Association of Rex-1 to target genes supports its interaction with Polycomb function


Abstract  Rex-1/Zfp42 displays a remarkably restricted pattern of expression in preimplantation embryos, primary spermatocytes, and undifferentiated mouse embryonic stem (ES) cells and is frequently used as a marker gene for pluripotent stem cells. To understand the role of Rex-1 in self-renewal and pluripotency, we used Rex-1 association as a measure to identify potential target genes, and carried out chromatin immunoprecipitation assays in combination with gene specific primers to identify genomic targets Rex-1 associates with. We find association of Rex-1 to several genes described previously as bivalently marked regulators of differentiation and development, whose repression in mouse embryonic stem (ES) cells is Polycomb Group-mediated, and controlled directly by Ring1A/B. To substantiate the hypothesis that Rex-1 contributes to gene regulation by PcG, we demonstrate interactions of Rex-1 and YY2 (a close relative of YY1) with Ring1 proteins and the PcG-associated proteins RYBP and YAF2, in line with interactions reported previously for YY1. We also demonstrate the presence of Rex-1 protein in both trophectoderm and Inner Cell Mass of the mouse blastocyst and in both ES and in trophectoderm stem (TS) cells. In TS cells, we were unable to demonstrate association of Rex-1 to the genes it associates with in ES cells, suggesting that association may be cell-type specific. Rex-1 might fine-tune pluripotency in ES cells by modulating Polycomb-mediated gene regulation.

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Introduction

After undergoing a first differentiation step, the preimplantation blastocyst is divided in Inner Cell Mass (ICM), which gives rise to the embryo proper, and trophectoderm (TE), an external epithelium that contributes to the placenta. Selfrenewing stem cells that can be maintained in culture for an apparent indiscriminate number of cell divisions can be derived from each of these lineages (Smith, 2005) and are referred to as embryonic (ES) and trophectoderm (TS) stem cells, respectively. TS cells are derived from polar TE, and retain the capacity to differentiate into all trophoblast subtypes of the placenta in quimeric embryos (reviewed in Rossant (2007)). Embryonic stem cells are characterized by two defining properties: selfrenewal and pluripotency. Pluripotency refers to the ability to differentiate into cell lineages of all three primary layers of the embryo, in fact all cell types present in an organism.

Molecular mechanisms that maintain/govern this pluripotent selfrenewing state operate at different levels and include (but are not limited to) signaling by LIF and BMP4, inhibition of ERK signaling (Nichols and Smith, 2009), cooperating networks of transcription factors and epigenetic mechanisms (Boyer et al., 2006a; Surface et al., 2010). Oct4, Sox2 and Nanog have been identified as essential for the formation and/or maintenance of both the ICM during mouse preimplantation development and murine ES cells in culture (Nichols et al., 1998; Avilion et al., 2003; Chambers et al., 2003; Mitsui et al., 2003). These three transcription factors constitute a core transcriptional network to maintain pluripotency through mutual positive regulation (Boyer et al., 2006a) and collaborative regulation of target genes. Acting either in parallel or downstream, additional circuits and regulatory factors are operative in pluripotency (Xu et al., 2008; Hu et al., 2009). Downstream of Oct4, repression of developmental regulators and stem cell maintenance also depend on Polycomb Group (PcG) proteins.

The Polycomb family comprises a set of structurally diverse proteins which assemble into chromatin-associated complexes of variable and context-dependent composition (e.g., differentiation status) with chromatin-modifying activities. Three principal PcG multi-protein complexes have been identified and characterized: Pho repressive complex (PhoRC) in Drosophila melanogaster, and across species Polycomb Repressive Complexes 1 and 2 (PRC1 and PRC2) (Schwartz and Pirrotta, 2007; Müller and Verrijzer, 2009). In mammals, Eed, Ezh2 and Suz12 participate in a core PRC2 complex that catalyzes trimethylation of histone H3 at lysine 27 (H3K27), which in turn is thought to provide a recruitment site for PRC1 (Cao and Zhang, 2004). The core PRC1 is composed of orthologs of Drosophila Polycomb (Cbx2, Cbx4 and Cbx8), posterior sex combs (Mel18 (Pcgf2) and Bmi1), sex comb extra (Ring1A and Ring1B, also known as Ring1 and Rnf2) and polyhomeotic (Phc1, Phc2 and Phc3). Polycomb genes were first identified in Drosophila as negative regulators of homeotic gene expression. In vertebrates, the PcG proteins play a role not only in the specification of the antero-posterior axis but also in X chromosome silencing, genomic imprinting, stem cell renewal or cell differentiation and cell fate (Schuettengruber et al., 2007; Pietersen and van Lohuizen, 2008).

Polycomb genes and Ring1B/Rnf2 in particular repress expression of developmental regulator genes in ES cells, that are characterized by so called "bivalent domains", a unique configuration of histone marks, carrying simultaneously histone marks associated with gene activity [histone H3 lysine 4 trimethylation (H3K4me3) and modifications associated with PcG-mediated repression (H3K27 trimethylation) (Azuara et al., 2006; Bernstein et al., 2006; Boyer et al., 2006a; Boyer et al., 2006b; Spivakov and Fisher, 2007). Repression in ES cells depends on both Oct4 (or other members of the core circuit) and Ring1B, in such a way that engagement at target genes is mutually interdependent (Endoh et al., 2008). Although only a fraction of bivalent genes (about 40%) is bound by Ring1B/Rnf2 in ES cells, a high percentage of Ring1B/Rnf2 targets is bivalent (Ku et al., 2008), and Ring1B-occupancy of these genes correlates with functional repression.

Apart from PRC1, Ring1 proteins participate in BcoR or Fbx10–BcoR complexes with RYBP/YAF2 (Gearhart et al., 2006; Sánchez et al., 2007). RYBP was first characterized as an interacting partner of the Polycomb group (PcG) protein Ring1A (Garcia et al., 1999). Similar to its close relative YAF2 (Kalénik et al., 1997), RYBP also interacts directly with the sequence-specific DNA binding protein YY1 (Schlossio et al., 2002). YY1 is one of the mammalian orthologs of the Polycomb-group genes and transcription factors Pho and PhoL (Brown et al., 1998) and is required for implantation during early mouse development (Donohoe et al., 1999; Gordon et al., 2006). Apart from direct interactions with EED/esc (Satijn et al., 2001) and with EZH1 (Wang et al., 2004a), among PcG proteins YY1 also associates with Ring proteins, albeit indirectly (del Mar Lorente et al., 2000), as neither Pho nor YY1 have been co-purified with PcG complexes. YY1 also interacts genetically with Ring1A in compound loss-of-function mice (del Mar Lorente et al., 2000). As opposed to strictly Pho-dependent assembly of PRC complexes on well-defined Polycomb response elements (PREs) in D. melanogaster (Wang et al., 2004b; Mohd-Sarip et al., 2005; Klymenko et al., 2006; Surface et al., 2010), in mammals PREs are not easily delimited. PcG complexes in mammals do not bind DNA directly, but associate to chromatin via binding to modified histones, non-coding RNAs or through association with DNA binding transcription factors (reviewed in Simon and Kingston (2009)) such as Oct4 (Endoh et al., 2008), YY1 (Woo et al., 2010) or Jarid2 (Shen et al., 2009; Li et al., 2010; Pasini et al., 2010). Confirming a function for YY1 as a recruitment factor for PcG complexes in mammals (Garcia et al., 1999; Gordon et al., 2006), YY1 binding sites are necessary for the repressive function of a Polycomb response element (PRE) that regulates the HoxD locus in human ESCs through recruitment of RYBP and PcGs (Woo et al., 2010).

Among transcription factors implicated in stem cell biology based on specific expression patterns or integration in LIF and BMP signaling pathways, (Okita et al., 2007; Takahashi and Yamanaka, 2006), Rex-1 was first discovered as a result of its specific expression in pluripotent F9 embryonal carcinoma (EC) cells (Hosler et al., 1989). Rex-1 (for reduced expression-1, also known as Zfp42) was subsequently shown to be expressed in other pluripotent cell types, especially undifferentiated embryonic stem cells (Rogers et al., 1991), multipotent adult progenitor cells (Jiang et al., 2002) and amniotic fluid cells (Karlmark et al.,
in the germ cells of the testis and the ICM and early TE derivatives of the mouse embryo (Rogers et al., 1991). In accordance with the restricted expression in pluripotent self-renewing cells, Rex-1 expression has been positively linked to increased pluripotency in both mES cells (Takahashi and Yamanaka, 2006; Okita et al., 2007; Toyooka et al., 2008) and human ES and iPS cells (Brivanlou et al., 2003; Chan et al., 2009). In contrast, conflicting results have been reported regarding the functional role of Rex-1. Gene silencing by RNA interference results in loss of self-renewal in ES cells (Zhang et al., 2006) and overexpression of Rex-1 negatively affects self-renewal (D. Guallar, M. Sánchez and J. Schoorlemmer, unpublished). However, Rex-1 does not have to be provided for efficient reprogramming of differentiated cells towards iPSS (Takahashi and Yamanaka, 2006; Okita et al., 2007) and Rex-1 is dispensable for maintenance of self-renewing pluripotent ES cells (Scotland et al., 2009).

Rex-1 encodes a protein containing four Cys--His type zinc-fingers, that is localized in the nucleus in ES cells (Masui et al., 2008) and displays significant similarity to the YY1 transcription factor family in the DNA-binding zinc finger domains (Kim et al., 2007). Chip--chip studies have revealed Rex-1 association to a circuit of active genes implicated in protein metabolism, rather than in developmental processes, that coincides partially with Myc targets as opposed to protein metabolism, rather than in developmental processes.

As YY1 has the potential to recruit PcG to target genes, we set out to investigate a similar role for Rex-1. We investigated protein expression of Rex-1 in the blastocyst of mouse preimplantation embryos, in ES cells as well as in TS cells. We tested for both protein--protein interactions of Rex-1 with Polycomb--proteins and associated factors. We subsequently used chromatin immunoprecipitation assays to map association of Rex-1 to potential target genes, comparing association in ES and TS cells. Our data support the involvement of Rex-1 in control of PcG target genes during pluripotency or differentiation.

Results

Expression of Rex-1 in blastocyst and blastocyst-derived stem cells

We obtained rabbit αRex-1 serum after immunization with a Rex--GST fusion protein (see M&M). To confirm specificity of Rex-1 protein detection, we confirmed absence of epitope detection by the serum after saturation with excess binding protein, both on western blot (Supplementary Fig. 1A) and in morula (Supplementary Fig. 1B). We addressed Rex-1 expression and localization in preimplantation embryos by indirect fluorescence studies in confocal sections of stained embryos. Representative examples of stained blastocysts are depicted in Fig. 1. Consistent with previous reports on mRNA expression, we detected anti-Rex-1 immunoreactivity throughout preimplantation development (S. Alonso, M. Climent and J. Schoorlemmer, manuscript in preparation). In early, pre-expansion blastocysts immunoreactivity was detected in all cells in a pattern that comprises a combination of cytoplasmic, perinuclear and nuclear localization (Fig. 1, panels C and G). Nuclear staining was apparent, but weaker as compared to stronger staining in the cytoplasm. As expected, we detected staining in the ICM (arrows) and counterstaining with the trophectoderm marker Cdx-2 (Fig. 1, panels B and F) confirmed the presence of immunoreactivity against Rex-1 serum in TE cells (arrowheads, panels C and G).

Considering the apparent staining with αRex serum throughout the blastocyst, we wanted to probe Rex-1 expression in blastocyst-derived stem cells in tissue culture. To do so, we assayed expression in ES cells (line E14T) and TS cells (line B7) cultured in the absence of feeder cells by anti-Rex-1 staining followed by indirect immunofluorescence. Staining patterns were analyzed by confocal microscopy and representative images are depicted in Fig. 2A. No staining was observed in either cell type when pre-immune or no primary antibody was used (Supplementary Fig. 2). In ES cells, Rex-1 staining was apparent (A488, panel N), mostly confined to the nucleus as revealed by nuclear counterstaining (DAPI, panel M). This pattern was confirmed by staining of HA-Rex-1 in the nucleus of transfected cells (Fig. 5A), as reported (Masui et al., 2008). No specific staining was apparent in RA-treated ES cells (panels P–S), which do not express Rex-1 (Fig. 2C). Most if not all TS cells showed weak, but detectable reactivity with the anti-Rex serum (panel V). Similar to ES cells, staining was most intense in the nucleus (panels V and W). These results are compatible with the presence of Rex-1 throughout the blastocyst that we observed (Fig. 2A).

To further confirm the expression of Rex-1 in ES and TS cells, we analyzed mRNA levels in both cell types by PCR. Expression of His2AZ (Jeong et al., 2005) was used as a control for the quality of the RNA and cDNA used, and was well detected in both samples (lanes marked His2AZ). Similarly, Rex-1 mRNA was easily detected in both cell types (Fig. 2B). Rex-1 transcript levels were analyzed by quantitative PCR. Relative to expression levels in E14T ES cells (assigned arbitrarily a 100% level), we detected levels in TS cells at about 14%, while in RA-treated ES cells levels had dropped below 1% (Fig. 2C).

Rex-1 interacts with PcG proteins

RYBP was discovered as a candidate mediator between DNA-binding YY1 and PcG complexes, especially Ring-containing complexes PRC1 or BcoR (Sánchez et al., 2007). As the C-terminal Zinc Fingers of YY1 that are necessary and sufficient for direct association with YY1 and the related proteins YY2 and Rex-1 (Kim et al., 2007), we tested potential interactions of Rex-1 or YY2 with either RYBP/YAF2 or Ring proteins. For similar reasons, we included Eed in the analysis as a YY1-interactor (Satijn et al., 2001). As yeast dihybrid assays have been previously used to identify and show interactions between PcG proteins (Alkema et al., 1997; Schoorlemmer et al., 1997; Satijn et al., 2001), we decided to make use of this assay for our studies. To do so, cDNAs were inserted in DNA binding domain (DBD) vectors (baits) and probed for interactions with activation domain (AD)-fused proteins (preys) in yeast dihybrid assays. The assay is
based on induction of a GAL4-driven HIS gene in a so-
constructed yeast strain, an event competed by varying
concentrations of 3AT. Several combinations of bait and
prey produced a mix of viable proliferating clones and growth-
arrested clones. We have taken the repeated appearance
of clones capable of complementing TRP, Leu and His defi-
ciencies as an indicator of interaction.

We tested all combinations of YY1, YY2 and Rex-1 fused to
GAL4DBD (or GAL4DBD itself) with AD-fused YAF2, RYBP and
Eed. Yeast colonies are shown in Figs. 3A and B, and results
are summarized in Fig. 3C. Duplicate isolates of all strains
(except for the combination of GAL4 in the absence of fused
cDNAs) with either YAF2, RYBP or Eed tested positive for
viability (Fig. 3A). No interaction was observed for any DBD
fusion or AD-fusion in the absence of an interacting cDNA on
the other vector of the pair, as assayed by growth in -HIS
(Fig. 3B). Weak growth was observed in strains carrying YY1
and either RYBP or YAF2 proteins (Fig. 3B and data not
shown). Further analysis revealed these interactions to be
sustained in the presence of 2.5 mM 3AT, but hardly visible
in the presence of 5 mM 3AT (Fig. 3B). Whereas no His
complementation was observed when assaying the combi-
nation of YY2 with RYBP and YAF2, Rex-1 combinations with
either of these factors showed robust growth resistant to
5 mM 3AT. In contrast to GAL4-YY1, GAL4-YY2 interacted
with GAD-Eed to allow growth in -HIS, as opposed to the
absence of growth when GAL4-YY2 was combined with AD-
RYBP/YAF2. Similarly, GAL4-Rex-1 also interacted weakly
with AD-Eed, as measured by His complementation.

Although yeast dihybrid assays do not necessarily reproduce
genuine protein–protein interactions, we nonetheless believe
our analysis provides support for the association of at least
Rex-1 with other proteins that form part of PcG-related
complexes, particularly RYBP/YAF2 and Eed.

We next tested the possibility that Rex-1 or YY2 also
associate with Ring1 proteins when overexpressed in tissue
culture cells. To do so, we introduced plasmids that drive
expression of either HA-tagged YY1-family protein, together
with plasmids that express fusion proteins of GST (E. coli
glutathione-S-transferase) or cDNAs encoding Ring1 proteins.
Interacting proteins were identified by Western blot after
purification of GST fusion proteins on glutathione (GSH)-

Sepharose beads (Fig. 4). As reported previously (del Mar Lorente et al., 2000) YY1 was co-isolated with GST-Ring1 or GST-Rnf2 proteins (Fig. 4, lanes marked GST-R1A and GST-Rnf2). The formation of these complexes was dependent on the presence of Ring proteins, as HA–YY1 was not detected when assayed in extracts prepared from cells that expressed GST only.

Similar to the YY1–Rnf2 interaction, both YY2 and Rex-1 were co-purified with both GST–Ring1 and GST–Rnf2 proteins on GSH beads (Fig. 4, lanes marked YY2 and Rex-1), in contrast to the absence of retention on non-fused GST. These results indicate that in 293 T cells, YY2 or Rex-1 is capable of participating in Ring1/Rnf2-containing protein complexes.

Furthermore, these results reconfirm the potential association of YY1-family proteins with PcG proteins as reported before (Wang et al., 2006), in line with the observed association of Rex-1 to PcG target genes in ES cells.

**Association of Rex-1 to target genes in embryonic stem cells**

We intended to apply the rabbit αRex-1 serum in ChIP analysis to examine Rex-1 binding to potential target genes in mouse ES cells. The serum recognized the GST-fusion protein used for immunization on Western blot (data not shown).
Figure 3  Interactions of YY1 family proteins with Polycomb-related proteins. Yeast dihybrid assays. A yeast strain carrying a HIS reporter gene (GAL1-HIS3) was transformed with vectors carrying GAL4 DBD-fusions of YY1-family proteins YY1, YY2 or Rex-1 and AD-domain fusions of RYBP, YAF2, or Eed. Primary transformants were picked and serial dilutions were plated to test for growth. Activation domain fusion is indicated to the right of each figure. Viability of strains was assessed on -LEU/TRP plates (indicated as +HIS) in panel A and for complementation of -His phenotype (-HIS) in panel B in the presence of 5 mM 3-aminotriazol (3-AT). Complementation of -HIS phenotype was competed with different concentrations of 3-AT. Interactions resistant to 2.5 mM, 5 mM and 10 mM 3-AT are represented in (C) as +, ++ and +++ respectively.
Association of Rex-1 to target genes supports its interaction with Polycomb function

Figure 4  Association of Rex-1 and Ring2 proteins. Human kidney 293 T cells were cotransfected with plasmids expressing HA-tagged-intact YY1, YY2 or Rex-1 proteins, in combination with plasmids expressing either GST fused to full-length Ring1A protein (GST-R1A), Rnf2 protein (GST-R1B//Rnf2) or GST alone (GST). Proteins in total extracts were detected by GST blot (top panel) or HA blot (middle panel). Proteins were captured by GSH-Sepharose and analyzed by Western blot with monoclonal anti-HA antibody (bottom panel). Bands corresponding to HA-YY1//YY2//Rex-1 or GST-Ring/GST are marked on the right.

shown), and HA–Rex-1 (as opposed to HA-YY2) expressed in 293 T cells was immuno-precipitated from cell lysates by the serum (Fig. 5B). Similarly, HA–Rex-1 in transfected ES cells, easily detected by HA blot (Fig. 5C), was immunoprecipitated from extracts in the presence of anti-serum (Fig. 5C, lane αRex-1), but not by pre-immune serum (Fig. 5C, lane PreI). DNA–protein interactions in mouse ES cells were stabilized by formaldehyde crosslinking, and sonicated chromatin extract was prepared from lysed cells for immunoprecipitation using optimized conditions (see M&M), and association to selected loci was detected by gene-specific PCR. We compared amplification on amounts of chromatin obtained from the same number of cells after immuno-precipitation using either pre-immune serum or αRex serum.

Considering both the homology between Rex-1 and YY1, and the reported interaction between YY1 and Ring1 (del Mar Lorente et al., 2000), we investigated a potential association of Rex-1 to a subset of Ring1B-regulated developmental transcription factors in ChIP assays. While efficient amplification was observed using control templates for each of the genes analyzed (Fig. 5D, lane No IP), hardly any amplification was observed for the majority of markers after immunoprecipitation with pre-immune serum (lane PreI). By contrast, we observed reproducible association of Rex-1 to most Ring1B-bound bivalent regulators i.e. Gata6, Olig2, Sox17, Klf4, Fgf5 and T. Although Eomes shares the same characteristics, we were unable to detect Rex-1 association in our ChIP assay. To confirm that results reflect authentic binding sites, we compared binding from wildtype and transfected cells. Olig2 was immunoprecipitated weakly in wildtype cells by Rex-1 but not pre-immune serum (Fig. 5F), whereas no binding could be observed to Eomes. ChIP assays performed on HA-Rex transfected cells confirmed the results obtained before, as Eomes continued negative whereas the Olig2 signal was improved.

Among the genes differentially expressed in Rex-1 positive versus negative subpopulations (Toyooka et al., 2008), we found moderate association of Rex-1 to several genes preferentially expressed in Rex-1 negative populations of ES cells (Sox17, BrachyuryT and Fgf5) (Fig. 5E, see also

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### Figure 4

**Association of Rex-1 and Ring2 proteins.** Human kidney 293 T cells were cotransfected with plasmids expressing HA-tagged-intact YY1, YY2 or Rex-1 proteins, in combination with plasmids expressing either GST fused to full-length Ring1A protein (GST-R1A), Rnf2 protein (GST-R1B//Rnf2) or GST alone (GST). Proteins in total extracts were detected by GST blot (top panel) or HA blot (middle panel). Proteins were captured by GSH-Sepharose and analyzed by Western blot with monoclonal anti-HA antibody (bottom panel). Bands corresponding to HA-YY1//YY2//Rex-1 or GST-Ring/GST are marked on the right.

**Legend:**
- **GST-R1A**
- **GST-R1B(Rnf2)**
- **GST**
- **HA-YY1//YY2**
- **HA-Rex1**
- **HA-YY1//YY2**
- **HA-Rex1**

**Cell Line:** 293T cells
Table S2). We also tested Rex-association to genes whose expression is elevated and positively correlated with Rex-1-expressing subpopulations. Among these genes, we were unable to detect significant binding to Nanog (data not shown) and Eomes (Figs. 5E and F). By contrast, in the absence of amplification from chromatin immunoprecipitated with pre-immune serum, we observed a clear signal in anti-Rex-1 immunoprecipitation for Tcl1 and Klf4, Ring1B binding to which has been reported in at least one study (Endoh et al., 2008). We conclude that Rex-1 associated...
with most Ring1B target genes examined: Gata6, Olig2, Sox17, T, Fgf5, Klf4, Tcl1 (Figs. 5D and E) and Hoxa11 (data not shown). These results indicate that Rex-1 might contribute to repression of bivalently marked differentiation markers.

**Association of Rex-1 to target genes in trophectoderm stem cells**

As Rex-1 is expressed in both the trophectoderm of blastocyst stage embryos, and TS cell in culture (Fig. 2), we tested in this cell type association of Rex-1 to the same potential target genes identified in ES cells (Fig. 6). ChIP assays as applied before to ES cells were analyzed by gene-specific PCR. As before, we observed amplification of positive control chromatin (Fig. 6A, lane 1), and hardly a signal from chromatin immunoprecipitated using pre-immune serum (Fig. 6A, lane 2). However, neither weakly bound genes in ES like Tcl1 and Klf4, nor a consistent positive Sox17 was detectably associated with Rex-1 in TS cells (Fig. 6A, lane 3). As overexpression of HA-Rex had previously improved resolution of the assay in ES cells, we examined association to HA-Rex in transiently transfected TS cells also. Surprisingly, neither of the 3 genes was Rex-1 associated (lane 6) under conditions that easily allowed detection of association in ES cells (lane 9). We extended our analysis to a larger panel of genes, but were unable to detect significant association in TS cells of Rex-1 to most developmental regulators tested i.e. Sox17, Fgf5, T, Cdx2 or Hoxb8 (Fig. 6B, lanes 6), as opposed to ES cells (lanes 1–3, Fig. 6B). Although association was not as strong as observed in ES cells, Rex-1 weakly associated to Gata6 in TS cells (Fig. 6B, lane 6). We conclude that although Rex-1 is expressed in both ES cells and TS cells, association of Rex-1 to several target genes is different between two cell types.

**Discussion**

**Association of Rex-1 to target genes in embryonic stem cells**

Although Rex-1 shares extensive homology with the DNA-binding Zinc Fingers of YY1, we failed to demonstrate in ES cells appreciable binding of Rex-1 (M. Sánchez and J. Schoorlemmer, data not shown) to the promoters of genes deregulated in YY1-deficient cells (Affar et al., 2006), or to YY1 binding sites in imprinted genes (Kim et al., 2006a). We also failed to detect Rex-1 associated in ES cells with clusters of YY1 binding sites near the Hoxa5 and Hoxc8 genes identified in mouse embryos (Kim et al., 2006b).

Published data available on Rex-1 association to target genes are limited to the regulation of Tsix expression (Navarro et al., 2010) and genomic analysis (ChIP-chip) of chromatin-immunoprecipitation assays (Kim et al., 2008). It was proposed that Rex-1 targets segregate to a distinct cluster separate from Nanog, Sox2, Dax1, Klf4, Oct4 targets and represent active genes implicated in protein metabolism, rather than in developmental processes, that coincide partially with Myc targets (Kim et al., 2008). We provide here the first evidence of association of Rex-1 to a different set of target genes. Although it is tempting to speculate association is directly to DNA, we have no evidence that this is indeed the case.

We show that Rex-1 is significantly enriched at promoters of PRC1 target genes in ESC (Fig. 5D). Specifically, we observed binding of Rex-1, to Hoxb8, Hoxa11, Gata6, Olig 2, Cdx2, Fgf5 and T (Table S1). These genes were identified as bivalently marked developmental control genes, bound in ES cells by Ring1B/Rnf2 (Boyer et al., 2006b; Ku et al., 2008) and derepressed in Ring1B-deficient ES cells (Endoh et al., 2008). Among the bivalently marked Rex-1-bound genes, at least Gata6, Olig2, Sox17, Hoxa11 as well as T and Fgf5 are not expressed in ES cells, as opposed to Episc (Brons et al., 2007; Tesar et al., 2007). We do not know at present whether the association of Rex-1 extends to a wider subset of bivalently marked Ring1B targets, but conclude that Rex-1 might mediate DNA-binding of Ring1B to a distinct subset of bivalently marked genes. On the other hand, Rex-1 is not associated with all bivalent marker genes, notable exceptions being Hox genes (M. Sánchez and J. Schoorlemmer, data not shown) and Eomes (Table S1). We can only speculate at this point why particular Ring1B target genes are bound and potentially regulated by Rex-1 and not others. Potential mechanisms of regulation include the methylation status at H3K27 and H3K4 of the gene involved and proteins recognizing this configuration, the contribution of other PcG-associated proteins especially

**Figure 5** Rex-1 is recruited to Ring1B target genes in ES cells. (A) Immunofluorescent detection of HA-tagged Rex-1 in transfected cells. Indirect immunofluorescent detection of HA-Rex-1 in pseudo-confocal sections of E14T ES cells. From top to bottom panels show staining of membranes in Red (WGA-Alexa 594), anti-HA staining visualized with anti-mouse A488 (green), nuclear staining with DAPI, composite HA + DAPI + WGA image. (B and C) Western blots to detect HA-tagged Rex-1 in transiently transfected cells, either directly in cell lysates or after immunoprecipitation using α-Rex-1 serum. HA-Rex-1 was detected using αHA monoclonal antibody in extracts from 293 T cells (B, HA-Rex-1), after immunoprecipitation from 293 T cells (B) or ES cells (C). The serum does not immunoprecipitate HA-YY2 (B). HA-Rex-1 was not immunoprecipitated by pre-immune serum (C, Prel). HA-Rex-1 was also detected in lysates from transfected ES cells using α-Rex-1 serum (C, right panel). (D) Binding of Rex-1 to potential target genes in ES cells was assessed by gene-specific PCR analysis after chromatin immunoprecipitation by Preimmune serum (Prel), or α-Rex-1 serum (IP Rex-1). The figure shows gene-specific PCR amplification, using primers specific for the genes indicated on the right. PCR reactions without input DNA served as a negative control (No input). PCR reactions using a fraction of purified chromatin extract from the same lysate are shown as positive controls on the left (No IP). (E) ChIP assays as in D to detect binding of Rex-1 to establish Ring1B target genes in ES cells transiently transfected with a HA-Rex-1-expressing plasmid. (F) ChIP assays were performed as in D. Comparison of Rex-1 ChIP results obtained in wildtype ES cells and HA-Rex-1 transfected ES cells. Rex-1-association was assessed by gene-specific PCR to Eomes or Olig2. Transfection of tagged Rex-1 influences the amplitude but not the quality of the signal.
RYBP, or the presence of mono-methylated histones identified as targets for Pho complexes (Klymenko et al., 2006).

In ES cells with attenuated expression levels of Rex-1 (D. Guallar, A. Larraga and J. Schoorlemmer, unpublished data), we do not observe induction of Gata6, Fgf5, T expression levels. Similarly, differentiation markers Fgf5, BrachyuryT, Gata6, Nestin, PDGFRα and LaminB1 are normally expressed in Rex-1-deficient ES cells, but super-induced upon differentiation (Scotland et al., 2009). Although we did not directly test association of Rex-1 to the latter 3 genes, they do fit in the group of Ring1B-regulated developmental control genes repressed in ES cells (Table S1). These combined findings suggest that Rex-1 influences differentiation rather than self-renewal itself and consistent with the hypothesis that Rex-1 somehow locally modifies the regulated loci in such a way that subsequent induction upon differentiation is altered. Also, a tempting speculation is that Rex-1 initiates the repressed state of these genes in concert with PcG, being dispensable for maintenance. Further work is required to substantiate this issue.

We have observed weak association of Rex-1 in ES cells to Tcl1 and Klf4, genes whose expression is positively associated with Rex-1 expression (Toyooka et al., 2008, Tables S1 and S2). In preliminary assays, we were unable to reveal association of Rex-1 to the promoters of Sox2 and Nanog, the essential regulators of the pluripotency network (Boyer et al., 2006a; Loh et al., 2006), similar to results published by Kim et al. (2008). By contrast, we report here the association of Rex-1 to Sox17, T and Fgf5, several of the genes with maximum differential expression associated with Oct4+ Rex-subpopulations (Toyooka et al., 2008; Table S2). This observation points towards a repressing function of Rex-1 on these genes in pluripotent, ICM-like cells.

**Target genes in embryonic versus trophoblast stem cells**

Rex-1 expression during preimplantation development had been previously investigated by in situ hybridization in peri- and preimplantation embryos (Rogers et al., 1991). Expression was reported in the ICM of blastocyst, and trophectoderm-derived tissues i.e. ectoplacental cone. In addition, genomic studies have shown Rex-1 mRNA expression in blastocyst (Hamatani et al., 2004). We report expression of Rex-1 protein in blastocyst stage mouse embryos and ES and TS cells derived from the blastocyst. Our data show that αRex-1 immunoreactivity is present in all cells of the murine blastocyst in an unexpected pattern that comprises a combination of cytoplasmic, perinuclear and nuclear localization. This pattern is reminiscent of cytoplasmic/perinuclear staining described previously for several chromatin regulators i.e. Dnmt1 (Ratnam et al., 2002), Cbx (Ruddock-D'Cruz et al., 2008) and most interestingly YY1 (Donohoe et al., 1999). As opposed to embryos, in both cell types in culture staining of Rex-1 is predominantly nuclear. We do not have an explanation for this phenomenon at present. We do conclude however, that Rex is expressed and may contribute to developmental and transcriptional processes in both cell types of the early blastocyst.

To our surprise, we were unable to detect Rex-1 associated in TS cells to the same genes we identified in ES cells. Although these results may have been influenced by (over)-expression levels, they do suggest that association of Rex-1 to genomic targets may be cell-type specific, similar to ES-restricted Rex-1 association to Tsix regulatory elements (Navarro et al., 2010). These combined findings raise the possibility that Rex-1 function is different in the two cell types and may indicate that Rex-1 controls a different circuit of genes in trophoderm as opposed to ICM. Repressive

**Figure 6** Comparison of Rex-1 association in ES and TS cells. ChIP assays. Binding of Rex-1 to potential target genes in TS cells, either wildtype or transiently transfected with a HA-Rex-1-expressing plasmid. Transiently transfected ES cells were included as a positive control. Association was assessed by gene-specific PCR analysis after chromatin immunoprecipitation using PreImmune (PreI) or αRex-1 serum (lanes IP Rex-1). Positive and negative controls are included as above (indicated as No input and No IP, respectively). Association was assayed for Sox17, Tcl1, Klf4 (panel A), and Gata6, Fgf5, T, Hoxb8 and Cdx2 (panel B).
mechanisms in placenta involve Polycomb group complexes (Umlauf et al., 2004), although bivalent domains are rather ES cell specific (Rugg-Gunn et al., 2010). As Ring1B-regulated repression of bivalent genes is restricted to ES cells as opposed to TS cells (Rugg-Gunn et al., 2010), we surmise that a specific epigenetic context restricts Rex-1 association with Polycomb to ES cells. Alternatively, the contribution of Rex-1 to TE-specific gene expression may be independent of PcG, as Eomes is not bound by Rex-1 in mES (Figs. 5E/F, Table S1). We are presently investigating the potential Rex-1 target genes in TS cells by ChIPseq approaches.

Rex-1 interaction with Polycomb-related protein complexes

Neither YY1 nor its fly homologs Pho and PhoL have been found as components of PRC1 or PRC2 PcG complexes (Schwartz and Pirrotta, 2007; Müller and Verrijzer, 2009). However, YY1 interacts with RYBP (Schlisio et al., 2002) and complexes containing YY1 and Ring proteins can form (del Mar Lorente et al., 2000; Wang et al., 2006). As the association of YY1 to this complex(es) relies at least in part on Zinc Finger sequences that are highly conserved in YY2 and Rex-1, the observed interaction between Rex-1 and Ring1 proteins was rather expected.

We employed different approaches to prove potential interaction between Rex-1 and Eed and RYBP (yeast dihybrid assays) or between Rex-1 and Ring1 proteins (co-immunoprecipitation from overexpressing mammalian cells). It should be noted that not all assays employed are capable of detecting all genuine or meaningful interactions. In fact, a comparison of 5 different assays for protein-protein interactions revealed that no single assay was capable of detecting more than 36% of a set of well established interactions (Braun et al., 2009). We hence feel rather confident that the association of Rex-1 with other proteins that participate in PcG-like complexes, particularly Ring proteins, RYBP, YAF2 and Eed, are meaningful in a particular context. We have been unable so far to demonstrate in vivo interactions between Rex-1 and either RYBP or Ring proteins in ES cells, due in part to the relative insolubility of Rex-1 and its tight association with chromatin (J. Schoorlemmer et al., unpublished results). The Rex-1 interaction with RYBP and PRC1 or PRC2 components may only take place on chromatin, preventing solubilization and subsequent biochemical analysis.

Rex-1 had been identified as a nuclear protein, and we now identify the potential of Rex-1 to associate with Ring1B, a Polycomb group protein with a central function in regulation of bivalently marked genes in ES cells. Not being the principal repressor, Rex-1 might fine-tune pluripotency in embryonic stem cells by modulating regulation of PcG-repressed developmental control genes.

Materials and methods

Generation of a rabbit α-Rex serum

Sequences encoding Rex1/Zfp42 (amino acids 1 to 62; accession N° NM95556) and YY2 (amino acids 1 to 62; accession N° AK036071) were cloned into pGEX4T-1 (Amersham). Glutathione S-transferase (GST) fusion proteins were produced in Escherichia coli BL21pLys. Expression of the fusion proteins was induced for 2–3 h at 37 °C with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were resuspended in 0.05 M Tris–HCl, pH 8.0, buffer containing 0.15 M NaCl, 1 mM DTT, 0.1% Triton X-100, 10% glycerol and protease inhibitors (Complete®, Boehringer Mannheim) and sonicated until clear. The cell lysate was centrifuged at 14000 g for 10 min at 4 °C and the supernatant collected. GST fusion proteins were purified from bacterial extracts by affinity chromatography using glutathione-Sepharose 4B (Amersham) as recommended by the manufacturer and further purified by SDS–PAGE (when necessary for concentration) for use in immunization of rabbits according to standard procedures (Harlow and Lane, 1998). Sera obtained were tested for affinity and specificity standard IgG purification.

Immunological reagents

Antibodies used include rabbit α-GST (Schoorlemmer et al., 1997), monoclonal α-HA (clone 12CA5) was obtained from Roche, α-HA (clone ZBP-1 H5) from Euromedex, α-Cdx2 (1:100, Biogenex, MU392A-UC, CDX2-88, BioGenex). Nuclei were stained using Draq5 (10 μM in PBS; Biostatus Ltd.) or DAPI (0.1 μg/ml; Sigma).

Plasmid construction

Generation of epitope-tagged vectors. Rex-1/Zfp42 cDNA was cloned from E14T ES cell mRNA by RT-PCR using oligonucleotides 5′-GGATCCATGTCCGAGAGGAAGTGTCG-3′ and 5′-GAATTCATTCAAGTGATCCCATCAGC-3′. A YY2 cDNA was derived in a similar fashion from mouse embryonic fibroblast mRNA, using primers 5′-CCTGATATGCTGAGTTGCGClocked into pGEX4T-1 (Amersham). CTDAGAGACAGAAGAACACT and TTTAATTGTGATCTGGCTT- TAAATGGGTGTTAAG. PCR Products were cloned in pGEM, verified by sequencing and transferred to plasmids pSG5HA for HA tagging. A human YY1 cDNA was excised from pCS2MycYY1 (García et al., 1999) and inserted into pSG5HA. Mammalian vectors expressing GST-fusions to Ring1A and Ring1B/Rnf2 were based on pEBG and have been described previously (del Mar Lorente et al., 2000). GST or GST–fusion proteins were expressed in eukaryotic cells from the pEBG plasmid (Spanopoulou et al., 1996; Garcia et al., 1999). cDNA from pSG5HA were excised and cloned into the chicken β-actin promoter-driven expression vector pCAGIP (Niwa et al., 1998) and used to transiently transfect murine ES cell lines. Further details of all plasmids used are available on request, and all constructs were verified by DNA sequencing. Plasmids used for transfections were purified on PureLink™ kits and columns (Invitrogen).

ES and TS cell culture and transfections

ES cell lines E14T (Aubert et al., 2002) was maintained on tissue culture dishes coated in 1% gelatin (Sigma-Aldrich, St Luis, MO) in ES medium (GMEM; Dulbecco’s modified Eagle’s medium) supplemented with 10% fetal calf serum, 10−4 M 2-mercaptopethanol, 2 mM l-glutamine, 0.1 mM non-essential
a 0.1 mM non-essential amino acid, 0.1 mM non-essential amino acid (GIBCO) 11140.

We seed 0.8×10⁶ cells per 60 mm dish. At least 2 h before transfection the medium was refreshed. 4 μg of each plasmid was mixed with lipids in OptiMEM(GIBCO), the mixture was left on the cells for 4 h, and then replaced with fresh medium. Cells were harvested 16–24 h after transfection.

**Immunofluorescence and confocal microscopy**

Mouse embryos (CD-1, Charles River) were obtained from superovulated females using standard methods (Nagy et al., 1992) and Torres-Padilla et al. (2006). Embryos were washed in PBS for 5 min, fixed at RT for 20 min in 2.5% paraformaldehyde in PBS (pH 7.4), permeabilized with 0.2% Triton-X100 in PBS for 15 min at RT and blocked/permeabilized in PBS/0.2% Triton-X100/10% FBS for 1 h at RT. All further incubations and washes were performed in PBS/0.2%Tx100/2% FBS. Fixed and permeabilized embryos were then incubated overnight at 4 °C with primary antibodies in PTF (PBS containing 2% FBS, 0.2% Tx100). Dilution of antibodies αCdx2 (1:100), αRex-1: 1:800. Double antibody stainings were carried out by mixing the appropriate primary and secondary antibodies for simultaneous incubation. Embryos were washed three times for 20 min before incubation with secondary antibodies. For detection, anti-rabbit biotin (1:300), anti-mouse biotin (1:300), streptavidin–rhodamineB (Molecular probes, S871), anti-mouse IgG-Alexa 488, secondary antibodies were diluted 1:200 in PTF solution and embryos were incubated for 1–2 h at RT followed by three washing steps in the dark. Embryos were adapted to increasing concentrations of glycerol in PBS and mounted in glycerol/DABCO 2.5%/Tris pH8.6 (DTG).

Nuclei were counterstained with DraQ5 (Cell Signaling Technology). Confocal sections were obtained in Leica confocal microscopy SP2 AOBS with 40× objective. Images were pseudocolored as follows: Rex-1 in red (embryos) and green (cells), Cdx-2 in green (embryos) and DraQ5 in blue (cell nuclei).

ES cells were cultured on gelatin-coated coverslips for at least 4 h to attach. Cells were fixed with 2% parafomaldehyde in PBS (pH 7.4), permeabilized in 0.5% Triton-X100 for 5 min and blocked for 30 min in 0.1% Tween-20 in PBS containing 2% BSA and 5% normal calf serum at RT. Incubation with primary and secondary antibodies as well as mounting was performed as described above. Slides were counterstained with DraQ5 (Invitrogen) or DAPI and mounted in DTG as above. Membranes were counterstained using a Wheat Germ Agglutinin Conjugate (Alexa Fluor 594, Molecular Probes), dilution 1:1000.

**RT-PCR analysis**

Cells were washed with PBS, cells were scraped and total RNA was extracted using TRiZol® reagent (Invitrogen). After digestion of genomic DNA (RQI RNase-Free DNase, Promega), phenol-chloroform extraction and ethanol precipitation were performed to obtain total RNA. Absence of RNA degradation was confirmed by visualization in agarose gel. RNA concentration was determined with Nanodrop (Thermo Scientific). cDNA was synthesized from 2 μg of RNA either with Oligo dT or random hexamer primers (ThermoScript® RT-PCR System, Invitrogen). RNA was degraded with RNase H treatment (Invitrogen kit). cDNA was analyzed by PCR (Taq DNA polymerase, Roche) or quantitative PCR (Platinum® SYBR® Green qPCR SuperMix-UDG, Invitrogen) on a ABI Prism 7000 Real-Time PCR system and analyzed using the accompanying SDS Software version 1.2.3. Table 2 lists the primers used. PCR products were visualized in 2% agarose gel. Control primers were taken from His2AZ (Jeong et al., 2005).

**Chromatin immunoprecipitation (ChIP)**

5×10⁶ cells were chemically crosslinked by the addition of 1% of final volume of formaldehyde solution for 10 min at room temperature. Cells were rinsed twice with 1× ice-cold PBS and scraped in 1 ml of ice cold PBS. Cells were pelleted at 2000 rpm at 4 °C for 5 min. The cell pellet was resuspended in lysis buffer (50 mM Tris–HCl pH 8.1, 1% SDS, 10 mM EDTA) and sheared to an average length of 800 bp by sonication on ice in a Diagenode Bioruptor® UCD-200 with the following settings: high duty cycle, 8 cycles of 30 s ON/30 s OFF. The sample was then centrifuged at 13000 rpm for 10 min at 4 °C to remove membrane and nuclear debris. The supernatant was diluted 10 times with dilution buffer (16.7 mM Tris–HCl pH 8.1, 167 mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA). For No IP control we take a 5% of total chromatin supernatant and proceed to crosslink reversal as described below.

Protein G magnetic Dynabeads (Dynal-Invitrogen) (100 ml were preloaded with 75 mg anti-Rex-1 IgG for 2.5 h at 4 °C in rotation in lysis/dilution (1:9) buffer. The beads were washed twice and incubated overnight with diluted chromatin at 4 °C on a rotating wheel. In control experiments, αRex-1 serum was replaced by an equal amount of Preimmune
serum or Rabbit Anti-mouse IgG (Jackson IR lab, No. 315-001-003).

Beads were successively washed with buffer I (20 mM Tris–HCl, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA), buffer II (20 mM Tris–HCl, 500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA), buffer III (10 mM Tris–HCl, 250 mM LiCl, 0.1% SDS, 1% Triton X-100, 1 mM EDTA) and buffer IV (50 mM Tris–HCl, 2 mM EDTA, 0.2% sarkosyl).

Elution was carried out in 1% SDS, 0.1 M NaHCO₃ buffer for 30 min and crosslink were reversed by adding 200 mM NaCl and overnight heating at 65 °C, now including No IP control. Then the samples were treated with proteinase K and RNAse, followed by phenol/chlorophrom purification and ethanol DNA precipitation. DNA pellets were resuspended in TE buffer and stored at −20 °C or processed for PCR.

PCR was performed on chromatin immunoprecipitated from equivalent amounts of cells using a Biometra TGradient thermal cycler using the following parameters: 36 cycles of (94 °C 45 s., 60 °C 45 s., and 72 °C 45 s) primers used are listed in Table 1. The PCR products were loaded on 2% agarose gels, visualized using ethidium bromide and photographed on a Gel Doc transiluminator (BioRad).

Yeast two hybrid screen and interaction assays

DNA binding domain and activation domain fusion proteins were expressed in yeast from the plasmids pAS2-1 and pGAD10 (Clontech), respectively. PCR products or cDNAs were digested with BamH1 and EcoRI, and ligated in frame to the GAL4 DNA binding domain into the yeast expression vector pAS2-1 (CLONTECH) or fused in frame to the GAL4 DNA binding domain. The junctions were sequenced to verify the reading frame. pAS2-1 has TRP1 as a selectable marker, allowing growth in the absence of tryptophan. Detailed information about all plasmids used is available from the authors on request.

Bait and prey plasmids were introduced by LiAc transformation into the Saccharomyces cerevisiae Strain HF7c [MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4-542, gal80-538, LYS::GAL1-HIS3, URA3::(GAL4 17-mers)]:CYC1-lacZ] as described previously (Schoorlemmer et al., 1997). After overnight recovery in yeast complete medium (-Trp-Leu ), the transformants were plated on selective medium for histidine prototrophy (Trp-Leu-His). Cells were grown in medium lacking tryptophan and then transfected with a AD-domain fusion. Transfectants were grown on SD/-trp/-leu/-his/3-AT selection plates lacking tryptophan, leucine and histidine and containing 3 mM 3- aminotriazol (3-AT). After standard transformation, single colonies were picked and duplicates of each bait-prey pair tested for viability on -Trp-Leu medium, and for complementation of the His mutation on -His plates in the presence of 3AT.

Co-immunoprecipitation

In vivo GST pull-down assays were adapted from a previous description (Garcia et al., 1999). Human embryonic kidney 293 T cells were transiently co-transfected on PolyDLysin-coated dishes with a mixture of plasmids expressing HAtagged YY1, YY2 or Rex-1 and GST-tagged Ring1A and Rnf2 constructs using FuGene Transfection reagent (Roche). The empty pEBG vector was used as a negative control. The day after transfection, cells were rinsed once with 1× ice-cold PBS and cells were scraped in 40 mM HEPES pH 7.6, 200 mM NaCl, 0.1% NP40, complete protease inhibitors without EDTA (Roche). Cells were lysed by standard sonication and lysates were spun at 4 °C for 10 min in an Eppendorf centrifuge at 12,000 rpm. The supernatant was preclared for 1 h with protein G-Sepharose. The supernatants were mixed with Table 2

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward (5’→3’)</th>
<th>Reverse (5’→3’)</th>
</tr>
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<tbody>
<tr>
<td>Rex-1/Zfp42</td>
<td>AAGCCGTATACGTCGACGCTGCAGGCT</td>
<td>ATGGGTGTTAATCCCGATGCTGTCTCAT</td>
</tr>
<tr>
<td>His2AZ</td>
<td>CGTATCACCCTCGTCCTTT</td>
<td>AAGCCCTACCTCGTCCTAAA</td>
</tr>
</tbody>
</table>
40 μl of GSH-Sepharose (50% packed volume) previously incubated with 0.5% non-fat dried milk. After incubation for 1 h at 4 °C with continuous rotation, the beads were washed in lysis buffer, and bound proteins eluted in Laemmli sample buffer, separated by SDS-PAGE prior and analyzed by standard Western blot.

A similar immunoprecipitation procedure was applied to E14T ES cells except that only HA-tagged gene constructs were used for transient transfection, and extracts were prepared according to the ChIP method outlined above.

Supplementary materials related to this article can be found online at doi: 10.1016/j.scr.2011.02.005.

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References


Association of Rex-1 to target genes supports its interaction with Polycomb function


