc, Psc, Ph and Sce families, in line with the *Drosophila* paradigm [6]. However, both the mammalian and *Drosophila* complexes appear to be considerably larger than predicted from these four subunits alone suggesting the presence of ancillary proteins. Indeed, there are several genetically defined *polycomb*-related genes in *Drosophila* that have yet to be functionally accommodated within the mammalian PcG arena. To our knowledge, these do not include an RNA helicase. On the contrary, the fly homolog of *MOV10*, *armitage,* was originally identified in a genetic screen for effects on the silencing and localization of *oskar* RNA. *Armitage* mutations cause a failure in the silencing of *stellate* by endogenous RNAi, in part because of impaired maturation of the RISC complex [34,36]. However, the influence of *armitage* mutations on embryonic patterning cannot be fully explained by RNA location and turnover and Armitage has also been implicated, along with other RNAi associated proteins, in repeat associated gene silencing [50,51].

The intracellular distribution of Armitage has not been fully explored but here we show that the majority of the endogenous MOV10 in human cells is nuclear, as judged by both cell fractionation and immunofluorescence, and a substantial proportion is bound to chromatin. This would agree with a recent report that found MOV10 associated with telomeric repeat sequences [52]. Our observations were made possible by the generation of antibodies that detect the endogenous protein rather than relying on ectopic expression of epitope-tagged protein. However, our results by no means exclude a role for MOV10 in RISC-associated events in the cytoplasm.

Interestingly, MOV10 has also been implicated in the replication of hepatitis delta virus, potentially by remodelling the incoming genomic RNA, but the intracellular venue for this process has not been directly addressed [46]. This system provides the best evidence thus far that MOV10 functions as an RNA:RNA helicase but it will be important to establish whether MOV10 operates as a multifunctional helicase in several aspects of gene regulation.

The broad distribution of MOV10 in gel filtration analyses argues against a unique association with PRC1 complexes. Similarly, although the ChIP data support a direct association of MOV10 with the *INK4a/ARF* locus, there was no obvious correlation with the localization of PRC1 complexes or H3K27me3. Nevertheless, shRNA-mediated knockdown of MOV10 resulted in up-regulation of *INK4a* at both the RNA and protein level, accompanied by displacement of PRC1 components and H3K27me3 from the promoter region and first exon of *INK4a.* To our knowledge, this is the first evidence for the involvement of an RNA helicase in PcG-mediated events and lends further support to the idea that ncRNAs are involved in targeting PRC complexes to specific loci.

Invoking an RNA component in the regulation of *INK4a/ARF* and the adjacent *INK4b* gene is an attractive proposition and could in part explain the unusual evolution of the locus [5]. However, the nature and origins of this RNA remain obscure. The complicated organization of the locus, with three promoters producing overlapping primary transcripts, provides opportunities for *cis* interference effects. In addition, antisense transcription has been detected in several settings, notably long range transcripts emanating from the nearby *MTAP* gene [53], the ANRIL transcript that is believed to originate from bidirectional transcription from the *ARF* promoter [54], and antisense transcription across the *INK4b*

promoter [55]. Moreover, complex mechanisms have been proposed for the binding of CDC6 and PcG complexes to a regulatory domain adjacent to *INK4b* that is dependent on antisense transcription from *MTAP* [56,57]. One attractive possibility would be that outlying mutations and polymorphisms in the chromosome 9p21 region that have been linked to chronic disease states and cancer predisposition impact on these long ncRNAs.

The tacit assumption in these studies is that the non-coding RNA or transcription per se is acting in *cis*. This would be similar to the effects of non-coding RNAs at imprinted loci and intergenic transcription in the *Drosophila bithorax* complex, but a different scenario has been proposed for the myriad of long ncRNAs identified in mammalian cells [13,14]. The few examples studied to date, such as HOTAIR and TUG1, appear to act in trans on distant target genes. If this applies to the regulation of *INK4b-ARF-INK4a*, identifying the relevant ncRNA presents a considerable challenge for the future. In virtually all of the precedents reported thus far, the ncRNA component is associated with the recruitment of the PRC2 complex rather than PRC1. Finding an RNA helicase involved specifically in PRC1-mediated events opens up new avenues for investigation as well as prompting re-evaluation of the functions of MOV10 homologs in other settings.

METHODS

Plasmids and shRNA

The full length cDNAs of human CBX2, CBX4, CBX6, CBX7 and CBX8 were subcloned into pcDNA6-based vectors (Invitrogen) in which the multiple cloning site was modified to introduce a C-terminal Flag or Flag/HA epitope (details available on request). IMP1 was PCR amplified, cloned into pcDNA3, sequence verified and transferred into the MCS of a modified pcDNA6 plasmid to introduce an N-terminal Flag tag. MOV10 coding sequences were transferred from a MYC-MOV10 plasmid [38] into a modified pcDNA6 MCS to incorporate an N-terminal HA tag.

The following lentiviral shRNAs were obtained from SIGMA: MOV10 shRNA1, NM_020963.1-1390s1c1; MOV10 shRNA2, NM_020903.1-2591s1c1; MOV10 shRNA3, NM_020903.1-191s1c1; CBX7 shRNA1, NM_175709.1-736s1c1; CBX7 shRNA2, NM_175709.1-153s1c1. An additional shRNA against MOV10 (shRNA #4), targeting nucleotides 2045-2054 of human MOV10, was constructed in the pRetroSuper vector.

Cell culture, viral transduction and transient transfections

HEK293T cells and primary human fibroblasts (Hs68, FDF and Leiden strains) were cultured in Dulbecco-modified Eagles medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 100 IU ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. Lentiviral and retroviral transductions were performed as previously described [6]. Transient transfections were performed with calcium phosphate using a total of 20 μg of plasmid DNA (10 μg of each construct or empty vector) and cells were harvested 36 h or 48 h later. In some experiments, HEK293T cells were transiently transfected with shRNA-encoding plasmid vectors (2 μg) using Effectene (Qiagen 301425). Transfection was repeated after 48 h and the cells were harvested 96 h after the first transfection.

Immunoprecipitation and immunoblotting

Cell extracts were prepared in lysis buffer containing 1% (v/v) NP40, 10 mM Tris.HCl pH7.5, 150 mM NaCl, 5 mM EDTA, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 10% v/v glycerol, 1 mM PMSF, and proteinase inhibitor (Roche) and clarified by high speed centrifugation. For Flag-tagged proteins, immunoprecipitation was performed as previously described [6]. Endogenous MOV10 was precipitated using 10 μg of the

recombinant His-Flag tagged anti-MOV10 antibody (AbD08355, Serotec) in combination with either Ni-NTA agarose (Qiagen) or with anti-Flag M2 coupled agarose (Sigma). The extracts were first pre-cleared with the agarose beads (Ni-NTA or FLAG-M2) and the precipitations were performed in the lysis buffer. When using Ni-NTA agarose beads, the lysis buffer was made with 0.5 mM EDTA instead of 5mM. Endogenous BMI1 was immunoprecipitated with 5 μg of anti-BMI1 antibody (clone F6, Millipore). Anti β-actin (A5441, Sigma) was used as a negative control.

Protein samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The following primary antibodies were used for immunoblottng: anti-MOV10 (Ab13, this paper or ab60132, Abcam), anti-p16 (JC8), anti-MEL18 (ab5267, Abcam), anti-BMI1 (05-637, Millipore), anti-RING2 (MAb provided by H. Koseki), anti-CBX7 (ab21873, Abcam), anti-CBX8 (Bethyl Laboratories), anti-GAPDH (ab9482-100, Abcam) and anti-TFIID (sc-204, Santa Cruz). The signals were detected by ECL (GE Healthcare) after incubation with the following secondary antibodies diluted 1:2000: donkey anti-rabbit HRP (GE Healthcare), sheep anti-mouse HRP (GE Healthcare) and anti-goat HRP (GE Healthcare).

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitations were performed as described previously [58]. MOV10 ChIP was performed using magnetic beads (Dynabeads Pan Mouse IgG, #110.41, Invitrogen) and the anti-Flag antibody (F3165-5MG, Sigma), alone for the negative control or in combination with the His-Flag tagged human anti-MOV10 antibody (AbD08355, AbDSerotec). Precipitated DNA was analyzed by real-time PCR using specific primer sets that span the human locus [49].

Cell fractionation and nuclei purification

HEK293T and FDF cells were harvested and washed twice in phosphate buffered saline (PBS) and the cytosolic fraction was extracted in buffer A comprising 10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M Sucrose, 10% (v/v) glycerol, 1 mM DTT and Roche protease inhibitor. The pellet (consisting of nuclei) was then treated with buffer C containing 20 mM HEPES, 1 mM EDTA, 1 mM EGTA, 0.4 M NaCl, 1 mN DTT, 0.5 mM PMSF and protease inhibitor to obtain the nuclear proteins after high-speed centrifugation.

Purified nuclei were obtained from HEK293T cells using the nuclei isolation kit (NUC-201) from Sigma. Nuclei were then incubated with or without RNAse A (2 $\mu g\ \mu l^{-1}$, Abcam) or RNAse H (0.5U μl^{-1}, Ambion) in CSK buffer containing 10 mM PIPES pH6.8, 100 mM NaCl, 300 mM sucrose, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 0.1% (v/v) Triton-X100 and Roche protease inhibitor. Chromatin and nucleoplasmic fractions were then separated by centrifugation. To solubilize chromatin bound proteins, CSK buffer was used with higher NaCl concentrations.

RNA extraction, quantitative reverse transcription and quantitative PCR

Total RNA was prepared using the Ultra Pure RNA extraction Kit from Roche and the cDNA was generated using 0.25–1 μg of RNA using MultiScribe reverse transcriptase and random hexamer primers (Applied Biosystems). The cDNAs were analyzed and quantified by real time PCR as described previously [6]. MOV10 transcripts were amplified using the following primers: Forward 5′- ACATTCTACATTGCCCGCTTCTTG -3′, Reverse 5′- CTCCTTCCTCTATCCGATTGGTCAC -3′. Selected HOXD genes were detected using published primer sets [13],[14].

Gel filtration

HEK293T and FDF cells were washed twice in PBS and nuclei were prepared by extraction with the buffer A, as above. Nuclear extracts were prepared in CHAPS buffer containing 0.5% (w/v) CHAPS, 1 mM EDTA, 50 Tris.HCl pH 8, 150 mM NaCl, and protease inhibitor. Samples (3 mg of protein) were loaded on a Superose 6 column (GE Healthcare 17-5173-01) equilibrated in CHAPS buffer. Fractions of 0.5 ml were precipitated with 10% (w/v) trichloroacetic acid, fractionated by SDS-PAGE and immunoblotted. Size calibration was performed using thyroglubulin (669 kDa), ferritin (440 kDa) catalase (232 KDa), aldolase (158 KDa) and albumin (67KDa).

Immunofluorescence

Primary human fibroblasts (Leiden and FDFs) were grown in Lab-Tek chamber slides (Nunc). The cells were fixed for 15 min in 3.7% (v/v) formaldehyde, permeabilized for 15 min in 0.1% (v/v) Triton-X100 and the blocking was performed for 2 h in 1% w/v BSA. The slides were incubated in Image iT-Fx signal enhancer solution (I36933, Molecular Probes, Invitrogen) and then with the anti-MOV10 antibody (ab60132, Abcam) overnight. The slides were incubated with the Alexa Fluor 555 (Molecular Probes, Invitrogen) secondary antibody and finally mounted in ProLong Gold antifade reagent with DAPI (P36931,Molecular Probes, Invitrogen).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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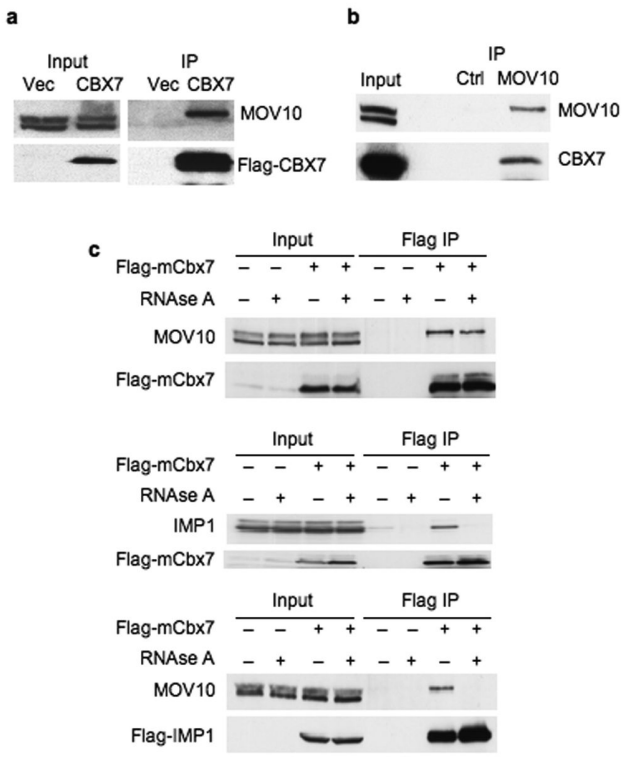

Figure 1.

Interaction of MOV10 with CBX7. (**a**) Co-immunoprecipitation of endogenous MOV10 (upper band of the doublet) with exogenous Flag-CBX7 in transiently transfected 239T cells. (**b**) 293T cell lysates were immunoprecipitated with a Flag-tagged recombinant monoclonal antibody against MOV10 or with anti-Flag beads alone (Ctrl) and immunoblotted with rabbit antibodies against CBX7 or MOV10. (**c**) Co-immunoprecipitation of endogenous MOV10 and transiently expressed Flag-mCbx7 with (+) or without (−) RNAse treatment. Analogous experiments were conducted with Flag-mCbx7 and endogenous IMP1 and with Flag-IMP1 and endogenous MOV10.

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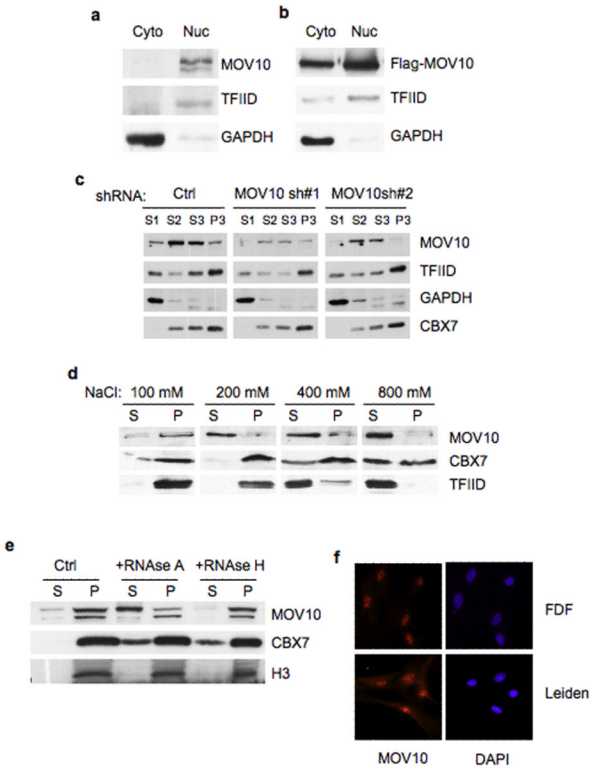

Figure 2.

MOV10 is predominantly nuclear and associated with chromatin. (**a**) Cytoplasmic and nuclear extracts from 293T cells were immublotted with antibodies against MOV10 (Ab13). TFIID and GAPDH were used as controls for the nuclear and cytoplasmic proteins respectively. (**b**) A similar experiment was performed with 293T cells transfected with a vector encoding Flag-tagged MOV10. (**c**) 293T expressing a lentiviral control shRNA (Ctrl) or two independent shRNAs targeting MOV10 (sh1 and sh2) were subjected to biochemical fractionation. The cytosolic S1, nuclear soluble fractions S2 and S3 and the chromatin-enriched fraction P3 were separated by SDS-PAGE and immunoblotted with the indicated antibodies. (**d**) Purified nuclei from 293T cells were extracted with increasing concentrations of NaCl, as indicated, and the proportion of MOV10 in the supernatant (S) or pellet (P) was determined by immunoblotting. CBX7 and TFIID were used as controls. (**e**) Purified nuclei were incubated with RNAse A, RNAse H or buffer alone (Ctrl) and the nucleoplasmic (S) and chromatin-enriched (P) fractions were immunoblotted for endogenous MOV10, CBX7 and histone H3 (as a control). (**f**) Immunofluorescence detection of endogenous MOV10 (red) in the FDF and Leiden strains of primary fibroblasts. Nuclei were visualized with DAPI.

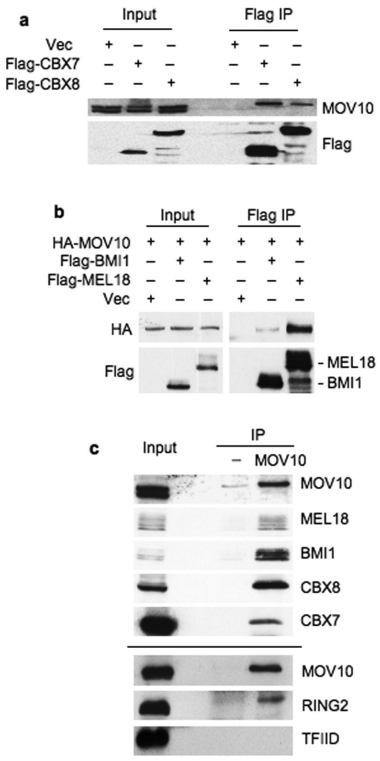

Figure 3.

MOV10 co-precipitates with multiple PRC1 components. (**a**) Co-immunoprecipitation of endogenous MOV10 with Flag-tagged CBX7 and CBX8 from transiently transfected 293T cells. Input represents 5% of the total cell extract used for each IP. The right panel shows the anti-Flag precipitates immunoblotted for the Flag-tagged proteins and endogenous MOV10. (**b**) Co-immunoprecipitation of HA-MOV10 with Flag-tagged BMI1 and MEL18 from transiently transfected cells. The left panels show the immunoblots for the input samples. The right panels show the Flag-IPs blotted with an anti-Flag or an anti-HA antibody. (**c**) Co-immunoprecipitation of endogenous PRC1 components with endogenous MOV10. Nuclear extracts from 293T cells were immunoprecipitated with a His-Flag tagged recombinant antibody against MOV10, using either NiNTA (upper panels) or anti-Flag M2 beads (lower panels). The (−) track refers to beads alone. The precipitated proteins were then immunoblotted with antibodies against the indicated proteins.

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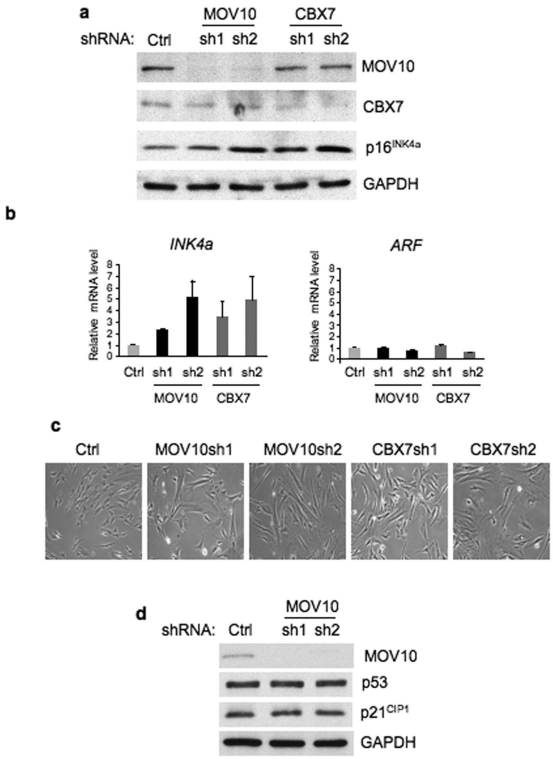

Figure 4.

MOV10 contributes to the regulation of *INK4a* in primary fibroblasts. (**a**) FDF cells were infected with lentiviruses encoding a control shRNA (Ctrl) and two independent shRNAs against MOV10 and CBX7 respectively (sh1 and sh2). The knockdown efficiency and the effect on $p16^{INK4a}$ were assessed by immunoblotting with antibodies against MOV10, CBX7 and $p16^{INK4a}$. GAPDH was used as a loading control. (**b**) Effects of MOV10 and CBX7 shRNAs on *INK4a* and ARF RNA levels as determined by qRT-PCR. Error bars, s.d.; n=3. (**c**) Phase contrast photographs showing the enlarged and flattened appearance of cells expressing the MOV10 and CBX7 shRNAs. (**d**) Following knockdown of MOV10 (as in panel **a**), cell lysates were immunoblotted for p53 and $p21^{CIP1}$ as indicated. GAPDH was used as a loading control.

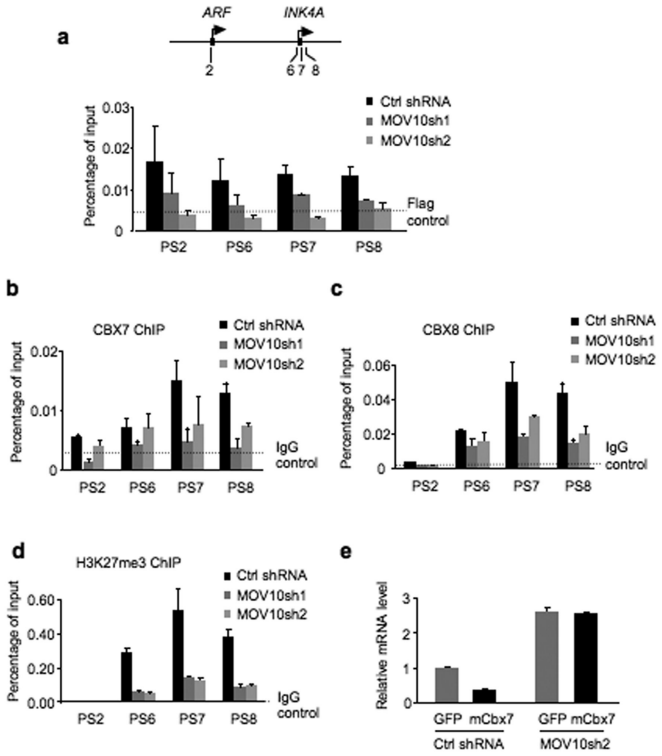

Figure 5.

MOV10 contributes to the transcriptional repression of *INK4a* by the Pc proteins. (**a**) Chromatin was prepared from FDF cells expressing a control shRNA (Ctrl) and two independent shRNAs against MOV10 (sh1 and sh2). Chromatin immunoprecipitation was performed using either Flag-beads or with a Flag-tagged recombinant antibody against MOV10. The precipitated DNA was analyzed by qPCR with the indicated primer sets corresponding to the promoter and first exon regions of *INK4a* and *ARF*, as described previously [6]. The same chromatin preparations as in panel **a** were precipitated with rabbit antibodies against CBX7 (**b**), CBX8 (**c**), and H3K27me3 (**d**) and analyzed by qPCR. In each case, an irrelevant IgG was used as a negative control. (**e**) FDF cells expressing an shRNA control (Ctrl) or an shRNA against MOV10 were super-infected with retroviruses expressing either GFP (as a control) or mCbx7. The relative *INK4a* mRNA levels were analyzed by qRT-PCR. Error bars, s.d.; n=3.

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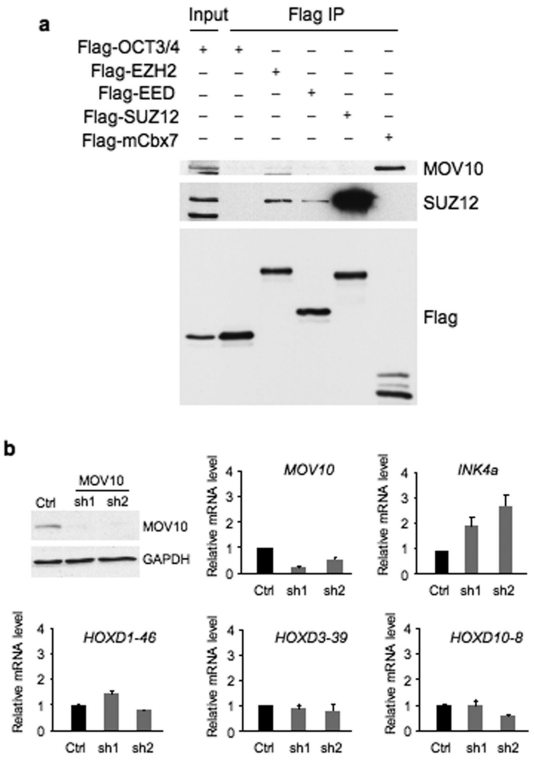

Figure 6.

Lack of interaction between MOV10 and PRC2 components. (**a**) 293T cells were transiently transfected with Flag-tagged versions of EZH2, SUZ12, EED and OCT3/4 with Flag-mCbx7 as a positive control. Samples of cell lysate were either analyzed directly (Input) or after immunoprecipitation with Flag antibody. Immunoblotting for endogenous MOV10 showed no evidence for an interaction with the PRC2 proteins but a clear association with mCbx7. Immunoblotting for SUZ12 confirmed that the PRC2 proteins co-precipitate in this setting. (**b**) FDFs were transduced with control shRNA (Ctrl) or two independent MOV10 shRNAs and their effects on the expression of *MOV10*, *INK4a*, *HOXD1-46*, *HOXD3-39* and *HOXD10-8* were assessed by qRT-PCR. Error bars, s.d.; n=3.

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