Histone H3 lysine 9 trimethylation is required for suppressing the expression of an embryonically activated retrotransposon in *Xenopus laevis*

Sarah Herberg, Angela Simeone, Mami Oikawa, Jerome Jullien, Charles R Bradshaw, Marta Teperek, John Gurdon & Kei Miyamoto†

Transposable elements in the genome are generally silenced in differentiated somatic cells. However, increasing evidence indicates that some of them are actively transcribed in early embryos and the proper regulation of retrotransposon expression is essential for normal development. Although their developmentally regulated expression has been shown, the mechanisms controlling retrotransposon expression in early embryos are still not well understood. Here, we observe a dynamic expression pattern of retrotransposons with three out of ten examined retrotransposons (αααα, λ-olt z-1 and xretpos(L)) being transcribed solely during early embryonic development. We also identified a transcript that contains the long terminal repeat (LTR) of λ-olt z-1 and shows a similar expression pattern to λ-olt z-1 in early *Xenopus* embryos. All three retrotransposons are transcribed by RNA polymerase II. Although their expression levels decline during development, the LTRs are marked by histone H3 lysine 4 trimethylation. Furthermore, retrotransposons, especially λ-olt z-1, are enriched with histone H3 lysine 9 trimethylation (H3K9me3) when their expression is repressed. Overexpression of lysine-specific demethylase 4d removes H3K9me3 marks from *Xenopus* embryos and inhibits the repression of λ-olt z-1 after gastrulation. Thus, our study shows that H3K9me3 is important for silencing the developmentally regulated retrotransposon in *Xenopus laevis*.

Transposons are mobile DNA elements that have the ability to propagate or translocate in the genome. Retrotransposons use a reverse-transcribed RNA intermediate for their transposition. They can be divided into two major categories: retrotransposons with or without long terminal repeats (LTR- and non-LTR retrotransposons, respectively). For example, after transcription of LTR retrotransposons the produced RNA is reverse-transcribed, leading to the formation of a double-stranded DNA including complete LTRs. This DNA can then be reintegrated into the genome. In order to protect the integrity of the genome, transposable elements (TEs) ought to be silenced in cells. The transcription of many TEs is repressed by DNA methylation or by repressive histone modifications, such as histone H3 lysine 9 trimethylation (H3K9me3). TEs are also silenced post-transcriptionally, for example by RNA interference or by piwi-interacting RNAs in germ line cells.

However, various findings have shown that TEs are not completely silenced in early mouse embryos. Between 15–20% of the early embryonic mouse transcriptome consists of repetitive elements, with LTR
retrotansomons representing 33–49% of these repetitive elements. Furthermore, transposition of the human or mouse L1 retrotansomon was found to occur in transgenic mice and rats during embryogenesis, leading to somatic mosaicism. Additionally, LTRs of retrotansomons can function as alternative promoters and first exons during mouse embryogenesis, forming chimeric transcripts of retrotansomons and protein coding genes. Chimeric transcripts formed by this means are not expressed at the same developmental stages as their conventional non-chimeric counterparts, suggesting that retrotansomons can control developmentally regulated expression of these transcripts.

Some studies found that the expression of retrotansomons in early mouse embryos is important for embryonic development. For example, murine endogenous retrovirus-like (MuERV-L), a mouse LTR retrotansomon that is transcriptionally activated 8–10 h after fertilization, is needed for the development of mouse embryos to the 4-cell stage. Moreover, MuERV-L was found to form chimeric transcripts in mouse embryos and marks the sub-population of embryonic stem cells, which carry the property of 2-cell stage embryos. Another retrotansomon that seems to be important for the embryonic development of mice is LINE-1. The microinjection of morpholino antisense oligonucleotides targeting LINE-1 irreversibly arrests embryonic development of mice. Since the expression of specific TEs in early embryos is essential for normal development, it is important to understand the mechanisms leading to TE activation in early embryonic stages and subsequent TE silencing. DNA demethylation does not seem to play a major role in TE activation in mouse embryos. Instead, histone modifications were shown to regulate expression of TEs. In mouse embryos, retrotansomons are marked by the repressive histone mark H3K9me3 and by the activating histone mark histone H3 lysine 4 trimethylation (H3K4me3), which is lost in parallel to transcriptional repression of retrotansomons.

In contrast to regulation of retrotansomons in mammalian embryos, almost nothing is known about epigenetic mechanisms of retrotansomon regulation in Xenopus embryos. It has been shown that Xenopus has very few bivalent domains marked by both H3K4me3 and histone H3 lysine 27 trimethylation (H3K27me3) to regulate expression of developmentally important genes, being different from mammals and zebrafish embryonic chromatin. Therefore, it is possible that different regulation mechanisms of embryonically active retrotansomons may exist in Xenopus. Moreover, Xenopus embryos allow the collection of a large number of in vivo embryonic cells when zygotic transcription starts. In gene expression database several retrotansomon transcripts of Xenopus laevis could be identified. In addition, the expression of a few retrotansomons was reported to be developmentally regulated. Based on this knowledge, we further investigated the expression of retrotansomons in Xenopus laevis and found retrotansomons whose expression is upregulated in early embryos. These retrotansomons are marked by H3K4me3 and H3K9me3 on their LTRs at different embryonic stages. Enzymatic removal of H3K9me3 marks from Xenopus embryos leads to the enhanced expression of a specific retrotansomon, demonstrating a role of H3K9me3 in the transcriptional repression of the developmentally regulated retrotansomon.

Results
The transcript levels of the retrotansomons 1a11, λ-olt 2-1 and xretpos(L) are upregulated after midblastula transition and downregulated after the gastrula stage. To identify retrotansomons that are specifically activated during early embryonic development, we first performed RNA-sequencing (RNA-Seq) analysis of gastrula embryos (stage 10.5–11.5) and calculated RPKM (reads per kilobase per million mapped reads) values of putative retrotansomons, whose transcript sequences are available in NCBI database (Supplementary Table S1). Their transcripts were detected in gastrula embryos (Supplementary Table S1). Among them, the expression of 10 different putative retrotansomons was examined in eggs and embryos (stage 1–37/38) by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (Fig. 1a,b, Supplementary Fig. S1). Using this method, we are able to determine overall transcript levels of retrotansomons existing in numerous copies at different genomic loci (see Discussion). Three retrotansomons were found, whose transcript levels are upregulated after the midblastula transition and downregulated after the gastrula stage (Fig. 1b,c). These three retrotansomons are 1a11 (GenBank accession number: L11263.1), λ-olt 2-1 (GenBank accession number: AF145965.1) and xretpos(L) (GenBank accession number: AF057166.1). λ-olt 2-1 and xretpos(L) show 95% nucleotide sequence identity in 1595 nucleotides. These retrotansomons start to be activated soon after the midblastula transition. Then, the transcript levels of 1a11, λ-olt 2-1 and xretpos(L) in whole Xenopus laevis embryos increase from the blastula stage (stage 8) to the gastrula stage (stage 12) and gradually decrease after the late gastrula stage (stage 12.5) (Fig. 1b). For this analysis all values were normalized to the total RNA concentrations of each samples. Normalization to the transcript level of cell was calculated based on the total cell number estimation (Supplementary Fig. S3). These results also
Figure 1. Developmentally regulated expression of retrotransposons 1a11, λ-olt 2-1 and xretpos(L) during *Xenopus laevis* embryonic development. (a) Schematic diagram of expression analysis during embryonic development. Embryos were collected at different stages. After RNA extraction and reverse transcription the transcript level was determined by qPCR. The container was drawn by S.H. (b) Relative changes of the transcript levels of 1a11, λ-olt 2-1 and xretpos(L) during embryogenesis in comparison to their transcript levels at the one-cell stage (St1 = 1) (n = 3–14). *pwp1* is a constantly expressed gene that was used as control (n = 1–4). All values were normalized to the overall RNA concentration of the respective sample. Sample RT- is a negative control. Error bars represent SEM. **P = 0.005, *P = 0.021. (c) Schematic diagram of the accumulated transcript levels of 1a11, λ-olt 2-1 and xretpos(L) during embryogenesis, explaining that their expression is upregulated after midblastula transition and shows the highest level at the late gastrula stage. (d) 1a11, λ-olt 2-1 and xretpos(L) are mainly detected as sense transcripts although antisense transcription can also be found. Forward primers were used for reverse transcription to analyze the expression of antisense transcripts from retrotransposons (blue bars), while reverse primers were used as a control (red bars). Upper graphs summarize antisense transcription at stages 1 and 12.5. Lower graphs represent the comparison between sense and antisense transcription although antisense transcription is almost invisible due to its much weaker expression than sense transcription. Relative transcript levels were compared to the transcript levels at the one-cell stage (St1 = 1) (n = 4). (e) Normalization by the cell number also indicates the upregulated expression of retrotransposons after midblastula transition and the downregulated expression after the gastrula stage. Relative changes of the transcript levels of 1a11, λ-olt 2-1, xretpos(L) and *pwp1* per cell during embryogenesis in comparison to their transcript levels at the one-cell stage (St1 = 1) (n = 3–13). Error bars represent SEM. Only values from stage 7 onwards are shown, since the increase of the cell number is less constant during the first six stages (Supplementary Fig. S3).
showed that the transcript level per cell increases after stage 8 and decreases after stage 12.5 (Fig. 1e). Thus it can be concluded that retrotransposons 1a11, λ-olt 2-1 and xretpos(L) show dynamic changes in transcription activities during embryonic development and the highest transcript levels of these are detected at the late gastrula stage.

**The LTR of λ-olt 2-1 is found in a transcript, which contains a high homology sequence with mars2.** It was previously shown that retrotransposons can regulate gene expression in early mouse embryos by functioning as alternative promoters and first exons4. Therefore, we asked if the three retrotransposons 1a11, λ-olt 2-1 and xretpos(L) also form chimeric (fusion) transcripts in early Xenopus embryos. To investigate this, a blast search for the three retrotransposons against a transcriptome assembly mapped to the *Xenopus laevis* genome 6.115 was performed. The transcriptome was generated from the RNA-seq analysis of gastrula embryos (stage 10.5–11.5) using paired-end sequencing with Illumina HiSeq2000. We found a transcript consisting of three exons, with the first exon and its surrounding region showing 97% sequence identity to the LTR of λ-olt 2-1 (Fig. 2a). The third exon shows no sequence identity to any reported gene. The second exon of this transcript, however, shows 87% sequence identity to the third exon of a gene called mars2. This mars2 gene encodes a mitochondrial methionyl-tRNA synthetase, which is needed for the translation of mitochondrial genes. Interestingly, tRNA is involved in the transposition of retrotransposons16. Hereafter, the transcript containing the LTR of λ-olt 2-1 as first exon (Fig. 2a, middle transcript) is referred to as mars2-like. The mars2 transcript found under the NCBI Reference Sequence NM_001086369.1 (Fig. 2a, lower transcript) is referred to as conventional mars2. The splicing junction between λ-olt 2-1 and mars2-high homology sequence was validated by continuous sequencing reads over the splicing junction (Supplementary Fig. S4). Moreover, we amplified mars2-like transcripts using primers located at the beginning of exon 1 and in exon 2, and sequenced the PCR product. This sequencing analysis further validated that mars2-like contains both retroelement λ-olt 2-1 (exon1) and mars2 homology sequence (exon 2) (Supplementary Fig. S5).

**mars2 expression during embryonic development.** Previous studies using mouse have shown that chimeric transcripts containing retrotransposons are specifically expressed in early embryos4. We therefore asked if the expression pattern of mars2-like, which contains the LTR of the retrotransposon, during embryonic development differs from that of the conventional mars2. To test this, different pairs of qPCR primers were designed to specifically detect either mars2-like or conventional mars2 (Fig. 2a). The transcript level of conventional mars2 (primer 2) is highest at the first three embryonic stages and then gradually decreases until the blastula stage (stage 9) (Fig. 2b). At this stage, the transcript level of mars2-like (primer 1) starts to increase (Fig. 2b). Interestingly, it seems that mars2-like transcript is substituting the transcript of conventional mars2 (Fig. 2b, the bottom merged graph). The expression of mars2-like (primer 1) resembles the expression of λ-olt 2-1 itself (Fig. 1b). These results suggest that λ-olt 2-1 may regulate the expression of mars2-like transcript.

**1a11, λ-olt 2-1, xretpos(L) and mars2-like are newly transcribed by RNA polymerase II.** Oocytes, direct precursors of eggs, contain some messenger RNAs (mRNAs) that have no or only very short poly(A) tails. During oocyte maturation and early embryonic development these short poly(A) tails are elongated, which facilitates translation17–19 and the amount of poly(A) tailed RNAs increase20,21. The transcript level of conventional mars2-like transcripts (primer 1) resembles the expression of λ-olt 2-1, xretpos(L) and mars2-like at the early embryonic stages is only an artifact, caused by the incapability of amplification by reverse transcription. In order to test if 1a11, λ-olt 2-1, xretpos(L) and mars2-like are newly transcribed or if their transcripts undergo changes in lengthening their poly(A) tails, α-amanitin was injected into fertilized one-cell embryos at two different concentrations (2 ng and 0.1 ng) (Fig. 3a). At the lower concentration (0.1 ng injection) α-amanitin can be used as a specific inhibitor for RNA polymerase II, while the higher α-amanitin concentration such as 20 μg/ml also inhibits RNA Polymerase III22. Injected, normal-looking embryos were collected at the blastula stage (stage 9) and samples for qPCR were prepared (Fig. 3a). As a control, the transcript level of sox17 and pwp1 was measured. sox17 expression, which is embryonically activated, was significantly inhibited by the addition of α-amanitin (P = 0.00002, Fig. 3b), indicating that α-amanitin treatment inhibits embryonic transcription. In contrast, the transcript level of pwp1 was not influenced by the injection of α-amanitin (Fig. 3b), suggesting that pwp1 transcripts are maternal transcripts. Even at the low α-amanitin concentrations the transcript levels of 1a11, λ-olt 2-1, xretpos(L) and mars2-like were significantly (P < 0.05) reduced by the injection of α-amanitin (Fig. 3c). In contrast, the transcript level of conventional mars2 does not seem to be influenced by the injection of α-amanitin. These results indicate that all three retrotransposons and mars2-like are newly transcribed by RNA polymerase II in embryos, while conventional mars2 seems to be a maternal transcript.

**1a11, λ-olt 2-1 and xretpos(L) are marked by H3K4me3 and H3K9me3.** We next investigated mechanisms leading to the developmentally regulated expression of 1a11, λ-olt 2-1 and xretpos(L)
transcription during embryogenesis (Fig. 1b, c). It was shown that retrotransposons in mouse embryos are marked by the active histone mark H3K4me3 and by the repressive histone mark H3K9me3. Repression of those retrotransposons was accompanied by the loss of H3K4me3, rather than the gain of H3K9me3. Therefore, we tested if LTRs of λ-olt 2-1, mars2-like and conventional mars2 are marked by H3K4me3 and H3K9me3 before and after the gastrula stage, at which transcriptional levels of those retrotransposons become highest. We collected embryos at the blastula stage (stage 8), the early gastrula stage (stage 10/11), the late gastrula stage (stage 12.5) and the neurula stage (stage 16–18) to perform chromatin immunoprecipitation (ChIP) analysis. Non-specific rabbit immunoglobulin G (IgG) was used as a negative control and precipitated 0.15–0.006% of input DNA (Supplementary Fig. S6a) while histone H3 as a positive control precipitated up to 27% of input DNA (Supplementary Fig. S6b). Both H3K4me3 and

---

**Figure 2.** The differential expression pattern between mars2-like and conventional mars2 during embryonic development. (a) The structure of λ-olt 2-1, mars2-like and conventional mars2. Grey boxes represent exons and the white box represents the LTR of λ-olt 2-1. The first exon of mars2-like and its surrounding region show 97% sequence identity to the LTR of λ-olt 2-1; the second exon shows 87% sequence identity to the third exon of conventional Xenopus mars2; the third exon shows no sequence identity to any reported gene. The full nucleotide sequence of mars2-like is shown in Supplementary Figure S5. Arrows represent binding sites of primer pairs used for qPCR. The forward primer of primer pair 1 binds to the splicing junction between exon 1 and exon 2 of mars2-like. Primer 2 binds to exon 1 of conventional mars2. (b) Changes in the transcript levels of mars2-like and conventional mars2 during embryogenesis in comparison to their transcript levels at stage 28/29 (St28/29 = 1). Stage 28/29 was used as a reference here instead of stage 1, since the transcript level of conventional mars2 is already very high at stage 1. The figure at the bottom shows a merge of mars2-like (primer 1) and conventional mars2 (primer 2). All values were normalized to the overall RNA concentration of the respective sample. Sample RT- is a negative control. All error bars represent SEM. n = 3–7.
H3K9me3 were enriched on LTRs of retrotransposons (Supplementary Fig. S6a). Relative enrichment of H3K4me3 to total histone H3 indicates that H3K4me3 levels increased up to stage 12.5 on all three retrotransposons (Fig. 4a, blue bars, *P < 0.05). A significant increase of H3K9me3 during developmental progression was observed in λ-o1t-2-1 (Fig. 4a, red bars, **P = 0.00008). These results suggest that the increase of H3K9me3 levels is correlated with transcriptional repression of retrotransposons and therefore might be important for the suppression of retrotransposon expression.

Figure 3. 1α11, λ-o1t-2-1, xretpos(L) and mars2-like are transcribed by RNA polymerase II. (a) Schematic diagram of the transcriptional inhibition experiment. 0.1 ng or 2 ng of α-amanitin were injected into one-cell stage embryos. Embryos were collected at the blastula stage (stage 9) and RT-qPCR was performed. The container was drawn by S.H. (b) and (c) The effect of α-amanitin injection on the transcript levels of sox17, pwp1, 1α11, λ-o1t-2-1, xretpos(L), and mars2-like. Transcript level of non-injected embryos were used as a reference (non = 1). All values were normalized to the RNA concentration of the respective sample. Sample RT- is a negative control. All error bars represent SEM. n = 3. *P < 0.05, **P < 0.005.
Figure 4. KDM4d overexpression removes H3K9me3 in embryos and upregulates λ-olt 2-1 at the neurula stage. (a) ChIP analysis indicates enrichment of H3K4me3 and H3K9me3 on LTRs of retrotransposons at different stages of embryogenesis. The precipitated DNA/input DNA of 1a11, λ-olt 2-1 and xretpos(L) was determined by qPCR. (H3K4me3 and H3K9me3: n = 3–4, IgG: n = 2). All values of modified histone and control IgG were normalized to H3 (Supplementary Fig. 6b). All error bars represent SEM. **P = 0.00008, *P < 0.05. Blue = H3K4me3, red = H3K9me3, and green = IgG. (b) KDM4d mRNA was injected into fertilized embryos to remove H3K9me3 during embryogenesis. At the gastrula stage (stage 10) and at the neurula stage (stage 17) embryos were collected and prepared for western blotting or qPCR. Myc-GFP mRNA was used as a control. The container was drawn by S.H. (c) Removal of H3K9me3 by KDM4d mRNA injection was verified by western blot. The same samples were used to detect the expression of HP1β in Xenopus embryos. Histone 4 (H4) was used as a loading control. (d) The effect of KDM4d overexpression on retrotransposons expression and on the expression of mars2-like and conventional mars2 at the gastrula (St10) and the neurula stages (St17). Transcription was measured by RT-qPCR. The relative change of the transcript level in comparison to the transcript level of control myc-GFP mRNA-injected embryos is shown. The transcript level of myc-GFP mRNA-injected embryos at stage 10 was set 1. All values were normalized to the RNA concentration of the sample. All error bars represent SEM. n = 3–5. *P < 0.05. Blue bars represent myc-GFP mRNA-injected embryos while red bars are KDM4d mRNA-injected embryos.
KDM4d overexpression removes H3K9me3 and releases transcriptional repression of \( \lambda\)-olt 2-1 at the neurula stage. Since the results of the ChIP analysis indicate that H3K9me3 is enriched on retrotransposons during embryonic development (Fig. 4a), we asked if this enrichment of H3K9me3 is required for retrotransposon repression in early and late embryonic stages. In order to reduce H3K9me3, mRNA of lysine-specific demethylase 4d (KDM4d), which specifically removes histone H3 lysine 9 trimethylation\(^{23,24}\), was injected into fertilized embryos. KDM4d mRNA-injected embryos were collected at the early gastrula (stage 10/10.5) and neurula (stage 16/17) stages and subjected to western blotting (Fig. 4b). The results showed that, in comparison to myc-GFP mRNA-injected control embryos, H3K9me3 was strongly reduced in KDM4d mRNA-injected embryos (Fig. 4c). Thus, H3K9me3 mark is removed in the gastrula and neurula embryos by KDM4d overexpression. Surprisingly, the removal of H3K9me3 mark did not cause any apparent phenotype in *Xenopus* embryos at least up to the neurula stage (stage 17).

Next, we asked if KDM4d overexpression influences the transcription of *1a11*, \( \lambda\)-olt 2-1 and *xretpos(L)*. For this reason, KDM4d mRNA-injected embryos were used for qPCR (Fig. 4b). Neither the transcript level of *1a11* nor that of *xretpos(L)* was affected by the injection of KDM4d mRNA (Fig. 4d). However, interestingly \( \lambda\)-olt 2-1 trancripts were significantly (\( P = 0.044 \)) upregulated in KDM4d mRNA-injected embryos at the neurula stage (stage 17), but not at the early gastrula stage (stage 10) (Fig. 4d). This indicates that H3K9me3 removal by KDM4d overexpression is associated with derepression of \( \lambda\)-olt 2-1 after gastrulation. KDM4d overexpression did not significantly affect the expression of other putative retrotransposons listed in Supplementary Table S1 (Supplementary Fig. S7), although Tx1L (AF027962.1)\(^{25}\) seems to be upregulated by the H3K9me3 removal.

Since *mars2-like* expression seems to be driven by \( \lambda\)-olt 2-1 (Fig. 2), we also measured the transcript level of *mars2-like* in embryos depleted of H3K9me3. We found that the KDM4d mRNA injection significantly (\( P = 0.040 \)) enhances *mars2-like* transcription in neurula embryos (stage 17) in good agreement with the transcriptional upregulation of \( \lambda\)-olt 2-1 (Fig. 4d). In contrast, the expression of conventional *mars2* was not affected by KDM4d mRNA injection (Fig. 4d). These results suggest that *mars2-like* is also regulated by H3K9me3 and support the idea that \( \lambda\)-olt 2-1 is involved in transcriptional regulation of *mars2-like*.

Even though H3K9me3 was also detected on \( \lambda\)-olt 2-1 LTR at early gastrula stages (stage 10) (Fig. 4a), KDM4d mRNA injection only had an effect on the expression of \( \lambda\)-olt 2-1 and *mars2-like* during the neurula stage (stage 17). A possible reason for this difference might be that some factors, which are associated with H3K9me3 and needed for heterochromatin establishment, are not yet expressed during gastrulation. These factors could for example be the Heterochromatin binding protein 1, which functions in heterochromatin mediated gene silencing and directly binds to H3K9me3\(^{26,27}\). By western blotting we showed that HP1 could not be detected in embryos at early gastrula stage (stage 10), but is expressed during neurula stages (stage 17) (Fig. 4c). It is therefore possible that the lack of HP15 during gastrulation may explain the insensitivity of \( \lambda\)-olt 2-1 and *mars2-like* expression to H3K9me3 removal in gastrula embryos (Fig. 4d).

**Discussion**

We examined the expression of ten putative retrotransposons during *Xenopus* embryogenesis and found that the expression of three of these retrotransposons (*1a11*, \( \lambda\)-olt 2-1 and *xretpos(L)*) is upregulated after midblastula transition and downregulated after the gastrula stage. The LTR of \( \lambda\)-olt 2-1 also forms a fusion transcript with a sequence that showed high similarity to *mars2*. All three retrotransposons are marked by the histone modifications H3K9me3 and H3K4me3. The KDM4d mRNA injection, which results in H3K9me3 removal, causes an increase of the \( \lambda\)-olt 2-1 and *mars2-like* transcript levels at the neurula stage, indicating that H3K9me3 plays a role in the repression of \( \lambda\)-olt 2-1 after gastrulation.

All three retrotransposons, *1a11*, \( \lambda\)-olt 2-1 and *xretpos(L)*, have been found as LTR retrotransposons in *Xenopus laevis*\(^{13,14}\). *1a11* encodes a 71 kDa protein, which shows similarity to the viral structure protein Gag. It was estimated that the *Xenopus* genome contains hundreds of sequences related to the LTR of *1a11*, and 20–30 sequences related to its open reading frame\(^{13}\). The published expression analysis data also indicate transcripational repression in late embryonic development, in good agreement with our qPCR data\(^{15}\) (Fig. 1b). The *Xenopus* genome seems to contain approximately 200 copies of *xretpos(L)* LTR, 15 copies of the *xretpos(L)* open reading frame, and 50 copies of the complete *xretpos(L)*\(^{16}\). Again, the published expression analysis data of *xretpos(L)* support our qPCR data\(^{15}\) (Fig. 1b). Moreover, it was shown by RNA and antisense RNA injections that *xretpos(L)* has a posterior-ventralizing activity during *Xenopus* embryogenesis\(^{28}\). All three retrotransposons have in common that they show no amino acid homology to the conserved pol gene. Since the pol gene encodes proteins needed for transposition (protease, reverse transcriptase, and integrase), it is likely that *1a11*, \( \lambda\)-olt 2-1 and *xretpos(L)* have lost their ability to transpose\(^{14}\).

We found that the LTR of \( \lambda\)-olt 2-1 can function as the first exon and forms a transcript with a sequence that has a high homology with *mars2* during *Xenopus* embryogenesis (Fig. 2a, called as *mars2-like*). However, we were not able to determine whether \( \lambda\)-olt 2-1 is located near conventional *mars2* gene due to an incomplete understanding of *Xenopus* genome. The second exon of *mars2-like* transcript shows high nucleotide sequence homology to the third exon of conventional *mars2*. Conventional *mars2* encodes a mitochondrial methionyl-tRNA synthetase. Mutations of the human homologue of this gene
cause a neurodegenerative disease called Autosomal Recessive Spastic Ataxia with Leukoencephalopathy (ARSAL). It is likely that retroelements-derived repetitive sequences surrounding human Mars2 (e.g. Line 1 and Line2 elements) are mediating genomic rearrangements that cause ARSAL.27 The methionyl-tRNA synthetase encoded by mars2 is needed for the translation of mitochondrial genes. Interestingly, tRNAs are also used as primer for the reverse transcription of retrotransposons or retroviruses. Methionyl-tRNA for example functions as a primer of yeast and plant retrotransposons like Ty1, Ty2, Ty3, Ty5 and Ta1.16 It is also suggested that xretpos(L) is using arginine-tRNA as primer for reverse transcription.22 23 Therefore, the developmentally regulated mars2 expression might be related to activities of transposable elements in embryos.

Our RT-qPCR data suggest that λ-olt 2-1 is driving mars2-like transcription (Fig. 2b). In this case, the LTR of λ-olt 2-1 might be used as a promoter to allow mars2-like expression (Fig. 2b). Several cases have been reported where LTR sequences act as alternative promoters.14 30 One of the most relevant cases has been reported in mouse embryos at the 2-cell stage, where LTR sequences of MuERV-L regulate stage-specific expression of some protein coding genes.30 However, in the case of mars2-like, it is unlikely that this gives rise to a meaningful protein since many stop codons are found in all reading frames. We therefore assume that mars2-like is more likely to be a long non-coding RNA (lncRNA) or a pseudo-gene product. lncRNAs are defined as non-coding RNAs longer than 200 nucleotides. These lncRNAs take on various functions, such as recombination, cell-cell signaling, post-transcriptional regulation, or regulation of chromatin states.31 The transcript level of conventional mars2 is strongly reduced from stage 9 to stage 10; at the same time the transcript level of mars2-like strongly increases (Fig. 2b). This implies that mars2-like RNA might be involved in the regulation of conventional mars2 expression.

Our RT-qPCR data suggest that λ-olt 2-1 is driving mars2-like transcription (Fig. 2b). In this case, the LTR of λ-olt 2-1 might be used as a promoter to allow mars2-like expression (Fig. 2b). Several cases have been reported where LTR sequences act as alternative promoters.14 30 One of the most relevant cases 8 10

Methods

Animals. All experiments using frogs were carried out following requirements of the UK Home Office. All experimental protocols were approved by the UK Home Office.

RNA extraction, reverse transcription and qPCR. Three oocytes, eggs or embryos per sample were collected and frozen at −80 °C. For RNA extraction, RNeasy Mini Kit (QIAGEN) was used according to the manufacturer’s protocol. RNA was eluted in 35 μl of the provided RNase-free water. For reverse transcription, 12 μl of the RNA solution was mixed with 0.5 μl 100 μM Oligo-dT-Primer and 0.5 μl RNase Inhibitor Murine and incubated at 65 °C for 5 min. All samples were then immediately placed on ice for more than 1 min. Afterwards, 6.6 μl of a reverse transcription mix (4 μl of 5 x First Strand buffer, 1 μl of 0.1 M DTT (dithiothreitol), and 1.6 μl of 10 mM dNTP-mix) were added to the RNA solution. For negative control (RT-), 4 μl of this reaction mix were transferred to a new tube. After adding 0.5 μl of Superscript III Reverse Transcriptase to the reaction mix, all samples were first incubated at 50 °C for 60 min and then at 70 °C for 15 min. All samples were diluted by 7.25-fold with ddH2O. For Fig. 1d, gene-specific forward primers (reverse primers as a control) were used for reverse transcription in order to examine whether retroelements are transcribed from both strands. Primer sequences are listed in Supplementary Table S2.
MEGAscript SP6 (Life Technology).

KDM4d was cloned into pCS2 vector and the linearized vector was subjected to mRNA synthesis using KDM4d or myc-GFP encoding mRNA (1 μg/ml), the Drummond Nonoject II Injector was used. Mouse stage was set as 1.

In some experiments when one-cell stage values are undetectable, the following expression changes of retrotransposons during early embryonic development, values of one-cell stage to calculate RPKM values.

Transposable elements. These counts were normalized by transcript length and total read counts in order to calculate RPKM values.

Identification of mars2-like. To identify the mars2-like transcript a BLAST search was performed, using a RNA-Seq dataset from Xenopus embryos at stage 10.5–11.5. The transcript sequence of mars2-like was originally found in transcriptome assembly mapped to the Xenopus laevis genome 6.1. The mars2-like map including exon information shown in Fig. 2a was captured from Xenopus laevis 6.1 genome. Moreover, mars2-like transcript was amplified using primers binding to exon 1 (Primer sequence: GGACAAGACATGTGGAGGAATG) and to exon 2 (Primer sequence: ggccacgcttTCTCTCTAC-TACCCCTTTTCT or ggccacgcgtAGTAAATAGCATGGAAGAAT). The resulting PCR product was used for DNA sequencing.

Injection of α-amanitin or KDM4d-encoding mRNA into fertilized embryos. Eggs were in vitro fertilized by distributing them in a solution of smashed testis in L15-Medium. After 10 min 5 ml of ddH₂O was added to the solution and the eggs were incubated for further 20 min. Fertilized eggs were then dejellied in 40 ml of 2% cysteine solution (40 ml of 0.1 x MMR, 0.8 g of Cystein, and 640 of ddH₂O was added to the solution and the eggs were incubated for further 20 min. Fertilized eggs were then dejellied in 40 ml of 2% cysteine solution (40 ml of 0.1 x MMR, 0.8 g of Cystein, and 640 of ddH₂O was added to the solution and the eggs were incubated for further 20 min. Fertilized eggs were then dejellied in 40 ml of 2% cysteine solution (40 ml of 0.1 x MMR, 0.8 g of Cystein, and 640 of ddH₂O was added to the solution and the eggs were incubated for further 20 min. Fertilized eggs were then dejellied in 40 ml of 2% cysteine solution (40 ml of 0.1 x MMR, 0.8 g of Cystein, and 640 of ddH₂O was added to the solution and the eggs were incubated for further 20 min. Fertilized eggs were then dejellied in 40 ml of 2% cysteine solution (40 ml of 0.1 x MMR, 0.8 g of Cystein, and 640 of ddH₂O was added to the solution and the eggs were incubated for further 20 min. Fertilized eggs were then dejellied in 40 ml of 2% cysteine solution (40 ml of 0.1 x MMR, 0.8 g of Cystein, and 640 of ddH₂O was added to the solution and the eggs were incubated for further 20 min. Fertilized eggs were then dejellied in 40 ml of 2% cysteine solution (40 ml of 0.1 x MMR, 0.8 g of Cystein, and 640 of ddH₂O was added to the solution and the eggs were incubated for further 20 min. Fertilized eggs were then dejellied in 40 ml of 2% cysteine solution (40 ml of 0.1 x MMR, 0.8 g of Cystein, and 640 of ddH₂O was added to the solution and the eggs were incubated for further 20 min. Fertilized eggs were then dejellied in 40 ml of 2% cysteine solution (40 ml of 0.1 x MMR, 0.8 g of Cystein, and 640 of ddH₂O was added to the solution and the eggs were incubated for further 20 min. Fertilized eggs were then dejellied in 40 ml of 2% cysteine solution. The dejellied, fertilized eggs were washed 10 times with 0.1 x MMR (100 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES pH 7.4) to carefully remove the cysteine solution. Then stage 1 embryos were transferred to injection medium 41 (6% Ficoll and 0.4 x MMR) and incubated at 14 °C for at least 15 min. For injection of 4.6 nl of α-amanitin or KDM4d-encoding mRNA into fertilized embryos.

Protein extraction and western blotting. For protein extraction, 100 μl of extraction buffer (500 mM Tris pH 6.8, 500 mM NaCl, 1% NP40, 0.1% SDS, 1% β-Mercaptoethanol and protease inhibitor cocktail) was used to homogenize 3 embryos per sample. To separate the resulting suspension, samples were centrifuged for 10 min at 4 °C and 16,000g. The clear middle layer (80 μl) was transferred to a fresh tube, mixed with 20 μl of 5x loading buffer (0.3 M Tris-HCl, pH 6.8, 50% (v/v) glycerol, 1% (w/v) SDS, 0.05% (w/v) bromphenol blue and 3.2% (v/v) β-mercaptoethanol) and boiled at 100 °C for 5 min. All samples were centrifuged for 3 min at room temperature (16,000 g). The supernatant was then transferred to a new tube.

Polyacrylamid gels (12% SDS) were loaded with 20 μl of this supernatant. Gel electrophoresis and western blots were performed according to standard protocols. For blotting PVDF membranes, a
semi-dry transfer system was used (30 min, 25 V). For the protein detection, following primary antibodies were used: anit-H3K9me3 (1:1000, ab8898, Abcam), anti-H4 (1:1000, ab10158, Abcam) and anti H1P1 (1:5000, MAB3448, Chemicon). Anti-rabbit or anti-mouse IgG Alexa Fluor 680 (Invitrogen) and/or anti-rabbit IRDye 800CW (LI-COR) were used as secondary antibodies (1:10,000, 1 h at room temperature). The blots were scanned using the imaging system Odyssey (LI-COR).

**Statistical analysis.** To test whether there is a statistically significant difference in the mean transcript level, a two-tailed T-test was used. The asterisk is shown if P < 0.05. All error bars represent the standard error of the mean (SEM).

**Primers.** All primers used for qPCR are summarized in Supplementary Table S2.

**References**
1. Gifford, W. D., Pfaff, S. L. & Macfarlan, T. S. Transposable elements as genetic regulatory substrates in early development. Trends Cell Biol. 23, 218–226 (2013).
2. Yang, N. & Kazazian, H. H., Jr. L1 retrotransposition is suppressed by endogenously encoded small interfering RNAs in human cultured cells. Nat. Struct. Mol. Biol. 13, 763–771 (2006).
3. Aravin, A. A., Sachidanandaman, R., Giraud, A., Fejes-Toth, K. & Hannon, G. J. Developmentally regulated piRNA clusters implicate MILL in transposon control. Science. 316, 744–747 (2007).
4. Peaston, A. E. et al. Retrotransposons regulate host genes in mouse oocytes and preimplantation embryos. Dev. Cell. 7, 597–606 (2004).
5. Kano, H. et al. L1 retrotransposition occurs mainly in embryogenesis and creates somatic mosaicism. Genes Dev. 23, 1303–1312 (2009).
6. Fadloun, A. et al. Chromatin signatures and retrotranscription profiling in mouse embryos reveal regulation of LINE-1 by RNA. Nat. Struct. Mol. Biol. 20, 332–338 (2013).
7. Kigami, D., Minami, N., Takayama, H. & Imai, H. MuERV-L is one of the earliest transcribed genes in mouse one-cell embryos. Biol. Reprod. 68, 651–654 (2003).
8. Macfarlan, T. S. et al. Embryonic stem cell potency fluctuates with endogenous retrovirus activity. Nature. 487, 57–63 (2012).
9. Beraldi, R., Pittoggi, C., Sciamanna, I., Mattel, E. & Spadafora, C. Expression of LINE-1 retroelements is essential for murine preimplantation development. Mol. Reprod. Dev. 73, 279–287 (2006).
10. Inoue, A., Matoba, S. & Zhang, Y. Transcriptional activation of transposable elements in mouse zygotes is independent of Tet3-mediated 5-methylcytosine oxidation. Cell Res. 22, 1640–1649 (2012).
11. Akkers, R. C. et al. A hierarchy of H3K4me3 and H3K27me3 acquisition in spatial gene regulation in Xenopus embryos. Dev. Cell. 17, 425–434 (2009).
12. Voigt, P., Tee, W. W. & Reinberg, D. A double take on bivalent promoters. Genes Dev. 27, 1318–1338 (2013).
13. Greene, J. M., Otani, H., Good, P. J. & Dawid, I. B. A novel family of retrotransposon-like elements in Xenopus laevis with a transcript inducible by two growth factors. Nucleic Acids Res. 21, 2375–2381 (1993).
14. Shim, S., Lee, S. K. & Han, J. K. A novel family of retrotransposons in Xenopus with a developmentally regulated expression. Genesis. 26, 198–207 (2000).
15. James-Zorn, C. et al. Xenbase: expansion and updates of the Xenopus model organism database. Nucleic Acids Res. 41, D865–870 (2013).
16. Marquet, R., Isel, C., Ehresmann, C. & Ehresmann, B. tRNAs as primer of reverse transcriptases. Biochimie. 77, 113–124 (1995).
17. McGrew, L. L. & Richter, J. D. Xenopus oocyte poly(A) RNAs that hybridize to a cloned interspersed repeat sequence are not translatable. Dev. Biol. 134, 267–270 (1989).
18. Rosenthal, E. T., Tansey, T. R. & Ruderman, J. V. Sequence-specific adenylation and deamination accompany changes in the translation of maternal messenger RNA after fertilization of Spisula oocytes. EMBO J. 3, 267–270 (1984).
19. Slater, D. W., Slater, I. & Gillespie, D. Post-fertilization synthesis of maternal messenger RNA after fertilization of Spisula oocytes. Nucleic Acids Res. 21, 309–327 (1993).
20. Sagata, N., Shiokawa, K. & Yamana, K. A study on the steady-state populaton of poly(A)+RNA during early development of Xenopus laevis. Dev. Biol. 77, 431–448 (1980).
21. Shiokawa, K., Kurashima, R. & Shingo, J. Temporal control of gene expression from endogenous and exogenously-introduced DNAs in early embryogenesis of Xenopus laevis. Int. J. Dev. Biol. 38, 249–255 (1994).
22. Stancheva, I. & Meehan, R. R. Transient depletion of Dmmt1 leads to premature gene activation in Xenopus embryos. Genes Dev. 14, 313–327 (2000).
23. Whetstine, J. R. et al. Reversal of histone lysine trimethylation by the JMJD2 family of histone demethylases. Cell. 125, 467–481 (2006).
24. Krishnan, S. & Trievel, R. C. Structural and functional analysis of JMJD2D reveals molecular basis for site-specific demethylation among JMJD2 demethylases. Structure. 21, 98–108 (2013).
25. Pont-Kingdon, G., Chi, E., Christensen, S. & Carroll, D. Ribonucleoprotein formation by the ORF1 protein of the non-LTR retrotransposon Tx1L in Xenopus oocytes. Nucleic Acids Res. 25, 3088–3094 (1997).
26. Bannister, A. J. et al. Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature. 410, 120–124 (2001).
27. Lachner, M., O'Carroll, D., Rea, S., Mechtcher, K. & Jenuwein, T. Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature. 410, 116–120 (2001).
28. Shim, S., Bae, N. & Han, J. K. Bone morphogenetic protein-4-induced activation of Xretos is mediated by Smads and Olf-1/Ebf-associated zinc finger (OAZ). Nucleic Acids Res. 30, 3107–3117 (2002).
29. Bayat, V. et al. Mutations in the mitochondrial methyltransferase catalyze a neurodegenerative phenotype in flies and a recessive ataxia (ARSAL) in humans. PLoS Biol. 10, e1001288 (2012).
30. Cohen, C. J., Lock, W. M. & Mager, D. L. Endogenous retroviral LTRs as promoters for human genes: a critical assessment. Gene. 448, 105–114 (2009).
31. Geisler, S. & Coller, J. RNA in unexpected places: long non-coding RNA functions in diverse cellular contexts. Nat. Rev. Mol. Cell Biol. 14, 699–712 (2013).
32. Lehnmert, B. et al. Suv39h-mediated histone H3 lysine 9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin. Curr. Biol. 13, 1192–1200 (2003).
33. Matsui, T. et al. Proviral silencing in embryonic stem cells requires the histone methyltransferase ESET. Nature. 464, 927–931 (2010).
34. Rowe, H. M. et al. KAP1 controls endogenous retroviruses in embryonic stem cells. Nature. 463, 237–240 (2010).
35. Walsh, C. P., Chalillet, J. R. & Bestor, T. H. Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. Nat. Genet. 20, 116–117 (1998).
36. Schneider, T. D. et al. Stage-specific histone modification profiles reveal global transitions in the Xenopus embryonic epigenome. PLoS One. 6, e22548 (2011).
37. Furano, A. V., Duvernell, D. D. & Boissinot, S. I1 (LINE-1) retrotransposon diversity differs dramatically between mammals and fish. Trends Genet. 20, 9–14 (2004).
38. Hellsten, U. et al. The genome of the Western clawed frog Xenopus tropicalis. Science. 328, 633–636 (2010).
39. Miyamoto, K. et al. Nuclear Wavel is required for reprogramming transcription in oocytes and for normal development. Science. 341, 1002–1005 (2013).
40. Trapnell, C., Pachter, L. & Salzberg, S. L. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics. 25, 1105–1111 (2009).
41. Smith, S. J., Fairclough, L., Latinkic, B. V., Sparrow, D. B. & Mohun, T. J. Xenopus laevis transgenesis by sperm nuclear injection. Nat. Protoc. 1, 2195–2203 (2006).
42. Gentsch, G. E. et al. In vivo T-box transcription factor profiling reveals joint regulation of embryonic neuromesodermal bipotency. Cell Rep. 4, 1185–1196 (2013).

Acknowledgements
We are grateful to Dr. E Hörmanseder for her kind gift of primers and to Mr N Garrett for his experimental help. K.M. is a Research Fellow at Wolfson College and is supported by the Herchel Smith Postdoctoral Fellowship. Gurdon laboratory is supported by grants from the Wellcome Trust (RG69899) and MRC to J.B.G.

Author Contributions
S.H., M.O., J.J. and K.M. performed research; K.M. designed research; J.B.G. supervised research; J.J., M.O. and M.T. provided experimental materials; S.H., A.S., C.R.B. and K.M analyzed data; K.M., S.H., C.R.B. and J.B.G. wrote the paper.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Accession codes: The RNA-seq data have been deposited in NCBI (SRA279316).

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Herberg, S. et al. Histone H3 lysine 9 trimethylation is required for suppressing the expression of an embryonically activated retrotransposon in Xenopus laevis. Sci. Rep. 5, 14236; doi: 10.1038/srep14236 (2015).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/