Epigenetic marking and repression of porcine endogenous retroviruses

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INTRODUCTION

Endogenous retroviruses (ERVs) are remnants of retroviral germ line infections and have been identified in all mammalian genomes investigated so far (Weiss, 2006). In response to deleterious effects exerted by replicating ERVs and other transposable elements, a wide range of counteracting defence mechanisms have evolved. DNA methylation was the first epigenetic modification that has been associated with transcriptional ERV silencing (Yoder et al., 1997). Chemical inhibition of DNA methylation in cultured murine embryonic fibroblasts (MEFs) was shown to increase expression levels of intracisternal type-A particle (IAP) and virus-like 30 (VL30) ERVs (Brunmeir et al., 2010).

More recently, DNA methylation-independent ERV repression mechanisms have been described (Leung & Lorincz, 2012). In murine embryonic stem (ES) cells, ERVs were found to be marked by repressive trimethylations of lysine-9 on histone 3 (H3K9me3) and lysine-20 on histone 4 (H4K20me3) (Mikkelsen et al., 2007). Depletion of the histone methyltransferase SETDB1 in murine ES cells resulted in a strong reduction of the H3K9me3 and H4K20me3 marks at ERV genomes (Matsui et al., 2010). Although knockout of the two H4K20 histone methyltransferases in murine ES cells led to a loss of the H4K20me3 mark at ERVs, the H3K9me3 marks remained largely unaffected and no ERV upregulation was observed (Matsui et al., 2010). ERV silencing by histone methylation seems to be cell-type specific since the H3K9me3 and H4K20me3 marks were not significantly enriched at ERVs in MEFs and neural progenitor cells (Mikkelsen et al., 2007). Recently, knockdown of the transcriptional co-repressor TRIM28 (KAP1/TIF1β) was shown to increase expression of various ERVs in murine ES cells (Rowe et al., 2010). Interestingly, TRIM28 was also identified as a component of repressor complexes that silences exogenous retroviruses in murine embryonic carcinoma (EC) and ES cells through binding to the primer-binding site (PBS) of integrated proviruses (Wolf & Goff, 2007; Wolf et al., 2008).

Three supplementary tables are available with the online version of this paper.
Studies of ERV silencing have so far almost exclusively focused on mice due to limitations in the accessibility of suitable ES or ES-like cell models from other mammals. However, porcine ERVs (PERVs) are of special interest since viral particles derived from PERVs are able to infect and replicate in human cells (Czauderna et al., 2000; Patience et al., 1997). Although trans-species infections of PERVs to humans have not been reported in porcine-cell or organ-transplantation trials (Meije et al., 2010; Mueller et al., 2011), the potential trans-species infection of PERVs remains a major concern since xenotransplantations from pigs to humans are widely investigated to alleviate the shortage of human donor organs (Boneva & Folks, 2004). Three replication-competent endogenous gamma-retrovirus PERV-γ1 subgroups (PERV-A, -B and -C) have been identified in the pig genome (Le Tissier et al., 1997; Patience et al., 1997). Two groups have independently reported the existence of an additional PERV-γ family, designated PERV-E (Mang et al., 2001) or PERV-γ2 (Patience et al., 2001). Although other partial PERV sequences have been identified in the pig genome, these elements have not been analysed in detail (Ericsson et al., 2001; Klymiuk et al., 2002; Mang et al., 2001; Patience et al., 2001).

In this study, we attempt to investigate the effect of epigenetic repression on PERVs in cultured porcine cells. So far, no long-term porcine ES cell line with stable pluripotent characteristics has been established (Brevini et al., 2008; Keefer et al., 2007). We therefore included isolated porcine embryonic germ cells (pEGCs) in our analysis since these cells resembled ES cells in their morphology and showed alkaline phosphatase activity, a marker for pluripotency (Shim et al., 1997). However, a recent genome-wide transcriptome analysis of pEGCs revealed that, although the majority of pluripotency genes were expressed at levels comparable to murine ES cells, expression of some of the core pluripotency markers could not be detected (Petkov et al., 2011). Nevertheless, these pEGCs formed embryoid bodies in suspension culture and could be differentiated into various cell types (Petkov et al., 2011).

To gather the required sequence information for our further analysis, we first performed a genome-wide screen for retroviral sequences in the pig genome and characterized several groups of PERVs. We then tested potential PERV reactivation upon inhibition of DNA methylation and histone deacetylation, and analysed chromatin modifications at PERV elements. Finally, we investigated the repression activity of PBS sequences frequently used by replication-competent PERVs in porcine and murine cells in retroviral transduction assays.

**RESULTS**

**De novo identification of PERVs in the pig genome by LTRharvest**

Although the main object of this study was to investigate epigenetic repression of PERVs, we found it necessary to start with a computational analysis of PERV families. To identify known and possible new PERV families, we decided to perform a screen of the entire available pig genome using LTRharvest (Ellinghaus et al., 2008), an algorithm developed for the de novo identification of retrotransposons and ERVs in complex genomes. LTRharvest detects potential LTRs based on their sequence similarity and distance to each other and should therefore facilitate the detection of full-length ERVs in a relatively unbiased manner. Applying the criteria described in Methods, 2358 elements were retrieved from LTRharvest and screened for retroviral protein domains available in the Pfam database (Table S1, available in JGV Online). In some elements, more than ten retroviral Pfam domain families were identified, indicating that these selected domains are representative for ERVs and can be also found in PERVs. However, in 94.4% of the putative PERV elements, none or only up to two retroviral domains could be identified. We therefore excluded these elements and continued our analysis with the 156 elements that matched three or more retroviral protein domains.

These 156 candidate elements were screened for homologies to published PERV sequences and to each other in order to cluster these sequences into families. Besides elements matching the known PERV-γ1, PERV-γ2 and PERV-β3 families, several elements showed strong homologies to an ERV element in the Repbase Update database, which was deposited as ERV1N-2_Ssc-I and is described as a non-autonomous ERV1-type element (Table 1). Seven elements matched to other published PERV-γ and PERV-β sequences, but were only present in single or few copies. The remaining 66 candidate elements did not show strong homologies to known PERV sequences and could not be

<table>
<thead>
<tr>
<th>PERV family</th>
<th>No. of identified elements</th>
<th>tRNAs matching PBS</th>
<th>Mean LTR divergence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PERV-A</td>
<td>13</td>
<td>Gly</td>
<td>0.0063</td>
</tr>
<tr>
<td>PERV-B</td>
<td>8</td>
<td>Gly</td>
<td>0.0014</td>
</tr>
<tr>
<td>PERV-C</td>
<td>2</td>
<td>Pro</td>
<td>0.0017</td>
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<tr>
<td>PERV-γ2</td>
<td>36</td>
<td>Gly</td>
<td>0.0303</td>
</tr>
<tr>
<td>PERV-β3</td>
<td>10</td>
<td>Lys</td>
<td>0.0058</td>
</tr>
<tr>
<td>ERV1N-2</td>
<td>14</td>
<td>Thr</td>
<td>0.0321</td>
</tr>
</tbody>
</table>

Table 1. PBS usage and LTR divergence in identified PERV elements
clustered into families based on sequence similarity. The identified PERV-γ1 elements were further categorized into the three known PERV-A, PERV-B and PERV-C subgroups based on sequence similarities with published PERV-γ1 genomes.

Characterization of identified PERV families

As in previously sequenced PERV-γ1 genomes, the two identified PERV-C elements contained a PBS complementary to porcine proline-tRNAs, whereas all identified PERV-A and PERV-B elements contained PBS sequences complementary to glycine-tRNAs directly downstream of the 5' LTR. PBS sequences complementary to glycine-tRNAs were also identified in PERV-c2 elements. A BLAST search of the sequences adjacent to the 5' LTR against the 3' ends of pig tRNAs revealed the presence of 18 nt long PBS sequences complementary to several lysine- and threonine-tRNAs in PERV-β3 and ERV1N-2 elements, respectively (Table 1).

Next, we inferred consensus sequences for the four identified PERV families and screened these sequences for ORFs of at least 300 codons in length and retroviral Pfam domains. ORFs coding for retroviral Gag, Pol and Env proteins were found in the PERV-c1 and PERV-c2 consensus sequences (Fig. 1a, b). In PERV-β3, we identified an ORF in the region associated with Gag domains and an obviously truncated ORF covering some of the identified Pol domains (Fig. 1c). No ORFs could be determined in the ERV1N-2 consensus sequence or any of the 14 identified genomic ERV1N-2 copies (Fig. 1d). Interestingly, we found Gag, Pol and Env domains in PERV-γ1, PERV-γ2, PERV-β3 and ERV1N-2, but only Gag and Pol domains in PERV-β3 elements. Moreover, no homology to any known protein was found in the region downstream of the PERV-β3 integrase domain by tblastx search against all available protein sequences in the National Center for Biotechnology Information (NCBI) database.

Based on the ORFs identified in the consensus sequences, coding regions from individual PERV elements were determined. The majority of the identified PERV-β3 elements contained full-length coding regions for the Gag protein, but in six elements the pol gene was disrupted by a large deletion. Although the deletion in the pol gene was shared by several PERV-β3 elements, we did not find indications for chromosomal duplication events within this family (Fig. 1c). The coding regions within PERV-γ1 and PERV-β3 elements were mainly intact with only few premature stop codons (Fig. 1a, c) whereas, in accordance with a previous study (Nascimento et al., 2011), the majority of the PERV-γ2 coding regions were disrupted by numerous premature stop codons (Fig. 1b).

Since the 5' and 3' LTRs of newly integrated retroviruses are identical, the divergence between these regions can be used to determine the time that has passed since ERV integration (Martins & Villesen, 2011). Although this method is biased by non-uniform mutation rates and
recombination events (Hughes & Coffin, 2001, 2004), it should allow an approximate estimation of the differences in the integration times of different ERV families within a genome. In accordance with previous reports (Tönjes & Niebert, 2003), PERV-γ1 elements contained highly similar LTRs, indicating a relatively recent integration time of these proviruses (Table 1). Surprisingly, also PERV-β3 proviruses had highly similar, and in two elements even identical LTRs, indicating that these elements have integrated relatively recently in history. The mean PERV-γ2 and ERVIN-2 LTR divergences were about five to six times higher than the LTR divergences in PERV-A and PERV-β3 elements (Table 1).

**Up- or downregulation of PERVs in porcine cells treated with inhibitors**

Since little is known about the transcriptional activity of PERV-γ2 and PERV-β3 elements, we analysed expression levels of these PERVs in pEGCs, neonatal fibroblasts (YUC8) and PK15 cells, a porcine epithelial kidney cell line, using specific primer sets. The primers used to detect expression of PERV-γ1, PERV-γ2 and PERV-β3 elements were predicted by *in silico* PCR to amplify transcripts expressed from 22, 21 and 18 genomic loci, respectively (Table S2). As shown in Fig. 2(a), whereas PERV-γ1 elements were highly expressed in PK15 cells and moderately expressed in pEGC and YUC8 cells, PERV-γ2 and PERV-β3 elements were expressed at levels below the low-copy housekeeping gene *Hprt* in all tested cell types.

To investigate whether epigenetic modifications such as DNA methylation or histone deacetylation control PERV expression, exponentially growing porcine fibroblasts and PK15 cells were treated with the DNA methyltransferase inhibitor 5-aza-2’-deoxycytidine (Aza-dC) and Trichostatin A (TSA), an inhibitor of histone deacetylation. The efficiency of Aza-dC treatment was determined by RT-quantitative PCR (qPCR) analysis of Deleted in Azooospermia-Like (*Dazl*) mRNA expression, which was previously reported to be upregulated in porcine fibroblasts treated with Aza-dC (Linher et al., 2009). *Dazl* expression was undetectable or at the limit of detection in the three cell types, and was induced following treatment with Aza-dC or Aza-dC and TSA (Fig. 2b). Expression of the PERV-B subfamily was increased by about 3.5-fold in YUC8 fibroblasts treated with Aza-dC and increased by more than twofold in PK15 cells treated with 1.5 μM TSA or TSA and Aza-dC combined but not with Aza-dC alone (Fig. 2c). Interestingly, in YUC8 cells treated with TSA, PERV-A and PERV-C expression was strongly reduced, whereas PERV-B, PERV-γ2 and PERV-β3 expression remained relatively unaffected. Since we were unable to find a suitable positive control for TSA-induced gene expression in pig cells, MEFs and NIH/3T3 cells were treated with TSA from the same stock as used for the porcine cells at various concentrations. The previously reported (Brunmeir et al., 2010) IAP and VL30 reactivation could be readily detected at a wide range of TSA concentrations in these cells (data not shown).

**PERVs are marked by repressive histone methylations**

Endogenous retroviruses are marked by the repressive histone methylations H3K9me3 and H4K20me3 in murine ES cells (Matsui *et al.*, 2010; Mikkelsen *et al.*, 2007). However, these chromatin modifications have not been reported for PERVs yet. To analyse histone modifications at PERV loci we designed PERV-specific primers based on the sequence information obtained by our *in silico* analysis and performed chromatin immunoprecipitation (ChIP) in two porcine cell types. We were unable to perform ChIP successfully with primary porcine fibroblasts due to reproducible lack of appropriate sonication of these fibroblast samples. Cross-linked chromatin isolated from pEGC and PK15 cells was immunoprecipitated with antibodies against various histone modifications to detect enrichment of these marks at retroviral loci.

In both cell types, we detected enriched levels of the repressive histone methylations H3K9me3 and H4K20me3 at all tested PERV loci as compared with a region around the transcriptional start site of the *Gapdh* gene, which was included as a control region in open chromatin formation (Fig. 3a, b). Although the nucleosome density, as determined by ChIP with an antibody against histone 3 (H3), was generally higher at PERV loci (Fig. 3c), H3 enrichment was less pronounced than the enrichment of H3K9me3 and H4K20me3, indicating that the observed higher levels of the H3K9me3 and H4K20me3 marks at PERVs are not merely the result of the higher nucleosome density at these regions. Interestingly, PERV-γ1 elements were generally marked by higher levels of repressive histone methylations than PERV-γ2 and PERV-β3 in pEGCs (Fig. 3a, b). Acetylation of lysine-9 in H3 (H3K9ac) is a marker of open chromatin and gene expression. As expected, this histone modification was found at the promoter region of the constitutively expressed *Gapdh* gene but was mainly absent at PERV loci (Fig. 3d). However, low levels of H3K9 acetylation could be detected at the 5’ UTR and the *gag* gene of PERV-γ1 elements in PK15 cells.

**Transduction efficiency of retroviral vectors with modified PBS sequences**

In mice, a repressor complex targets the PBS of integrated retroviruses and induces transcriptional silencing in EC cells and MEFs (Barklis *et al.*, 1986; Haas *et al.*, 2003). Recently, the KRAB zinc finger protein ZFP809 was identified as the DNA-binding factor that recruits the repressor complex to a 17 bp-long repressor-binding site that overlaps with the retroviral PBS complementary to a proline-tRNA (Pro-PBS) (Wolf & Goff, 2009). We have previously shown that murine leukemia virus (MLV)-based retroviral vectors are able to utilize different PBS sequences to prime reverse transcription (Lund *et al.*, 1993), and most
of these PBS variants escape repression in murine EC cells (Modin et al., 2000). The Pro-PBS used by PERV-C elements is identical to the Pro-PBS that was shown to induce repression in mice. We therefore wanted to test whether active PERVs might be repressed via their PBS in pig cells. Since we did not find any potential ZFP809
homologue in the pig genome by BLAST search and it is possible that different PBS sequences are repressed in mice and pigs, we included two PBS (Gly1 and Gly2) sequences, which we found to be commonly used by the PERV-A, PERV-B and PERV-γ2 elements identified in our screen. Both of these sequences perfectly match to the 3′ end of different porcine, murine and human glycine-tRNAs.

To analyse PBS-dependent repression, we created retroviral plasmid vectors (pPBS-Gly1 and pPBS-Gly2) by replacing the Pro-PBS of pPBS-Pro, an Akv-MLV-derived retroviral vector with a neomycin resistance marker (Modin et al., 2000), with these new PBS sequences (Fig. 4a). A repression escape vector (pPBS-B2) that harbours a single mutation in the Pro-PBS (Barklis et al., 1986) was included as a control. Three primary porcine cell types and two murine cell lines (F9 EC cells and NIH/3T3 fibroblasts) were included in the transduction assay. F9 EC cells are known to repress the Pro-PBS vector but not the B2-PBS mutant, whereas NIH/3T3 cells are permissive for both vectors (Modin et al., 2000). Transductions in NIH/3T3 cells were therefore performed in parallel to normalize differences in viral particle production. The titre of a vector in NIH/3T3 cells divided by the titre of the same vector in a tested cell type indicated the normalized repression activity (hereafter referred to as repression index).

All four tested vectors transduced NIH/3T3 cells with comparable titres, showing that reverse transcription could be efficiently primed by glycine-tRNAs. As expected, the

![Fig. 3. ChIP-qPCR analysis of histone modifications at PERVs in pEGCs and PK15 cells. Cross-linked chromatin was immunoprecipitated with antibodies against (a) H3K9me3, (b) H4K20me3, (c) H3, (d) H3K9ac or (e) an unspecific control antibody (IgG). Enrichment of retroviral sequences or a control sequence within the Gapdh promoter region in the immunoprecipitated samples was determined by qPCR. Data shown are the mean ± SD ChIP enrichment values relative to the input control (n=3, technical replicates).](image-url)
PBS-Pro vector transduced F9 cells poorly, whereas the mutated PBS-B2 vector escaped repression (Fig. 4b). Comparable repression indices were determined for the PBS-Gly1, PBS-Gly2 and PBS-B2 vectors, indicating that these glycine PBS sequences were not immediately repressed in F9 cells. All four vectors transduced porcine fibroblasts and pEGC with similar titres and low repression indices (Fig. 4b), indicating that none of these PBS sequences induced immediate transcriptional repression in the tested porcine cells.

DISCUSSION

Previous studies have indicated that PERV-γ1, PERV-γ2 and PERV-β3 elements have entered the pig germ line after the divergence of the family Tayassuidae lineage about 20 million years ago, but before speciation within the family Suidae lineage (Niebert & Tönjes, 2005; Patience et al., 2001). Whereas PERV-γ1 remained active up to the present and may still increase in copy numbers by reinfection, all PERV-γ2 elements seem to have lost their capacity to replicate (Nascimento et al., 2011). Our ORF conservation and LTR divergence analysis of the PERV-β3 family suggests that, although these elements seem to have largely lost their transcriptional activity, at least some PERV-β3 elements may have remained active until recently or even to the present. The observed lack of coding regions for Env proteins in these elements further suggests that PERV-β3 may replicate via Env-independent replication mechanisms such as retro-transposition (Jungmann & Tönjes, 2008), co-packaging (Besmer et al., 1979) or in trans complementation of the Env protein by exogenous retroviruses (Magiorkinis et al., 2012).

It has been shown that the transcriptional activity of in vitro DNA methylated PERV-γ1 LTRs driving transient expression of a reporter gene is strongly reduced in PK15 cells (Park et al., 2010). However, in a previous study, no PERV-γ1 reactivation was reported in adult porcine islets and islet-like cell clusters upon 5-azacytidine treatment (Schmidt et al., 2005). Here, we have shown that chemically induced epigenetic chromatin modifications affect PERV expression in a specific manner. As shown for murine ERVs (Brunmeir et al., 2010), the potential for PERV reactivation seems to vary strongly between PERV families and possibly depends on yet-to-be identified cis-acting retroviral control elements and cellular factors, as discussed previously (Brunmeir et al., 2010). In pEGCs and PK15 cells, the lack of PERV reactivation upon Aza-dC treatment may be explained by the observed repressive histone methylations at these elements. Importantly, TSA inhibits histone deacetylases but not histone methylases and may therefore not be sufficient to induce ERV reactivation in cells that repress ERVs by histone methylation. Unfortunately, chemical inhibitors of H3K9 trimethylation that could be used to induce a potential PERV reactivation in these cells have not been identified so far (Wagner & Jung, 2012).

YUC8 fibroblasts and PK15 cells were derived from a Yucatan minipig and a Hampshire pig, respectively. Although these domestic European pig breeds are closely related and therefore are most probably derived from the same wild pig ancestor (Jiang et al., 2008), it is possible that they harbour polymorphic PERV-γ elements that differ in their transcriptional activities. Therefore, these two cell types cannot easily be compared in terms of PERV expression and reactivation. It

Fig. 4. Transduction efficiency of retroviral vectors using different PBS in murine and porcine cells. (a) Outline of the PBS exchange approach: primers P2 and P3 contain the new PBS sequence (dashed line) at the 5' end and anneal up- and downstream, respectively, of the PBS on the retroviral vector plasmid. PCR products amplified with the primer pairs P1/P2 and P2/P3 were connected using primers P1 and P4, which anneal to regions further up- and downstream, respectively, of the PBS. The original and newly introduced PBS sequences are shown on top of the vector graphic with nucleotides differing from the Pro-PBS marked in grey. (b) Murine NIH/3T3 and F9 EC cells, and porcine pEGC, primary fetal fibroblasts (YUC2) and primary neonatal fibroblasts (YUC8) were transduced in parallel with serial dilutions of vesicular stomatitis virus-G (VSV-G) pseudotyped viral particles containing vectors with different PBS sequences. Viral titres (c.f.u. ml⁻¹) were determined by counting resistant colonies after 10–14 days of selection with G418. The repression index for each vector was defined as the ratio of the viral titre in NIH/3T3 cells to the titre in the indicated cell line. Transductions were performed in triplicate; mean ± SD values are shown.
will be interesting to investigate whether fibroblasts derived from European and Asian pig breeds, which are believed to have originated from different wild pig ancestors (Kijas & Andersson, 2001), show differences in PERV reactivation on DNA demethylation and histone deacetylation.

We showed that PERVs are marked by the repressive histone methylations H3K9me3 and H4K20me3. These marks were not restricted to embryonic germ cells but were also apparent in porcine embryonic kidney cells, indicating that certain highly differentiated cell types may also retain the little understood cellular properties required for ERV silencing by histone methylations. Interestingly, the only presently active PERV family, PERV-γ1, was not only expressed at the highest levels in all tested cell types, but also generally marked by higher levels of histone methylation than PERV-γ2 or PERV-β3 elements. This observation supports the 'genome defence theory' which predicts that young, active ERVs are more strongly repressed than already inactivated elements (Huda et al., 2010). Also in murine ES cells, active ERVs were found to be marked by higher levels of H3K9me3 and H4K20me3 than other LTR elements (Mikkelsen et al., 2007). However, PERV-γ1 elements seem to partially escape repression in PK15 cells, as indicated by relatively high expression levels and some degree of H3K9 acetylation. It remains unclear whether the observed conjoined presence of H3K9 acetylation and trimethylation indicates that those two marks differ from cell to cell in a stochastic manner or is rather an effect of differences in the epigenetic status of individual insertions, possibly influenced by the surrounding chromatin environment and nearby cellular promoter and enhancer elements.

The observed lack of a PBS-targeted repression mechanism in porcine cells is inconclusive since putative PBS targeting proteins may merely not be sufficiently expressed in the pEGCs and porcine embryonic fibroblasts analysed in our study. Nevertheless, the absence of a ZFP809-like repressor that targets the Pro-PBS in pigs would not be unexpected since we found that only one subgroup of a low copy PERV-γ1 family (PERV-C) contains intact Pro-PBS sequences. PERV-C elements have entered the pig germ line relatively recently, as indicated by the observation that PERVs have not been fixed in pigs (Fujimura et al., 2008; Mang et al., 2001). Therefore, the presence of a Pro-PBS-binding repressor in pigs seems unlikely since the evolution of a target-specific retroviral restriction factor would require a selective pressure that constantly affects the fitness of a species for many generations. However, the oldest PERV-γ1 and PERV-γ2 elements are believed to have colonized the pig genome several million years ago (Tönjes & Niebert, 2003), yet their commonly used glycine-PBS sequences were not repressed in the analysed cell types. Although awaiting further confirmation, our results therefore indicate that PBS-targeting retroviral repression mechanisms may not have evolved in pigs.

In conclusion, we have shown that PERVs are selectively regulated by chemical inhibitors of DNA methylation and histone deacetylation and marked by repressive histone methylations in porcine cells. Our results and the ongoing progress in the generation of pluripotent porcine cell lines will facilitate further studies addressing functional and possibly medically relevant aspects of the epigenetic mechanisms that control PERV expression.

METHODS

**In silico analysis of PERVs in the Sus scrofa genome.** All sequenced chromosomes of the Sus scrofa genome assembly (Suscrofa9.2/susScr2) were downloaded from the University of California, Santa Cruz (UCSC) Genome Browser and analysed by LTRharvest (Ellingshaus et al., 2008). The program was run at default settings with the following changes: the minimum length of each putative LTR was set to 350 bp and the minimum distance between the 5’ and 3’LTR was increased to 4 kb. Candidate elements were screened by tblastx BLAST search against a local library of 21 domain families constituted by 1011 seed protein sequences that had been inferred from alignments of all family member sequences in the UniProt database and are available on the Pfam database (http://pfam.sanger.ac.uk) (Table S1). The library was based on Pfam domains used in a previous ERV screen (Steinbiss et al., 2009), but slightly modified. The maximum e-value for the tblastx BLAST search was set to 1 × 10⁻9. The following published partial PERV sequences were used for BLAST searches: PERV-γ1 (GenBank accession no. AF274705), PERV-γ2 (AF274706), PERV-γ3 (AF274707), PERV-γ4 (AF274708), PERV-γ5 (AF274709), PERV-γ7 (AF511111), PERV-γ8 (AF511112), PERV-γ9 (AF511113), PERV-γ10 (AF511114), PERV-β1 (AF274710), PERV-β2 (AF274711), PERV-β3 (AF274712), PERV-β4 (AF274713) and PERV-β5 (AF511115). Additional PERV sequences were downloaded as FASTA files from the Repbase Update database (http://www.girinst.org). All BLAST searches were performed using the CLC Main Workbench program. Sequence alignments were performed using the MUSCLE alignment tool in the MEGAS package (Tamura et al., 2007).

**Cell culture.** Feeder-free pEGCs (passage 8), derived from male Yucatan minipig embryos, porcine primary fetal (YUC2) and new-born fibroblasts (YUC8) were kindly provided by Dr Stoyan Petkov (Petkov & Anderson, 2008). pEGCs were grown for two to three passages on gelatin-coated culture plates in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% FBS and non-essential amino acids (NEAA) (Gibco) before analysis. Porcine primary fibroblasts were grown in DMEM supplemented with 15% FBS and NEAA. Murine F9 EC cells were grown on gelatin-coated culture plates in DMEM supplemented with 10% FBS. Mouse NIH/3T3 fibroblasts were cultivated in DMEM supplemented with 10% new born calf serum. The porcine kidney cell line PK15 (CCl-33) was purchased from the ATCC and cultivated in DMEM supplemented with 10% FBS. All culture media were supplemented with 1% penicillin and streptomycin (Invitrogen). A summary of the used cell lines is shown in Table S3.

**Inhibitor treatments.** TSA was applied to exponentially growing cells at concentrations of 0.5 or 1.5 μM for 24 h. Aza-dC was applied at concentrations of 1 or 3 μM for 24 h followed by additional incubation for 24 h in fresh medium without inhibitors. Higher concentrations of TSA and Aza-dC were tested but generally resulted in growth inhibition and cell death. Combined treatment with Aza-dC and TSA was performed by growing cells in medium with 1 μM Aza-dC for 24 h, followed by treatment with 0.5 μM TSA for an additional 24 h.

**PERV expression analysis.** Total RNA was extracted from exponentially growing cells using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RNA was purified from possible DNA contaminations using the DNA-free kit (Ambion). Reverse transcription was carried out with the RevertAid H Minus
First strand cDNA synthesis kit (Fermentas) and random hexamer primers. For expression analysis, quantitative PCR (qPCR) was performed on an Mx3000P thermocycler (Stratagene) using Platinum SYBR Green qPCR SuperMIX UDG (Invitrogen). Amplification efficiency of each primer pair was determined using dilution series of cDNA or DNA samples as template. Target specificity of primers was confirmed by amplicon size and melting curve analysis. Changes in expression were calculated based on cycle numbers (Ct values) necessary for detection of PCR products and primer efficiencies. Primer sequences are given in Table S2.

ChIP. Preparation of soluble chromatin was carried out using the MACNify Chromatin Immunoprecipitation kit (Invitrogen) according to the manufacturer’s instructions. Cross-linked chromatin was sheared for 15 min in a Covaris S2 sonicator under the following settings: 200 cycles/burst, 20 % duty cycle, intensity: 8. Successful chromatin sonication to fragments of 200–500 bp length was monitored by fragment length analysis of the reverse cross-linked chromatin samples on agarose gels. Sheared chromatin from 2 × 10^5 cells was used per ChIP. Following antibodies for immunoprecipitation of unmodified or modified histones H3 and H4 were purchased from Abcam: H3 (ab1791), H3K9me3 (ab8898), H4K20me3 (ab9053) and H3K9ac (ab4441). A rabbit IgG antibody (Invitrogen) was used as negative control to determine background signals caused by non-specific chromatin binding. Per ChIP, 1 μg (H3, H3K9me3, H4K20me3 and IgG) or 2 μg (H3K9ac) of antibody was applied. Enrichment of specific ERV sequences in the immunoprecipitated samples was determined by qPCR with Platinum SYBR Green qPCR SuperMIX UDG (Invitrogen) using a ChIP input control for normalization. Amplification efficiency of each primer pair was determined by qPCR using a dilution series of the input control. Primer sequences are given in Table S2.

Plasmids and vectors. The Akv MLV-derived retroviral plasmid vectors pPBS-Pro and pPBS-B2 were described previously (Modin et al., 2000). The vectors using PBS complementary to distinct ERV sequences were digested with EcoRI and ligated into the pRSV-Pio vector, containing the new PBS sequences at their 3’ end. Two PCR products were amplified from the pPBS-Pro vector template using the Gly1-PBS or Gly2-PBS containing primers and primers that anneal to the 5’ LTR (P1) and the 5’ UTR (P4) downstream of the promoter. The two amplicons were connected by PCR using equal amounts of the two PCR products as template and the primers P1 and P4. Merged amplicons containing the new PBS sequence were digested with EcoRI and SpeI and ligated into the digested pPBS-Pro vector. The following primers were used for the PBS exchange: P1 (GTGAATTCCTTACCTTACGTTTCCCCG); P2 (PTTGGAGACCCCCGCCCAGGG); P3 (PBS-AATGAAAGAC-CCCCAGGCTG); P4 (GTGAAGCTTGGCTGCAGGTCGA). The newly introduced PBS sequences are shown in Fig. 4(a). Successful cloning was confirmed by sequencing. The vectors expressing viral proteins for the production of retroviral particles in 293T packaging cells, pGag-pol-IRES-bsr’ (Morita et al., 2000) and pMDG (VSV-G Env protein) (Naldini et al., 1996) were kindly provided by Dr Toshio Kitamura and Dr Didier Trono, respectively.

Retroviral transduction assay. VSV-G pseudotyped viral vectors were produced in 293T packaging cells. For this, 2.5 × 10^6 293T cells were cotransfected with 4 μg pGag-pol-IRES-bsr, 5.5 μg pMDG and 1 μg of the retroviral plasmid vectors using the calcium phosphate transfection method as described elsewhere (Modin et al., 2000). Growth medium of 293T cells was renewed 1 day after transfection, and cells were incubated for 1 day more before harvesting viral particles. Virus supernatants were filtered through a 0.45 μm filter, and transferred serially diluted to target cells seeded at a density of 5 × 10^5 cells (pEGCs) or 1 × 10^5 cells (F9, NIH/3T3, YUC2 and YUC8) per well on six-well dishes 1 day prior to transduction] in the presence of 6 μg Polybrene (Sigma-Aldrich) ml^-1. G418-containing selection medium was added 24 h post-transduction at concentrations of 0.4, 0.6, 0.8 or 1 mg ml^-1 to F9, NIH/3T3, pEGCs and porcine fibroblasts, respectively. Resistant colonies were counted after 10–14 days of selection to determine viral titres (c.f.u. ml^-1). To confirm identity of the PBS in integrated vectors, resistant F9 and NIH/3T3 colonies were pooled and genomic DNA was extracted for vector sequencing as described previously (Modin et al., 2000).

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