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The Krüppel-associated Box Repressor Domain Can Trigger *de Novo* Promoter Methylation during Mouse Early Embryogenesis*[S]

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The Krüppel-associated box (KRAB) domain is a transcriptional repression module responsible for the DNA binding-dependent gene silencing activity of hundreds of vertebrate zinc finger proteins. We previously exploited KRAB-mediated repression within the context of a *tet* repressor-KRAB fusion protein and of lentiviral vectors to create a method of external gene control. We demonstrated that with this system transcriptional silencing was fully reversible in cell culture as well as *in vivo*. Here we reveal that, in sharp contrast, KRAB-mediated repression results in irreversible gene silencing through promoter DNA methylation if it acts during the first few days of mouse development.

The human genome encodes several hundreds of KRAB[4]-containing ZFPs, a vertebrate-specific family of DNA-binding factors that likely plays major roles in gene regulatory networks important for ontogenesis, differentiation, and cell growth (1–4). When tethered to DNA via their zinc finger motifs, KRAB-ZFPs recruit the KAP1 (KRAB-associated protein 1) corepressor (also known as TIF1β, transcription intermediary factor 1β; KRIP1, KRAB-interacting protein 1; or TRIM28, tripartite motif protein 28) via their KRAB domain. KAP1 is characterized by the presence of a RING finger, B-boxes, a coiled-coil region, a PHD finger, and a bromodomain (5–7). The first three of these motifs define the so-called RBCC or TRIM domain, which is both necessary and sufficient for homo-oligomerization and direct binding to KRAB. Upon recruitment to DNA loci, KAP1 functions as a scaffold for the formation of a multimolecular complex comprising HP1 (heterochromatin protein 1), histone deacetylases, and histone methyltransferases, which induces transcriptional repression through the formation of heterochromatin (8, 9). The primordial importance of this process from the earliest stage of mammalian development is supported by the embryonic lethality of the mouse KAP1 knock-out, which results in failure to gastrulate and death at day E5.5 (10). Considering the obvious importance of KRAB-mediated gene control, it is quite remarkable that only very few gene targets of the KRAB-containing ZFPs have so far been identified (11).

We described previously a conditional gene regulation system based on the use of the tetracycline-controllable trans-repressor tTRKRAB, a chimeric protein built by fusing KRAB with the DNA binding domain of the *Escherichia coli* tetracycline repressor (*tetR*). Upon binding tetracycline operator (*tetO*) sequences, tTRKRAB induces transcriptional repression, which can silence RNA polymerase I, II, and III promoters situated within a radius of a few kilobases, thus allowing for doxycycline-regulated gene expression and knockdown (12–15) (Fig. 1*A*). Using lentiviral vectors as vehicles for this system, we demonstrated that KRAB-induced silencing is fully reversible *in vitro* and *in vivo* even after prolonged periods of repression in diverse contexts such as in a variety of established human and murine cell lines, in primary cells, including mouse ES cells, and in the brain of rats injected with a vector expressing the tTRKRAB repressor and a *tetO*-controlled target (14). Here we present evidence revealing that, in sharp contrast to all these other settings, KRAB-mediated repression results in irreversible gene silencing through promoter DNA methylation if it acts during the first few days of mouse development.

During early embryogenesis, the mouse genome undergoes an orderly cycle of demethylation-remethylation (16–20). After fertilization of the oocyte, methylation is erased from both paternal and maternal alleles except in regions of imprinted genes, with DNA methylation levels reaching their lowest level in the blastocyst at embryonic day E3.5. After implantation, although the DNA of extra-embryonic cells remains hypomethylated, the bulk of the genome becomes hypermethylated in the embryonic ectoderm and mesoderm through active *de novo* methylation, except in so-called CpG islands (CGIs). CGIs are G + C-rich regions that overlap with

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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[4] The abbreviations used are: KRAB, Krüppel-associated box; ZFP, zinc finger protein; FACS, fluorescence-activated cell sorter; GFP, green fluorescent protein; TSA, trichostatin A; dox, doxycycline; 5-Aza, 5-azacytidine; LTR, long terminal repeat; CGI, CpG island; E, embryonic day; ES, embryonic stem; mES, murine embryonic stem; CMV, cytomegalovirus.

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either repeats such as Alu or with the transcriptional start sites of promoters (in which case they are called start CGIs), notably promoters active during early embryogenesis (21). The mechanisms behind this patterning remain incompletely characterized, although a critical role for the DNA methyltransferases DNMT3a and -3b in *de novo* DNA methylation has been demonstrated through gene knock-out experiments in the mouse (22). The data presented here raise the possibility that KRAB-containing ZFPs play an important role in shaping these DNA methylation patterns established during this early embryonic period.

EXPERIMENTAL PROCEDURES

Lentiviral Vector Construction and Production—The LVCT-tTRKRAB vector was described previously (14). LVC-tTRKRAB was constructed by first replacing the EF-1α promoter in pWPXL with the CAG promoter and then substituting the GFP cDNA for one encoding tTRKRAB. In LVUT.GFP, *tetO* sequences were introduced upstream of the ubiquitin C promoter (UbiC). For LVUT.GFP.IRES.tTRKRAB, an IRES-tTRKRAB cassette was placed downstream of the GFP marker of LVUT.GFP (23). The self-deleting lentiviral vector expressing CRE was described previously (24). Lentiviral vector particles were generated by transient transfection of 293T cells as described previously (14).

Transgenesis—LVCT.GFP, LVC.tTRKRAB, LVCT.tTRKRAB, and LVUT.tTRKRAB transgenic mice were generated by perivitelline injection of lentiviral vectors as described previously (14). The E14 ES cells were cultured as described (14). The blastocyst transfer was performed according to standard techniques (25). Doxycycline (dox) was administered in drinking water at a concentration of 2 g/liter supplemented with 5% sucrose. The generation and genotyping of floxed KAP-1 mice have been described previously (26).

Cell-based Experiments—Both mES E14 cell culture and ear fibroblast isolation have been described previously (14). For drug-based studies, fibroblasts were plated at low density in medium containing dox (1 mg/liter) and treated with 5-Aza (7.5 μM) or TSA (300 nM) for 24 h before analyzing by FACS for GFP 24 h later.

DNA and RNA Studies—Southern blot analyses were performed by subjecting tail DNA to BssHII and BglII digestion, before gel electrophoresis, transfer to membranes, and hybridization with ^{32}P-labeled probes specific for the RRE region, as described (14). Quantitative real time PCR using SYBR green was performed using standard procedures. Data were normalized against β-actin and glyceraldehyde-3-phosphate dehydrogenase mRNA levels. Bisulfite sequencing was done using the EpiTect bisulfite kit (Chemicon) according to the supplier's recommendations. For each PCR, 1 μl of bisulfite-treated DNA was used. PCR conditions were as follows: 94 °C for 15 min, followed by 45 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. PCR-amplified products were cloned into a TOPO cloning vector (Invitrogen), and between 12 and 20 clones from each PCR were then sent for sequencing (Microsynth, Switzerland). Sequence data were analyzed using BiQ analyzer (27). Primer sequences are available upon request.

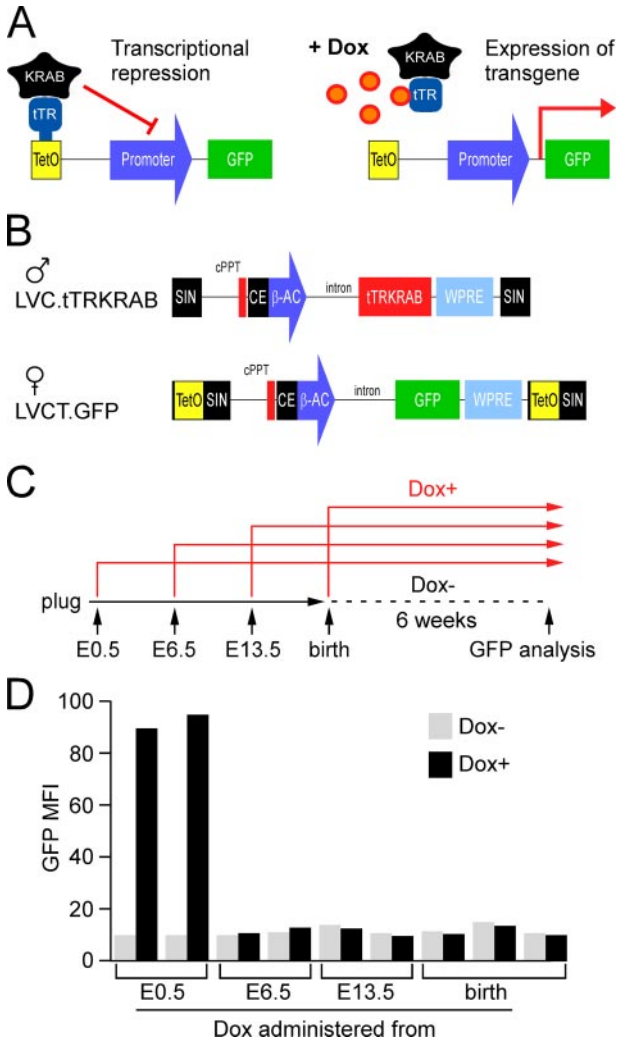

FIGURE 1. **KRAB binding during early development induces irreversible silencing.** *A,* mode of action of the doxycycline-controllable KRAB repressor tTRKRAB. *Left,* in the absence of dox tTRKRAB binds to *tetO* and suppresses transcription. *Right,* in the presence of dox, tTRKRAB is sequestered away from the DNA, allowing for gene expression. *B,* schematic representation of the integrated form of the lentiviral vectors LVC.tTRKRAB and LVCT.GFP that were used to generate transgenic mice. *C,* experimental scheme. LVC.tTRKRAB and LVCT.GFP transgenic mice were crossed, and doxycycline was administered in the drinking water at the indicated time points. *D,* GFP expression in fibroblasts isolated from dually transgenic mice cultured in the presence or absence of doxycycline. Each sample represents cells from one transgenic mouse. *SIN,* self-inactivating long terminal repeat; *cPPT,* central polypurine tract; *CE,* CMV enhancer; *β-AC,* chicken β-actin promoter; *WPRE,* woodchuck hepatitis post-transcriptional regulatory element.

RESULTS

tTRKRAB Induces Irreversible Silencing during Early Development—With the long term goal of engineering transgenic animals that allow for externally controllable gene knockdown and transgene expression, we previously exploited the tTRKRAB system within the context of lentiviral vectors (14, 15). We demonstrated that when such vectors were used to engineer transgenic mice by transduction of fertilized oocytes, the resulting animals exhibited controllable transgene expression as long as the KRAB-containing regulator was prevented from binding its target DNA during pregnancy. However, our preliminary analyses also revealed that if tTRKRAB was left to bind its target during early development, then the transgene

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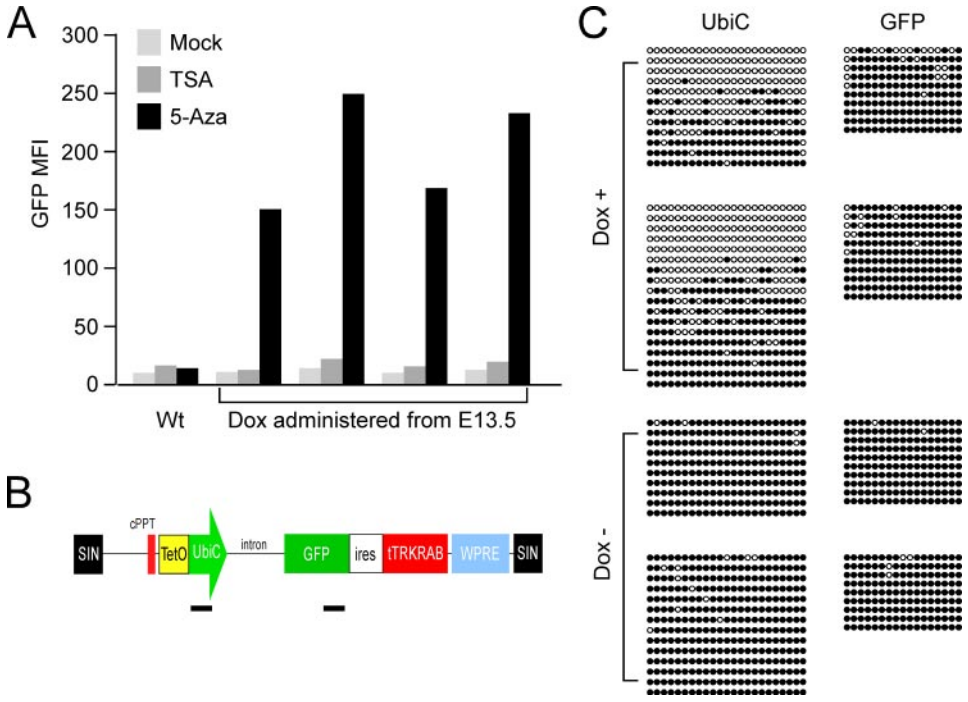

FIGURE 2. **KRAB-induced irreversible silencing is associated with promoter DNA methylation.** *A,* effect of 5-Aza or TSA treatment on dox-induced GFP expression in fibroblasts isolated from transgenic mice carrying both the LVC.tTRKRAB and LVCT.GFP vectors (Fig. 1). Each sample represents a single transgenic mouse. *B,* schematic representation of the integrated form of the LVUT.GFP.IRES.tTRKRAB vector. *C,* bisulfite sequencing analysis of the UbiC promoter and the GFP coding sequence from four mice. Two mice received doxycycline during development from E0.5 (*top*), whereas two animals did not (*bottom*). *Empty* and *full circles* represent unmethylated and methylated CpGs, respectively. *UbiC,* human ubiquitin promoter. Wt, wild type; *SIN,* self-inactivating long terminal repeat; *cPPT,* central polypurine tract; *CE,* CMV enhancer; *WPRE,* woodchuck hepatitis post-transcriptional regulatory element.

was irreversibly silenced (14). To investigate this phenomenon further, we injected a lentiviral vector expressing tTRKRAB under the control of the ubiquitous CAG promoter (LVC.tTRKRAB) into the perivitelline zone of fertilized oozytes, which we re-implanted into foster mothers as described previously (23, 28) (Fig. 1*B*). We then transduced ear fibroblasts from the pups with a lentiviral vector containing a GFP reporter downstream of a polymerase II promoter juxtaposed with *tetO* sites. Animals whose cells displayed fully doxycycline-controllable GFP expression, indicative of a functional tTRKRAB system, were kept for further studies (data not shown). In parallel, we generated transgenic mice with a lentiviral vector that expressed GFP under the control of the CAG promoter and contained *tetO* sequences in the LTR (LVCT.GFP). CAG is a chimeric promoter comprising the CMV immediate early enhancer linked to the chicken β-actin promoter and first intron, which was previously found to be strongly active in transgenic mice (28, 29). Accordingly, the LVCT.GFP mice displayed high levels of GFP expression in all tissues (data not shown). We generated dually transgenic animals by crossing LVC.tTRKRAB males with LVCT.GFP females (Fig. 1*A*). We divided the population into four groups, depending on the time point from which dox was administered (*e.g.* E0.5, E6.5, or E13.5 or from birth). Then we tested the dox responsiveness of GFP expression in ear fibroblasts harvested from 6-week-old pups from all groups (Fig. 1*C*). GFP was expressed only in cells derived from the group of animals that had received dox from E0.5, whereas it remained undetectable in cells from animals in the other groups that received the antibiotic only from later time points, even after prolonged dox treatment (Fig. 1*D*). These results suggested that binding of tTRKRAB to the provirus during the first days of development resulted in irreversible silencing of the CAG promoter.

KRAB-induced Irreversible Silencing Is Caused by DNA Methylation—To investigate the likely epigenetic mechanism of the silencing in GFP-negative animals, we treated their fibroblasts with selective inhibitors of DNA methyltransferases (5-aza-cytidine, 5-Aza) and histone deacetylases (TSA) (Fig. 2*A*). TSA had no effect, whereas 5-Aza restored dox-induced GFP expression in these cells, consistent with a primary role for DNA methylation in the silencing. In support of this hypothesis, Southern blot and PCR-based analyses of genomic DNA digested with a methylation-sensitive restriction endonuclease revealed that the provirus was methylated in regions corresponding to the CAG promoter in fibroblasts from all animals, except in those exposed to dox since day E0.5 (data not shown). To confirm these data with a more quantitative approach, we performed a bisulfite sequencing analysis of genomic DNA from mice harboring silenced or expressed proviruses. Because amplification of the CAG promoter turned out to be technically difficult, we generated new animals using the LVUT.GFP.IRES.tTRKRAB vector, which contains all parts of the KRAB system, including a *tetO* site and a human ubiquitin promoter-GFP cassette driving the expression of a tTRKRAB-ires-GFP cassette in a self-regulatory fashion, as described previously (Fig. 2*B*) (14). In these animals as in their LVC.tTRKRAB/LVCT.GFP counterparts, GFP was irreversibly repressed when dox was omitted during early development (data not shown). This result indicated that KRAB-induced silencing was not restricted to the CAG promoter. Bisulfite sequencing of the ubiquitin promoter region of the provirus revealed that in animals left off dox during early embryogenesis, there was an almost complete methylation of the 23 CpG dinucleotides contained in this fragment (Fig. 2*C*, *Dox*−, *left*; 96.4 and 98.3% methylated CpG dinucleotides in 14 and 10 clones, respectively, amplified from two mice). In contrast, the animals that had been kept on doxycycline all harbored completely unmethylated ubiquitin promoter sequences, albeit mixed with partially and even some completely methylated sequences (Fig. 2*B*, *Dox*+, *left*; 55% unmethylated CpG in 30 fragments amplified from two mice). We also performed bisulfite analysis of the transgene coding sequence (GFP). Interestingly, we found that this region was highly methylated in both sets of animals (Fig.

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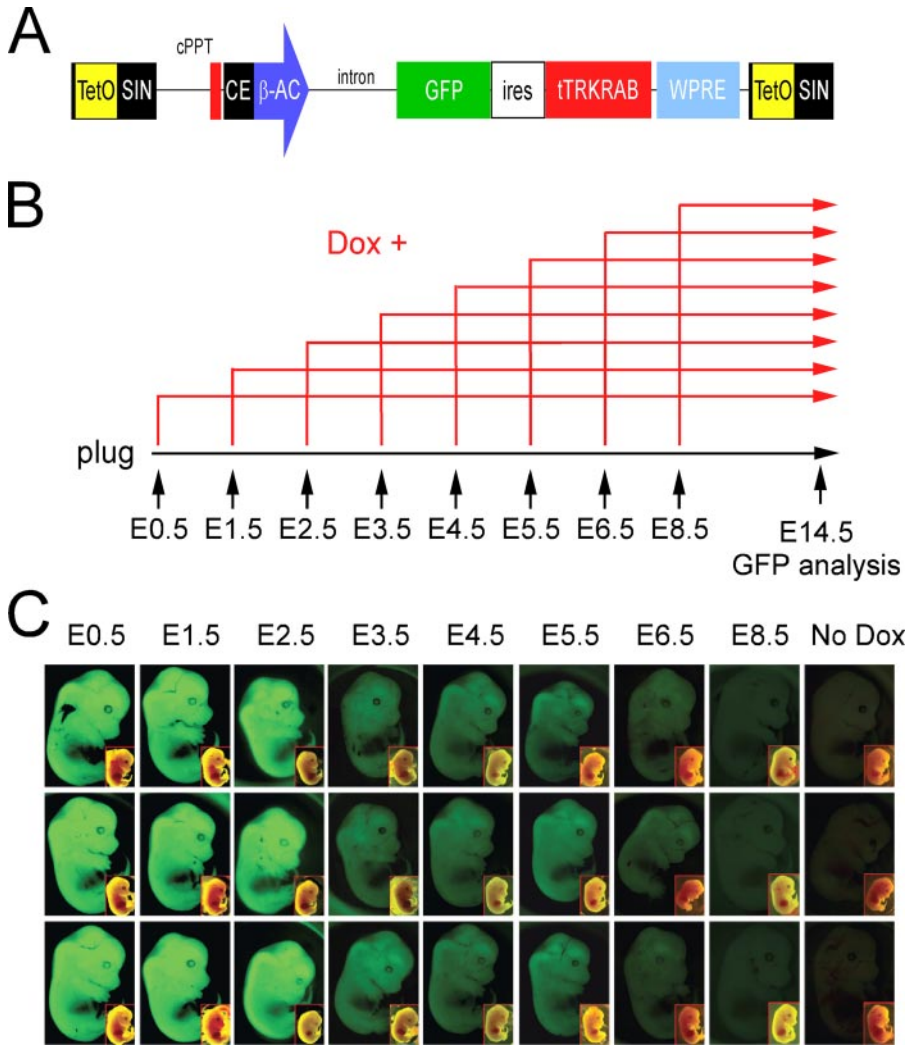

FIGURE 3. **Timing of KRAB-induced DNA methylation.** *A,* integrated form of the LVCT.GFP.IRES.tTRKRAB vector. *B,* experimental scheme. Transgenic males carrying the LVCT.GFP.IRES.tTRKRAB vector were crossed with wild type females that received doxycycline at the indicated time points. *C,* epifluorescence analysis of GFP expression in the F1 transgenic embryos retrieved at E14.5. *SIN,* self-inactivating long terminal repeat; *cPPT,* central polypurine tract; *CE,* CMV enhancer; *β-AC,* chicken β-actin promoter; *WPRE,* woodchuck hepatitis post-transcriptional regulatory element.

2*B*, *right*). We also found complete methylation of the GFP sequence in all LVC.tTRKRAB/LVCT.GFP mice, whether they had been kept "on" or "off" doxycycline during early development, and of the LTR region of the proviruses, in which transcriptional sequences were deleted because of the "self-inactivating" design of the vector, in both groups of animals (supplemental Fig. 1). These data indicate that, following lentivector-mediated transgenesis, the provirus becomes methylated over its entire sequence except in region(s) corresponding to active promoter(s). Our results also suggest that binding of a KRAB-containing repressor during the early embryonic period overrides factors that normally prevent the methylation of CpG-rich promoters.

tTRKRAB-induced DNA Methylation Is Restricted to the Earliest Days of Development—These results suggested that KRAB-induced DNA methylation of promoters contained in lentiviral vectors paralleled the physiological wave of genome-wide methylation taking place during the first days of mammalian embryogenesis. We performed a series of experiments to study this in more detail. We crossed wild type females with transgenic males generated with the LVCT.GFP.IRES.tTRKRAB vector, which contains both a *tetO*-CAG-GFP conditional expression cassette and the tTRKRAB repressor (14). We then treated the pregnant females with dox starting at day E0.5 or at each consecutive day up to E8.5, and we examined GFP expression in embryos harvested at E14.5 (Fig. 3, *B* and *C*). Embryos were strongly GFP-positive as long as dox had been administered at day E2.5 or earlier. With later time points of dox introduction, we found a progressive decrease of GFP expression with no GFP expression detected when doxycycline administration was started at E6.5 or later. Southern blot analysis revealed that this loss of GFP expression was accompanied by increased DNA methylation of the promoter sequences (data not shown). Together, these experiments show that if tTRKRAB binding is prevented before E3.5, GFP expression is fully restored at E14.5. However, if KRAB is left to bind between E0 and E3.5, GFP expression as measured at E14.5 is reduced, at levels that are inversely proportional to the duration of the dox-off, KRAB-bound period, with complete silencing if the repressor is allowed to bind until E7.5. Therefore, these results identify a time window during which KRAB-induced irreversible silencing occurs.

To confirm that KRAB-induced DNA methylation can be reversed during early development, we crossed LVCT.GFP.IRES.tTRKRAB-transgenic, GFP-suppressed males with wild type females, which we then treated with dox from the beginning of their pregnancy (Fig. 4*A*). The offspring expressed GFP at high levels (Fig. 4*B*). Correspondingly, Southern blot analyses confirmed that methylation of the CAG promoter, present in the fathers, was reversed in the F1 generation when dox prevented tTRKRAB binding during early embryogenesis (Fig. 4*C*). Of note, animals produced in this fashion exhibited fully reversible silencing of the transgene even after up to 12 months without doxycycline (data not shown).

ES Cells and ES Cell-derived Mice Exhibit Reversible KRAB-mediated Repression—These results demonstrate that KRAB induces *de novo* DNA methylation if tethered to promoter sequences during early embryogenesis. It is well established that *de novo* methylation can be carried out by the DNA methyltransferases DNMT3a and DNMT3b (22). These enzymes are

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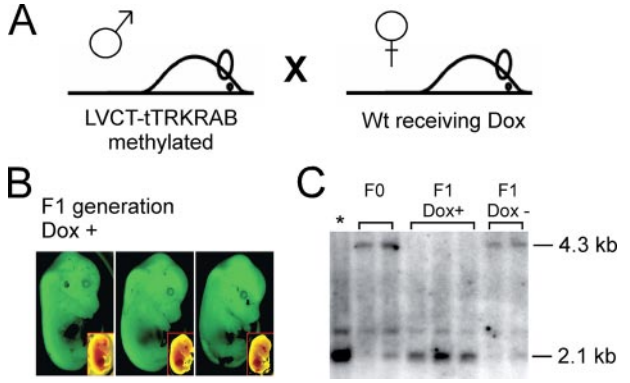

FIGURE 4. **KRAB-induced promoter methylation is reversed in the preimplantation embryo.** *A,* silenced LVCT.GFP.IRES.tTRKRAB mice were crossed with wild type (*Wt*) females receiving doxycycline throughout pregnancy. *B,* GFP expression in F1 embryos (E12.5) from pregnant females receiving doxycycline from E0.5. *C,* Southern blot analysis of BssHII/BglII-digested genomic DNA from transgenic F0 males and F1 embryos born from wild type females treated with or without doxycycline during pregnancy, using a probe complementary to a lentiviral specific sequence (RRE). In the F0 mice and F1 mice not receiving doxycycline, a 4.3-kb band representing methylated CAG promoter is easily detected. The 2.1-kb band represents a nonmethylated CAG promoter. *, control male that received doxycycline during development and then after birth was left off doxycycline for 12 months.

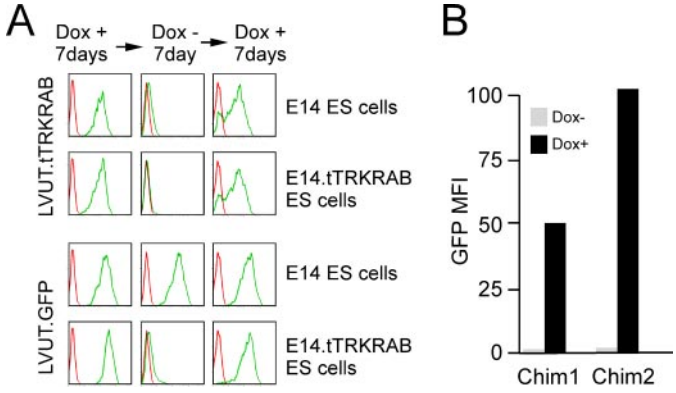

FIGURE 5. **KRAB-induced silencing is reversible in mES cells and ES cell-derived mice.** *A,* wild type ES cells or ES cells stably expressing tTRKRAB were transduced with either LVUT.GFP.IRES.tTRKRAB or LVUT.GFP and kept in the presence of doxycycline for 7 days. GFP-expressing cells were FACS-sorted and FACS-analyzed. Doxycycline was then removed from the cell culture media for 7 days. After subsequent FACS analysis, the cells were then once again treated with doxycycline for the next 7 days. *y* axis represents number of cells; *x* axis represents fluorescent intensity. *B,* mES cells transduced with the LVCT.GFP.IRES.tTRKRAB and kept in the absence of doxycycline were used for blastocyst injection. The foster mother did not receive doxycycline until midgestation. GFP expression in fibroblasts from chimeric mice (*Chim*) cultured in the presence or absence of doxycycline were then analyzed.

highly expressed in murine embryonic stem cells, a setting in which endogenous retroelements are irreversibly silenced through mechanisms that involve DNA methylation (30). However, we previously noticed that KRAB-induced silencing does not lead to DNA methylation in mES, even after prolonged repression (14). A concern with this experiment was that the autoregulatory design of the LVCT.GFP.IRES.tTRKRAB vector implies that levels of the trans-repressor are low in the silenced state. Thus, to confirm that tTRKRAB does not induce DNA methylation in ES cells, we generated clones of E14 mES cells stably expressing tTRKRAB (by transduction with LVC.tTRKRAB). These cells were then transduced with lentiviral vectors harboring *tetO* sites either in the LTRs or next to the internal promoter and expressing GFP from the CAG, the ubiquitin, or the human PGK promoters. In all cases we observed fully reversible, dox-mediated regulation, with no sign of irreversible silencing (Fig. 5*A*). We then injected mES cells transduced with the LVCT.GFP.IRES.tTRKRAB vector that had been kept off dox for 2 weeks into blastocysts, and we transferred those to foster mothers that we kept off doxycycline. We then examined the resulting pups. Blood samples from these chimeric mice contained GFP-expressing cells if the animals received dox after birth (data not shown). Furthermore, analysis of ear fibroblasts harvested from 6-week-old mice revealed fully dox-responsive GFP expression (Fig. 5*B*). Thus, KRAB-induced silencing is not accompanied by irreversible silencing when it is triggered in ES cells, even when these are re-implanted *in vivo*. It suggests that the epigenetic pathway responsible for KRAB-induced DNA methylation in early embryos is not present in ES cell lines, and it is not re-activated when these are placed in the environment of a pre-implantation blastocyst.

KAP-1 Is Essential for tTRKRAB-mediated Transcriptional Repression—It has been demonstrated that the KAP1 corepressor is essential for KRAB-mediated repression in human cells (9). To confirm that our experimental system, which relies on a fusion of the human KOX1 protein KRAB domain with the *tetR* from *E. coli*, functions through similar mechanisms, we generated primary fibroblasts from mice carrying loxP site-modified KAP1 alleles (26). To inactivate KAP1, we transduced these cells with a lentiviral vector expressing the CRE recombinase (24). Genotyping of the resulting cell population 1 week later confirmed that the LoxP-flanked sequence had been efficiently excised, which resulted in an almost complete loss of KAP1 transcripts, as assessed by real time PCR (Fig. 6, *A* and *B*). When we transduced these cells with the LVCT.GFP.IRES.tTRKRAB vector in the presence or absence of doxycycline, we did not observe any KRAB-mediated repression of the GFP transgene (Fig. 6*C*). This result confirms that our system completely depends on KAP1, as is thought to occur with endogenous KRAB-containing zinc finger proteins.

DISCUSSION

Here, we demonstrate that tethering a KRAB-containing protein to chromosomal DNA during the first few days of mouse embryonic development triggers CpG methylation in nearby promoters. These results were obtained in the context of lentiviral integrants subjected to control by a *tet* repressor-KRAB fusion protein. Considering that the natural targets of KRAB-containing ZFPs are mostly unknown, one can only speculate about the importance of this observation for the methylation events that occur during the earliest period of vertebrate embryogenesis. However, the strict KAP1 dependence of our system supports its physiological relevance.

It appears from our cumulated data that KRAB-mediated repression has distinct consequences depending on the context. Although we systematically observed that tTRKRAB induced irreversible promoter silencing because of promoter CpG methylation if tethered to a lentiviral integrant during the first few days of mouse embryogenesis, we always observed that tTRKRAB-induced repression was reversible outside of this time period. Similarly, in transduced murine or human cell

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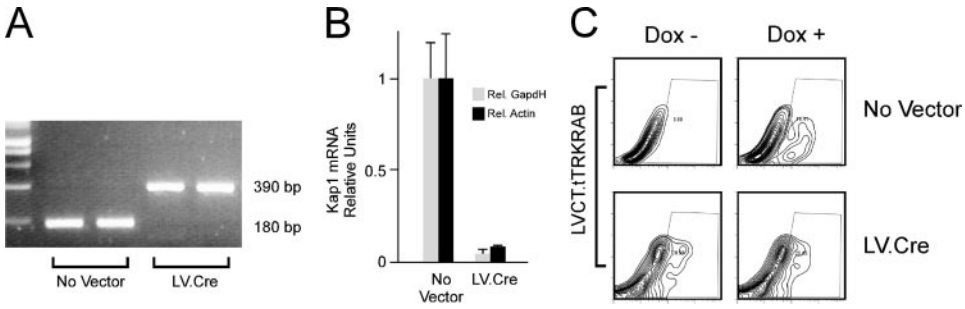

FIGURE 6. **tTRKRAB-induced repression is dependent on KAP1.** *A,* PCR analysis of fibroblasts derived from mice homozygous for a floxed KAP1 allele transduced with a lentiviral vector expressing the CRE recombinase. The 390-bp band represents a null-allele. *B,* quantitative real time PCR analysis of cDNA derived from the floxed KAP1 fibroblasts. *C,* FACS analysis of floxed KAP1 fibroblasts first transduced with the lenti-CRE followed by transduction with LVCT.GFP.IRES.tTRKRAB and cultured with or without doxycycline. *y* axis represents forward scatter; *x* axis represents fluorescent intensity.

lines or primary cells as well as in the brain of rats injected with a tTRKRAB-controllable lentiviral vector, target promoters could be turned on and off through addition or removal of doxycycline (14, 15). It is unclear why we found KRAB-induced irreversible silencing in embryos but not in mES cells considering that the *de novo* methyltransferases Dnmt3a and Dnmt3b are known to be highly expressed and active in mES cells (22). It may be that an as yet unidentified factor links KAP1 to the *de novo* DNA methylation machinery and that this factor is missing in mES cells.

Our results contrast with a previous report revealing that KRAB can induce CpG methylation and irreversible polymerase II promoter silencing in plasmid-transfected NIH3t3 cells (8). One explanation could be that our lentivector-based approach introduced at most two tTRKRAB-binding sites in the vicinity of targeted promoters, whereas in this other study stable cell lines were generated with plasmids, most likely resulting in the immediate juxtaposition of many KRAB-binding sites and in turn in heterochromatin-forming complexes, perhaps above a critical threshold. Furthermore, lentiviral vector-mediated transduction can yield several integrants per cell, increasing the chance that one at least will be fully regulatable.

Bisulfite sequencing revealed that even in the absence of tTRKRAB binding during development, only about half of the active promoters remained unmethylated. This suggests that lentiviral integrants are subjected to epigenetic modifications and that a significant fraction of them are inactivated by DNA methylation, along a pattern presumably influenced by the integration site of the vector. Interestingly, it was only in the promoter region that we found unmethylated proviral DNA, whereas the coding sequences and the inactivated LTRs were always methylated. Thus, the pattern of lentiviral provirus DNA methylation mimics that of cellular genes, where the coding sequence is usually methylated irrespective of the state of the promoter. The strong housekeeping promoters present in the lentivectors used in this study all harbor start CGI. It is unclear why CpG islands are not methylated during early development, although the correlation between start CGIs and expression during early embryogenesis suggests that expression *per se* might prevent this modification (21). Our data would support such an interpretation.

Because they were obtained in the unnatural context of the tTR-KRAB/*tet*O lentivector-based system, our results do not allow us to implicate formally KRAB-containing zinc finger proteins in the wave of DNA methylation that characterizes the mammalian early embryonic period. Noteworthy, many conditional transgenic mice lines have been established based on a *tetR*-containing transactivator, the functionality of which eliminates the possibility that the presence of a *tetR* domain be in itself responsible for triggering CpG methylation (31–34). Our results thus warrant experiments aimed at determining whether a lack of KRAB-mediated DNA methylation contributes to the early embryonic lethality of KAP1 knock-outs. More specific analyses will have to await the unequivocal identification of KRAB-KAP1 functional targets. No less than 7000 KAP1-binding sites were recently identified in the human genome through chromatin immunoprecipitation studies (35). However, the remarkably subtle phenotypes of several conditional KAP1 knock-out mice recently established in our laboratory[5] suggest that this number is a gross overestimation of *bona fide* KRAB/KAP1 functional targets.

Independently of these considerations, our data open interesting leads for the development of new genetic tools. For instance, variations of our system, *e.g.* through the site-specific introduction of tTRKRAB-binding sites by homologous recombination or via the fusion of KRAB with natural or artificial zinc fingers (36), could be used to induce methylation at virtually any place in the genome, whether to knock out genes or to try modulating the function of *cis*-acting elements such as enhancers or *trans-a*cting elements such as transposons (37, 38).

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