The battle inside our genome

Controlling transposable elements and the evolution of human gene regulatory networks

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Chapter 4

The Identification of SVA Controlling KZNF Proteins: ZNF91 and ZNF611 Bind SVA elements, but Only ZNF91 is Essential for Their Repression

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Submitted
Abstract

SVA elements are a great-ape specific class of transposable elements which gave rise to ~ 1500 human-specific SVA insertions since we diverged from our common ancestor with chimpanzees. In addition, SVA elements are still active in modern humans leading to hundreds of polymorphic SVA insertions in the human population and sporadic occurrence of SVA-insertion linked diseases. Importantly, SVA elements have been attributed cis-regulatory properties and may have contributed to the evolutionary innovation of human gene regulatory networks. Yet, in most tissues, SVA elements are kept in a repressed state through binding of specific members of the KRAB zinc finger (KZNF) gene family and the recruitment of co-repressor KAP1. Here we aimed to find which KZNF genes are required and sufficient for SVA repression. To this end, we identified ZNF91 binding sites through chromatin immunoprecipitation (ChIP) and re-analyzed ChIP-exo data of ten KZNF proteins that were suggested to bind SVA elements. We identified ZNF91 and ZNF611 as having the highest capacity to bind SVA elements. To elucidate the roles of ZNF91 and ZNF611 in controlling SVA elements we used CRISPR/Cas9 to delete ZNF91 and ZNF611 in human embryonic stem cells (hESCs). RNA sequencing revealed that SVA elements became transcriptionally active only upon ZNF91 deletion and no additive effect was observed if both ZNF91 and ZNF611 genes were deleted. Interestingly, we did observe a collective upregulation of KZNF genes upon deletion of either ZNF91 or ZNF611. This suggests that loss of KZNF-mediated SVA control elicits a genomic response which might act to counteract the aberrant activation of SVAs in our genome. Our data sheds light on the complex battle between SVA elements and KZNF genes in our genome possibly giving a glimpse of the pioneer response of our genome when invaded by TEs.
Introduction

Transposable elements (TE) are mobile pieces of DNA that can spread through a copy and paste mechanism and compose approximately 50% of the human genome (Cordaux and Batzer 2009). Although first discarded as junk DNA, they are now widely regarded as drivers of evolutionary innovation of gene regulatory networks. It has been hypothesized that through waves of insertions TEs contributed to the evolution of gene expression by providing individual genes new cis-regulatory elements, and connecting genes that have a TE insertion nearby on the regulatory level. Indeed, multiple examples exist where TEs function as species- and cell type-specific gene regulators (Wang et al. 2007; Kunarso et al. 2010; Xie et al. 2010; Lynch et al. 2011; Chuong et al. 2013; Sundaram et al. 2014; Notwell et al. 2015; Chuong et al. 2016; Ito et al. 2017; Trizzino et al. 2017, 2018; Pontis et al. 2019).

In this study we focus on a great-ape-specific TE family called SVA, named after it’s composite domains: SINE-VNTR-Alu. They show ~2700 fixed insertions in the human genome of which ~1500 are human-specific. SVA elements are the youngest active human retrotransposon and therefore polymorphic SVA insertions occur frequently in the human population (Ostertag et al. 2003; Savage et al. 2013, 2014; Ha et al. 2016). They are shown to function as transcriptional activators or repressors, depending on the TFs they are bound by and they act in a cell-specific manner (Jacobs et al. 2014; Trizzino et al. 2017, 2018; Pontis et al. 2019). Although they have a strong gene regulatory potential, it has been suggested that SVA insertions mostly negatively affect gene expression through the induction of local chromatin (Lippman et al. 2004; Rebollo et al. 2011; Jacobs et al. 2014). Namely, under most conditions SVA elements are repressed by members of the KRAB zinc finger gene family (Jacobs et al. 2014; Imbeault et al. 2017; Pontis et al. 2019) that recruit KAP1 (Nielsen et al. 1999; Sripathy et al. 2006) and repressive epigenetic modifiers (Schultz et al. 2001, 2002; Turelli et al. 2014).

The KZNF gene family expanded through segmental duplications and most KZNF genes reside in clusters (Huntley et al. 2006; Thomas and Schneider 2011). The correlation of waves of TE insertions with expansion of the KZNF clusters is in part explained by the evolutionary arms race model which proposes that KZNF genes emerge to repress new TE invasions (Thomas and Schneider 2011; Jacobs et al. 2014; Imbeault et al. 2017). However, it remains elusive how KZNF genes sense new TE invasions and how their response is mediated. It was shown recently that TE activation can trigger an innate immune response consisting
of induction of interferon stimulated genes and upregulation of KZNF genes (Kauzlaric et al. 2017; Tie et al. 2018). These findings were attributed to a TE-tran-
script induced immune response and cis-regulatory properties of activated TEs
controlling KZNF genes closeby. The mechanisms behind this and the conse-
quences of this first line of defense against TE activity remain to be established.

We previously showed that ZNF91 is able to bind and repress SVA elements
(Chapter 3, Jacobs et al. 2014). However, a recent study suggested that a
number of other KZNF proteins also bind SVA elements (Imbeault et al. 2017).
However, to what extent SVA elements are controlled by these SVA-binding
KZNF proteins and what their interrelation is, remains elusive. In this study we
identified ZNF611 and ZNF91 as the KZNF genes that have the highest binding
capacity to SVA elements. By genetic deletion of ZNF91, ZNF611 or both in
human embryonic stem cells (hESCs) we determined which of these KZNF is
required and sufficient for repression of SVA elements. In chapter 3 we extensi-
vely analyzed the impact of the deletion of ZNF91 on the epigenome and tran-
scriptome of SVA elements. In this chapter we extend this analysis by studying
the role of ZNF611 and the combined effect of ZNF611 and ZNF91.

Results

ZNF91 and ZNF611 bind SVA elements at the VNTR domain
To identify the repressor(s) of SVA elements we investigated the binding patterns
of several candidate KZNF proteins. We performed ZNF91-GFP overexpression
in HEK293 cells followed by chromatin immunoprecipitation and sequencing
(ChIP seq). We compared this to ChIP-exo data of ten KZNF proteins that were
suggested to bind SVA elements (Imbeault et al. 2017). Our analysis revealed
that ZNF91 and ZNF611 are the only KZNF proteins that bind all SVA subclasses
and show overall much higher coverage on SVA elements compared to the
other KZNF proteins (Fig 1A). Evolutionary young SVA elements (SVA-D/E/F)
are exclusively bound by ZNF91 and ZNF611 (Fig 1A), whereas the older SVA
subclasses (SVA-A/B/C) also show binding of other KZNF proteins, albeit to a
lesser extent (Fig 1A). 88% and 58% of SVA elements are bound by ZNF91 and
ZNF611 respectively and nearly all ZNF611 bound elements are also bound
by ZNF91 (Fig 1B). Interestingly, ZNF91 binding is found at two domains of
several SVA subclasses, whereas ZNF611 binding is confined to one region
(Fig1A+D). ZNF91 binding co-localizes with KAP1 peak-summits (Jacobs et
al. 2014) at the border of the Alu-VNTR domains (Fig 1D). ZNF611, on the
other hand, binds to a more central part of the VNTR (Fig 1D). To determine the core binding sites of ZNF91 and ZNF611 we generated motifs from top scoring peaks localized to SVA elements. For ZNF91 we generated two motifs; one derived from peaks localized to the Alu-VNTR domain and the other from peaks localized to the VNTR-SINE domain. Both ZNF91 motifs show overlap with each other and the ZNF611 binding motif in the VNTR part (Fig 1E). This indicates that the core binding site of both KZNF proteins localizes to the VNTR domain of SVA elements. Note that the small proportion of SVA elements not bound by ZNF91 or ZNF611 do contain either the ZNF91 or ZNF611 motifs in their DNA sequence. However, these SVA elements are much shorter and are likely truncated SVAs which contain less potential binding sites. (Fig 1F). The remaining group of SVA elements not bound by ZNF91 or ZNF611 is even more truncated and lost the KZNF binding sites completely (Fig 1F).

**Homininae-specific changes in ZNF domains of ZNF611 may have enabled it to recognize SVA elements**

To get more insight into the battle between SVA elements and KZNF proteins we studied the structural changes of ZNF91 and ZNF611 over the course of evolution in relation to the expansion of SVA elements. ZNF91 and ZNF611 both emerged in the last common ancestor (LCA) of old world monkeys (OWM). ZNF91 gained the ability to repress SVA elements after major structural changes consisting of the addition of seven zinc fingers as a result of segmental duplication of zinc fingers 18-23 in the LCA of gorillas, chimpanzees and humans (Jacobs et al., 2014, Fig 2A). A multiple sequence alignment of the ZNF611 protein sequence derived from different primate species revealed only small structural changes over the course of evolution (Fig 2A, Suppl Fig 1).

**Figure 1. SVA elements are bound by ZNF91 and ZNF611 at the VNTR domain.**

A) Profile plot showing ChIP coverage of putative SVA binding KZNF proteins (Except ZNF91, ChIP data from Imbeault et al. 2017). For ZNF91 the mean of two replicates is displayed. ZNF91 and ZNF611 are highlighted, since they have the strongest affinity for SVA elements. B) Pie charts showing the number of SVA elements bound by ZNF91 (green) and ZNF611 (yellow), Venn diagram showing the number of SVA elements bound by ZNF91, ZNF611, and both. C) Frequency plot showing fraction of SVA subclasses bound by ZNF611 (yellow), ZNF91 (green), both (light green) and neither (grey). D) Schematic of SVA-D with different domains indicated. In blue a pile up of KAP1 summits on the SVA-D consensus is displayed (Jacobs et al., 2014). In green and yellow a representative example of ZNF91 and ZNF611 coverage on an SVA-D are shown. Two ZNF91 binding motifs in SVA elements were identified localizing to the Alu-VNTR border and VNTR-SINE border and one ZNF611 motif in the VNTR. E) Comparison of ZNF611-motif (yellow) with ZNF91-motif1 and ZNF91-motif2 (both green) showing overlap at the VNTR part of the ZNF91 binding motifs. F) Length of SVA elements bound by ZNF91, ZNF611 or both (light green) and not bound by ZNF91 or ZNF611, but with any of the binding motifs present in their sequence (light grey) and not bound SVA elements without any of the motifs (dark grey). For statistiscal testing an unpaired wilcoxon rank sum test was used followed by an FDR correction (**** P<0.0001).
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However, there are several remarkable changes in the ZNF domains of ZNF611 in lineages towards human. We used ZNF611 binding motif prediction to identify the potential impact of these changes on the recognized DNA code (http://zf.princeton.edu, Persikov & Singh 2014, Fig2B). In several species belonging to OWM lineages, ZNF611 showed the exact same configuration, consisting of a KRAB domain and 17 zinc fingers (Suppl Fig 1). However, in the LCA of gorillas, chimpanzees and humans zinc finger 4 became dysfunctional as a consequence of the substitution of both histidine residues (Fig 2A+B). Note that two histidine residues, together with two cysteine residues, are essential for successful folding of the finger around a zinc ion (Frankel et al 1987). Four other zinc fingers showed structural changes in lineages towards human leading to alterations in the recognized DNA sequence (Fig 2A+B). The most remarkable change is probably the human and chimpanzee specific substitution of a DNA contacting amino acid residue in zinc finger 15, resulting in the addition of a C in to binding motif (Fig 2A+B). This part of the predicted motif showed striking overlap with the logo generated with non-TE sequences bound by human ZNF611 according the ChIP-exo data. This finding not only increases the trustworthiness of the predicted motifs, but it also suggests that the structural change of zinc finger 15 may have been important for ZNF611 to be able to recognize SVA elements (Fig 2B).

**SVA transcription is unaffected in ZNF611 ko hESCs**

To increase our understanding of the role of ZNF91 and ZNF611 in controlling SVA elements we first looked at their expression levels throughout the human body. In most tissues ZNF91 and ZNF611 are moderately to highly expressed, although ZNF91 is generally expressed at higher levels (Fig 3A). In hESCs both

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**Figure 2. Evolutionary history of ZNF611 and ZNF91.**

A) Schematic overview of structural evolution of ZN611, ZNF91 and SVA elements with KRAB and zinc finger domains indicated. Conserved Zinc fingers are indicated in yellow (ZNF611) and green (ZNF91). Zinc fingers that changed a contact residue (position -1, 2,3,6 of the alpha helix) in the lineage to human (light pink) or another lineage (dark pink) are shown. In purple zinc fingers that were predicted to be dysfunctional because of loss of zinc coordinating histidines or cysteines.

For ZNF91 light blue zinc fingers indicate that they were added as a result of segmental duplication events derived from zinc fingers 6 and 18-23 (indicated with grey arrows, Jacobs et al 2014).

For ZNF611 the amino acid sequence of zinc finger 4 and zinc finger 15 is displayed for different primate species. In red substituted histidines are indicated. In purple the cysteine and histidine residues are indicated and in pink the DNA contacting amino acid residues.

B) ZNF611 protein sequence of different primate species were used to predict the number of functional zinc fingers and the recognized DNA motif (http://zf.princeton.edu/index.php). The dark red boxes indicate part of the motif that shows differences between (the lineages to) human and the ancestral version that may have been relevant for the binding of SVA elements. At the bottom the human ZNF611 binding motif as identified with ChIP matched with the predicted motif.
The Identification of SVA Controlling KZNF Proteins: ZNF91 and ZNF611 Bind SVA elements, but Only ZNF91 is Essential for Their Repression

A

DNA binding residue change in lineage to human  Predicted dysfunctional ZNF
DNA binding residue change in other lineage  Segmental duplications of ZNF domains
Premature stop codon

Rhesus macaque
Gibbon
Orangutan
Gorilla
Chimpanzee
Human

ZNF611

ZNF91

SVA

KRAB

B

Predicted # ZNF

Rhesus macaque 17
Gibbon 17
Orangutan 17
Gorilla 10
Chimpanzee 16
Human 16

Human ChIP non-TE motif
genes are expressed, with ZNF91 expression levels being approximately twice as high as ZNF611 (Fig 3B). Expression of ZNF91 and ZNF611 was abolished through genetic deletion of their TSS in hESCs (Fig 3B). We reasoned that they could both be essential for SVA control, therefore we used one ZNF91 ko line to generate a ZNF91/ZNF611 double knockout (dko, Fig 3B). RNA sequencing of different ZNF91 ko, ZNF611 ko and ZNF91/ZNF611 dko clonal lines revealed that SVA transcription is induced only when ZNF91 was abolished (Fig3C). Differential expression analysis revealed 173 significantly upregulated SVA transcripts in ZNF91 ko versus wt cells. However, none of these elements showed upregulation in absence of ZNF611, although 75% of these SVA elements also showed ZNF611 binding (Fig 3D). On top of that, no additional effect on SVA transcription was observed when ZNF611 was deleted in ZNF91 ko cells (Fig 3C+D). Finally, no SVA driven chimeric transcripts, as identified in absence of ZNF91, could be found in ZNF611 ko cells (Suppl. Fig 2).

**ZNF91 and ZNF611 bind to different gene promoters**

The large overlap between ZNF91 and ZNF611 SVA binding motifs suggests that they may be binding similar non-SVA sequences as well. ZNF91 and ZNF611 motifs derived from peaks localized at promoter sequences are aligning to the SVA motifs and to a lesser extent to each other (Fig 4B and Suppl Fig 3A). Surprisingly, the number of gene promoters bound by both KZNF proteins at the same genomic location is very limited (29 genes, Fig 4A). It is noteworthy that 11/29 of these genes are recently evolved non-coding RNAs. ZNF91 binds to genes associated with chromosomal organization and localization, DNA conformation and regulation of cell cycle (Fig 4A). Genes bound by ZNF611, on the other hand, are related to limb development, sex differentiation and positive regulation of cell death (Fig 4A). Interestingly, two GO terms are shared in the top10, although they encompass different genes. The fact that ZNF91 and ZNF611 bind to 1062 and 461 gene promoters respectively suggests that both may directly regulate these promoters (Fig 4A). However, differential gene expression analysis using RNA seq data of ZNF91 and ZNF611 ko hESCs revealed no collective expression change of ZNF91 bound or ZNF611 genes respectively (Fig 4C). This may indicate that the KZNF binding data which is derived from KZNF overexpression in HEK293 cells, does not accurately represent the roles of the KZNF proteins in hESCs. Therefore, we restricted our analysis to gene promoters that showed a KAP1 peak at the same location in hESCs (data from Jacobs et al. 2014). 14% of ZNF91 and 19% of ZNF611 binding sites in gene promoters are bound by KAP1 in hESCs (Suppl Fig 3B). However, these genes do not show a collective expression change
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Figure 3. SVA transcription only upregulated in absence of ZNF91
A) Expression of ZNF91 and ZNF611 in different tissue expressed in transcripts per million (TPM), data from Gtex Portal. B) Schematic overview of ZNF91 and ZNF611 genes. gRNAs targeting the area around the TSS were used to generate ZNF91 ko, ZNF611 ko, ZNF91/ZNF611 dko hESCs using CRISPR/Cas9, coverage tracks of RNA seq displays expression of ZNF91 and ZNF611 in hESCs. C) Principal component analysis including all SVA transcripts in hESC lines with indicated genotype. D) Heatmap showing hierarchical clustering of SVA transcripts differentially expressed in ZNF91 ko hESCs (Log2FC >3) using Z-score of normalized counts of RNA seq data of different wt and ko hESC lines. Pie chart showing fraction of differentially expressed SVA elements that is bound by ZNF611.
in ZNF91 or ZNF611 ko hESCs either (Suppl Fig 3C). Overall we cannot find evidence for a direct gene regulatory role for ZNF91 or ZNF611 in hESCs.

**Upregulation of KZNF genes in ZNF91 and ZNF611 ko hESCs**

PCA of gene expression revealed that the impact of ZNF91 and/or ZNF611 deletion on gene expression is minimal (Suppl Fig 4A). To our surprise, however, we observed a collective upregulation of KZNF genes in both ZNF91 and ZNF611 ko hESCs (Fig 5A). It seems to be unlikely that they are regulated by ZNF91 and ZNF611 directly, since very few KZNF genes are bound by ZNF91 and/or ZNF611 at their promoter (Suppl Fig 4B). Subsequently, we asked if the location of KZNF genes was important for their upregulation in absence of ZNF91 or ZNF611. Most KZNF genes reside in clusters, but a small number of solitary KZNF genes exist in the genome. We found that KZNF genes in clusters are upregulated compared to solitary KZNF genes in ZNF91 ko, but not in ZNF611 ko hESCs (Fig 5B). Also, when we looked at individual clusters located on chromosome 19 the effect was restricted to a subset of clusters in the ZNF91 ko (clusters as described by Thomas & Schneider 2011, Suppl Fig 4C). The absence of an additive KZNF upregulation in ZNF91/ZNF611 dko cells compared to ZNF91 ko illustrates that the effect of ZNF611 ko is mild (Suppl Fig 4D). Recently it was shown that during early embryonic development evolutionary young KZNF genes are upregulated together with young TE classes, such as SVA elements (Pontis et al. 2019). Therefore we explored if upregulation of KZNF genes in ZNF91 and ZNF611 ko hESCs was related to evolutionary age. However, for neither KZNF ko this seems to be the case: none of the KZNF gene collections grouped by evolutionary age reached significance in ZNF611 ko hESCs and nearly all are significantly up in ZNF91 ko hESCs (Fig 5C, Suppl Fig 4E). Although SVA elements do not become transcriptionally active in the absence of ZNF611, it is possible that SVA elements are epigenetically derepressed leading to upregulation of KZNF genes in the neighbor-

**Figure 4 ZNF91 and ZNF611 bind to different gene promoters.**

A) Venn diagram showing the number of gene promoters bound by ZNF91 or ZNF611 and the 29 that are bound by both at the same location. 1Kb up- and downstream of the TSS was considered promoter sequence. SVA elements inside promoter sequences were excluded from this analysis. Two tables show the top 10 of GO terms associated with gene promoters bound by ZNF91 (left) and ZNF611 (right). Green high light shows GO terms that appear in both lists. B) Promoter binding motifs of ZNF91 and ZNF611 aligned. Promoter motifs were obtained with peak summits extended 50bp to each side localizing to 1kb up- and downstream of TSS of UCSC genes. For ZNF91 the top 200 peaks were included and for ZNF611 the top 100. C) Boxplots showing Log2 Fold Change of ZNF91 ko (left) and ZNF611 ko (right) of ZNF91 (green, 799 genes) and ZNF611 (yellow, 322 genes) bound gene promoters compared to a size matched random set of genes. For statistical testing an unpaired wilcoxon rank sum test was used (ns=not significant).
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**A**

Bound gene promoters

- **ZNF91**
  - 1032
  - 29

- **ZNF611**
  - 432

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**B**

Promoter motifs

**C**

- **ZNF91**
  - Log2FC: ns
  - Downregulated
  - Rs = 1

- **ZNF611**
  - Log2FC: ns
  - Downregulated
  - Rs = 1
hood. Because we do not possess information regarding the epigenetic status of SVA elements in ZNF611 ko hESCs we used the list of SVA elements that gain H3K4me3 in absence of ZNF91. Interestingly, KZNF genes located closer to an H3K4me3 positive SVA showed a stronger collective upregulation compared to a random set of genes than those that were located further away suggesting that indeed SVA elements may have become derepressed to a certain extent in the absence of ZNF611 (Fig 5D).

Figure 5. Upregulation of KZNF genes in ZNF91 and ZNF611 ko hESCs
A+B) Boxplots showing Log2Foldchange of KZNF gene expression and a similar number of random genes in ZNF91 ko (green) and ZNF611 ko (yellow) hESCs. A) All KZNF genes together (~300 genes), B) KZNF genes residing in clusters (~260 genes) or solo (37 genes). C) Boxplots showing log2Foldchange of KZNF genes grouped by evolutionary age compared to a group of randomly selected genes (random=26 genes, non-primate=162 genes, basal=18, simian=54, catarhine=47, hominoid=18). D) Boxplots showing Log2Foldchange of KZNF grouped per distance to nearest SVA that gained H3K4me3 upon ZNF91 deletion (random=82 genes, 0-50kb=112 genes, 50-100kb=60 genes, 100-150=46 genes, >150kb=85 genes). For all analyses only expressed (baseMean>10) genes were included. For statistical testing an unpaired wilcoxon rank sum test was used followed by an FDR correction in case multiple groups were compared (*P<0.05, **P>0.01, ***P<0.001, **** P<0.0001).
Discussion
ChIP data of tagged KZNF proteins revealed that ZNF91 binds SVA elements most strongly and frequently, followed by ZNF611 that also binds SVA elements of all subclasses. However, only ZNF91 appeared to be required and sufficient for SVA repression in hESCs. It was shown before that ZNF91 and SVA elements evolved in parallel driving each others evolution (Jacobs et al. 2014). This was illustrated by major structural changes of ZNF91 in the LCA of gorilla, chimpanzee and human that took place right after the emergence of SVA elements (Jacobs et al. 2014, Fig 2A). Here we showed that evolutionary pressure on ZNF611 seems to have been lower. However, recent structural changes in a few zinc fingers may have been a crucial step in optimizing ZNF611 to bind SVA elements. However, the lower levels of KAP1 at ZNF611 binding sites on SVA elements and the lower fraction of SVA elements bound indicate that it may be a suboptimal repressor in comparison to ZNF91. This is further supported by the fact that SVA elements only become transcriptionally active in the absence of ZNF91.

We are aware, however, that the effect of ZNF611 deletion could be mild and become invisible at the transcriptional level. It has been shown before that only a small fraction of epigenetically active SVA elements also show transcription, thus perhaps effects of ZNF611 deletion are limited to changes in chromatin status (Chapter 3, Pontis et al. 2019). Alternatively, ZNF611 may be required for SVA repression in other cell types or during other stages of development. Profiling of histone marks associated with active and repressed chromatin (e.g. H3K27ac, H3K4me3, H3K9me3) in ZNF611 ko hESCs and ZNF61-ko hESC-derived tissues could shed light on the role of ZNF611 in controlling SVA elements. Noteworthy is that a recent study showed that ZNF611 is capable of repressing H3K27ac positive SVA elements upon overexpression in naive hESCs (Pontis et al. 2019). This raises the possibility that, at least in hESCs, endogenous ZNF611 expression levels are too low to repress SVA elements effectively. ZNF611 may be overshadowed by ZNF91 which is expressed at a higher level and binds more SVA elements.

Unlike what is reported for other KZNF proteins (Oleksiewicz et al. 2017; Yang et al. 2017; Chen et al. 2019; Farmiloe et al. In press), we found no evidence for a strong effect of ZNF91 or ZNF611 binding to gene promoters directly, independent of SVA elements. Important to be aware of the fact that the ChIP data is obtained through KZNF overexpression in HEK293 cells, meaning that we cannot be certain that the identified binding sites are also occupied in hESCs. However, the fact that a subset of ZNF91 and ZNF611 peaks on gene promoters
co-localizes with KAP1 binding in hESCs is suggesting that, at least a fraction of, those peaks are genuine.

Striking is that although we find no transcriptional activation of SVA elements in the absence of ZNF611, we observed a collective upregulation of KZNF genes located close to SVA elements that gained H3K4me3 in ZNF91 ko hESCs. This is in line with data showing that in naive hESCs activated SVA elements connect to KZNF gene promoters and induces their expression (Pontis et al. 2019). In ZNF611 ko hESCs SVA elements may be activated only on the epigenetic level and this may just be enough to upregulate KZNF gene expression. More investigations into the chromatin status of SVA elements in ZNF611 ko hESCs would shed more light on this. If SVA elements are, to some extent, derepressed in ZNF611 ko cells these findings would further support a feedback loop between SVA activity and KZNF gene expression, as was observed for ZNF91 ko cells. A collective upregulation of KZNF genes in response to unleashed SVA elements could be a mechanism to enhance repressive capacities of KZNF proteins in defense against a potential TE outburst: Higher levels of KZNF protein may induce more aspecific binding, resulting in repression of sequences that are usually not bound with high affinity. This way the human genome would use the gene regulatory properties of SVA elements to enforce SVA repression. In support of this is a study that attributed upregulation of KZNF clusters in mouse KAP1 ko cells to unleashed TE enhancers in the vicinity (Kauzlaric et al. 2017).

Together our data show the complexity and dynamics of the interaction between KZNF genes and TEs. The co-evolution of SVA elements and ZNF91/ ZNF611 are a great example of how the human genome adapted to control the activity of a TE class with strong gene-regulatory potential. We showed that although SVA elements are bound by ZNF91 and ZNF611, ZNF91 seems to fulfill a more prominent role in controlling SVA elements in hESCs. On top of that, we obtained important insights into the adaptability of the human genome by unraveling a potential mechanism through which the epigenetic activation of TEs elicit the expression of KZNF genes as a way for our genome to establish a defense against the TE invasion.
**Materials & Methods**

**HEK293 cell culture and transfection**

HEK293 cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Invitrogen). 0.25% trypsin/0.01% EDTA (Invitrogen) in PBS was used to passage cells 1:10 every 3-4 days. One day prior to transfection, 2 million cells were seeded per 60mm dish to reach 70% confluency on the day of transfection. 4.1 μg pCAG.ZNF91-GFP or pCAG.GFP plasmid was used to transfect each 60mm dish using 5.5 μl lipofectamine 3000 (Invitrogen) and 8.3 μl P3000 (Invitrogen). 48 hours after transfection cells were isolated for chromatin immunoprecipitation (ChIP).

**Human embryonic stem cell culture**

H9 human embryonic stem cells were grown on matrigel (Corning) coated dishes. They were cultured in hESC medium that was incubated with mouse embryonic fibroblasts (MEFs) for 24 hours. hESC medium consisted of: DMEM/F12 supplemented with 2mM L-glutamine (Invitrogen), 20% knockout serum replacement (Gibco), penicillin/streptomycin (Invitrogen), non-essential amino acids (Invitrogen), 0.1mM 2-mercaptoethanol (Invitrogen). Medium was supplemented with basic fibroblast growth factor (sigma, 8ng/μl) and changed daily to secure pluripotency of hESCs. For maintenance of the culture, cells were grown in colonies and passaged manually by cutting the colonies with a needle. For transfection and clonal expansion hESCs were grown as single cells and passaged using accutase (sigma) at ratios 1:4-1:10. For single cell culturing, culture medium was supplemented with ROCK inhibitor Thiazovivin (2μM, Sigma) 1hr before dissociation and during plating.

**hESC transfection and clonal expansion**

Two gRNAs targeting a few hundred base pairs around the TSS of ZNF91 or ZNF611 were cloned into pX330 ([Suppl Table 1](#)). For transfection 400.000 hESCs per well were seeded on a 6 well plate one day prior transfection. Per well a total of 3 μg px330 (Cas9-gRNA), 1.5 μg of each of the two gRNAs and 20 ng of pCAG.GFP, was mixed with 5 μl P3000 reagent in a total volume of 50 μl opti-MEM (Invitrogen). This DNA mix was combined with a second mix containing 3.75 μl Lipofectamine 3000 (Invitrogen) and 46.25 μl opti-MEM (Invitrogen) and incubated for 15 minutes at room temperature. Medium was refreshed before adding 100 μl transfection mix dropwise. 48 hours after transfection GFP positive cells were sorted using fluorescence activated cell sorting (FACS) on a FACS Aria III with a 100 μm nozzle. Cells were plated at low density (5000-9000 cells) on MEF coated 60mm plates for clonal expansion.
Until colonies appeared, cells were cultured in recovery medium, consisting of hESC conditioned medium and MEF conditioned medium (1:1). For ZNF91 and ZNF611 wt and ko clonal lines were expanded in parallel. For ZNF91/ZNF611 dko cells one clonal ZNF91 ko line was used to generate ZNF611 wt and ko lines in parallel. To ensure clonality, colonies were passaged manually to a 96 well plate before they started to merge. All lines were frozen down and genotyped using PCR and sanger sequencing (genotyping primers see Suppl. Table 1). Lines were considered wild type if only one wt band was found with PCR and sequencing confirmed no insertions/deletions were present. Lines were considered knockout if only band with the deletion was found and sequencing confirmed that the start codon was deleted. A selection of wild type and knockout lines were expanded for mRNA level detection with qPCR (primers in Suppl Table 1) to select lines used for RNA seq.

**Plasmids**
For ZNF91-GFP ChIP experiments pCAG.ZNF91 from Jacobs et al. 2014 was used to generate pCAG.ZNF91-Glycinelinker-eGFP. For CRISPR/Cas9 deletion experiments gRNAs were cloned into pX330-SpCas9-HF1 (addgene #108301), pCAG.GFP (addgene #11150) was co-transfected for FAC-sorting of hESCs.

**RNA sequencing**
Samples of ZNF91 ko, ZNF611 ko and ZNF91/ZNF61 dKO were used for RNA sequencing (Suppl Table 2). Total RNA was isolated using Trizol according manufacturer’s protocol, followed by a DNase treatment and RNA purification using the RNA clean and concentrator kit (Zymo research). Ribosomal RNA was depleted from total RNA with the rRNA depletion kit (NEB# E6310) and subsequently prepared for RNA seq with NEB Next Ultra Directional RNA Library Prep Kit (NEB #E7420) at GenomeScan. Samples were sequenced at 150 bp paired end at an illumina Hiseq 4000 device.

**Mapping and analysis of RNA seq data**
A snakemake (Köster and Rahmann 2012) pipeline was written to quality check, trim, map and quantify RNA seq data. The complete pipeline can be found at DOI:10.5281/zenodo.2581199. Below a short description of the separate steps composing the pipeline. Paired end Illumina RNA seq fastq files were used as input. Read quality was assessed with FASTQC. Trimmomatic (Bolger et al. 2014) was used to clip adapter sequences and trim low quality reads. Reads were aligned to the human genome (Hg19 version) using STAR (Dobin et al. 2013) with default settings except: outFilterMismatchNmax=2 and outFilterMulti-
mapNmax=10, outWigType=bedGraph, outSAMtype=BAM SortedByCoordinate. Base-by-base coverage tracks were generated from each bedGraph file with bedGraphToBigWig. Coverage tracks were displayed on UCSC genome browser (session: http://genome-euro.ucsc.edu/s/ninaharing/Chapter_4). Raw read counts of genes were determined with FeatureCounts (Liao et al. 2014) using Hg19 KnownGenes.GTF from UCSC for annotation (downloaded 12th September 2016). Only properly paired reads were counted (-B) and the library was reversely stranded (-s 2). Genes were summarized at metalevel (default), whereas repeats were summarized at the feature level (-f).

Stringtie (Pertea et al. 2015) was used to build de novo transcript models from ZNF91 wildtype and knockout hESC samples mapped with STAR (bam files sorted by coordinate used as input). Predicted transcripts were included with a minimum length of 100bp (-m) and minimum read coverage of 1.5 (-c). One annotation file was generated from all separate stringtie transcript models that could be used by FeatureCounts to estimate raw counts of chimeric transcripts. DEseq2 (Love et al. 2014) was used to normalize raw counts, perform principal component analysis and differential expression analysis of genes, repeats, and chimeric stringtie transcripts. For differential expression analysis of SVA elements Log2FC >3 was considered differentially expressed, because SVA expression levels were too low to determine adjusted p values adequately. R packages ggplot2 (Wickham 2009) and ggpubr were used to generate plots and perform statistical testing. Heatmaps were generated with MeV (vs 4.8.1).

**Chromatin immunoprecipitation (ChIP)**

HEK293 cells were harvested the following way: 48 hours after transfection with ZNF91-GFP or GFP two replicates each, samples were harvested from a confluent 60mm dish by mechanical detachment with a cell scraper in cold PBS. Medium was replaced with 10 ml cold PBS. Cells were crosslinked immediately after harvest by adding 1 ml of 11x crosslinking buffer (50mM Tris-HCL pH 7.5, 100mM NaCl, 1mM EDTA, 0.5 mM EGTA, 11% Formaldehyde) to 10ml of cold cell suspension for 10 minutes at room temperature on a rocking platform. To quench formaldehyde glycine was added at a final concentration of 0.11 M and samples were incubated for 5 minutes at room temperature on a rocking platform. Cells were pelleted through centrifugation at 2000 rcf and 4 °C for 5 minutes. To remove all traces of crosslinking buffer, the cells were washed two times with 10 ml cold PBS. hESC samples were split in two for H3K4me3 and H3K27ac ChIP.

For cell lysis, 1 ml lysis buffer 1 (50 mM Heps-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP40, 0.25% Triton X-100) supplemented with
protease inhibitor cocktail (Roche) was added to cell pellets and incubated on a rocking platform for 10 minutes at 4 °C. Cells were pelleted again by centrifugation (5 min at 2000 rcf at 4 °C). 1 ml lysis buffer 2 (10mM Tris-HCL pH 8, 200 mM NaCl, 1mM EDTA, 0.5 mM EGTA) supplemented with protease inhibitor cocktail (Roche) was added and cell pellets and incubated at on a rocking platform for 5 minutes at 4 °C. Cells were pelleted again by centrifugation (5 min at 2000 rcf at 4 °C). The pellet was resuspended in 100 μl lysis buffer 3 (10 mM Tris-HCL pH 8, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-Deoxycholate, 0.5% N-Lauroylsarcosine) supplemented with protease inhibitor cocktail (Roche). Chromatin was sheared to fragments of ~500bp in 12 cycles of sonication (intensity high, 30 sec on and 1 min off) in a bioruptor. 450 μl lysis buffer 3 and 50 μl 10% Triton X-100 was added to the sonicated cell lysate. Samples were centrifuged max speed for 10 min at 4 °C. 50 μl supernatant was stored at -20 °C to be used as input sample and the rest was used for chromatin immunoprecipitation (ChIP). 50 μl of Dynabeads M-280 sheep anti-rabbit igG (invitrogen) were washed three times with 0.5% BSA in PBS before incubating with 5 μg rabbit anti-GFP (abcam, ab290) on a rotator at 4 °C for 4 hours. Excess antibody was removed by three wash steps with lysis buffer 3. Beads were re-suspended in 500 μl 1% Triton X-100 in lysis buffer 3 and added to the lysate. IP was performed on a rotator at 4 °C overnight. Immuno complexes were washed four times with 1ml RIPA buffer (50 mM Hapes-KOH pH 7.5, 500 mM LiCl, 1 mM EDTA, 1% NP40, 0.7% Na-Deoxycholate) supplemented with protease inhibitor cocktail (Roche) and once with 1 ml cold TBS (50mM Tris-HCL pH7.6, 150mM NaCl, GFP and H3K4me3) using a magnetic stand. After removal of all traces of TBS, the beads were resuspended in 200 μl elution buffer (50 mM Tris-HCLpH 8, 10mM EDTA, 1% SDS). 150 μl elution buffer was added to thawed Input sample (50 μl). Chromatin was eluted and crosslinking reversed by incubating at 65 °C on a rocking platform. 200 μl TE (10 mM Tris-HCLpH8, 1 mM EDTA) was added to each sample to dilute SDS in elution buffer. ChIP DNA was treated with Ambion RNase Cocktail (Invitrogen) for 30 min at 37 °C and Proteinase K (Invitrogen) for two hours at 55 °C. DNA was extracted with one step phenol/chloroform and two steps chloroform. Ethanol (100%) was used to precipitate DNA for 45 min at -80 °C. DNA was pelleted by centrifugation at 14000rpm for 45 min at 4 °C. Pellet was washed with 70% ethanol and centrifuged at 14000rpm for 10 min at 4 °C. DNA was dissolved in nuclease free water. Finally, extracted DNA was purified using DNA clean & concentrator-5 columns (Zymo research). QPCR using Quantitect SYBR green (Roche) was performed on a Roche Lightcyycler 480 II to check ChIP enrichment.
The Identification of SVA Controlling KZNF Proteins: ZNF91 and ZNF611 Bind SVA elements, but Only ZNF91 is Essential for Their Repression

ChIP seq library prep and sequencing
25-30 ng of ChIP DNA was used as starting material for Truseq ChIP sample prep (Illumina, Lot 20194738). Provided protocol was followed with the following adjustments: DNA clean & concentrator-5 kit (Zymo research) instead of beads was used for DNA purification. DNA fragment size selection with 2% agarose size selection gels (Invitrogen) using E-Gel sizeSelect II (Invitrogen) was done before and after PCR amplification of indexed ChIP DNA to obtain the 400-500bp fraction. ChIP samples were pooled and subjected to 75bp paired end sequencing at a depth of 130 million reads.

Mapping and analysis of ChIP seq data
Paired end ZNF91 ChIP data of two replicates were analyzed in parallel with single end ChIP-exo data from Imbeault et al. (2017). KZNF proteins described as SVA binding were included (ZNF28, ZNF30, ZNF141, ZNF202, ZNF257, ZNF558, ZNF611, ZNF641, ZNF649, ZKSCAN5). Read quality was assessed with FASTQC. Trimmomatic (version 0.38) (Bolger et al. 2014) was used to clip adapter sequences and trim low quality reads. Bowtie2 (version 1.0.1) (Langmead and Salzberg 2012) in --end-to-end --very-sensitive mode was used to map reads to the human genome (Hg19 version). Fragment length for valid paired end fragments was set to a minimum of 80 (-I) and a maximum of 500 (-X). The resulting sam files were converted to bam files sorted by coordinate using Samtools (version 1.7) (Li et al. 2009). The rmdup function of Samtools was used to remove potential PCR duplicates only in ZNF91 ChIP samples, because duplicates occur frequently and are usually genuine read in ChIP exo experiments. BamCoverage from the deeptools package (version 2.5.7) (Ramírez et al. 2016) was used to generate RPKM normalized base-by-base (--binSize 1) coverage bigwig file. BigwigCompare from the deeptools package was used to generate one coverage bigwig file displaying the mean of two ZNF91 replicates. Profile plots were generated with deeptools computeMatrix and plotHeatmap 2kb around the center of SVA elements using Galaxy (Galaxy deeptools version 3.1.2.0.1).

Peak calling was done with MACS2 (version 2.1.1.20160309) (Zhang et al. 2008). All tags at a given location were included (--keep-dup all) and subpeaks were determined (--call-summits). Individual peaks were assessed to find a cut off representing only genuine peaks. We used the following cut offs: ZNF91 rep1 MACS score>=50, ZNF91 rep2 and ZNF611 MACS score >=100. For ZNF91 only peaks occurring in both replicates were included. This resulted in 7220 genuine ZNF91 peaks and 4142 genuine ZNF611 peaks.
Motif analysis
MEME suite (version 5.0.4) (Bailey et al. 2009) was used for motif discovery (MEME), motif search (FIMO) and motif comparison (TomTom). For motif discovery sequences of peak summits extended with 50bp to both sides were retrieved and used as input for MEME. For ZNF91 SVA motif discovery SVA elements were split in half and intersected with ZNF91 peaks. Top200 peaks localizing to each half were used to generate two motifs. For ZNF611 SVA motif discovery top 500 peaks localizing to SVA elements were used to generate a motif. For promoter motif discovery top200 and top100 of ZNF91 and ZNF611 peaks overlapping with gene promoters (TSS plus/minus 1kb) were used respectively. For both motifs the orientation of SVA elements and genes were taken into consideration.

Evolutionary history of ZNF611
Human ZNF611 protein sequence was downloaded from hg19 and separate exons were blatted to different primate genomes (chimp (panTro6), gorilla (gorGor5), orangutan (ponAbe3), Gibbon (nomLeu3), crab-eating macaque (macFas5), Rhesus macaque (RheMac8), baboon (papAnu4)). Primate DNA sequences were BLAT back to the human genome to verify that it was truly ZNF611 and translated to protein. Protein sequences of primate species were used as input for multiple sequence alignment using clustal Omega (Madeira et al. 2019). Prediction functional zinc finger domains and DNA motifs bound by ancestral forms of ZNF611 was done using an online prediction tool (http://zf.princeton.edu/index.php, Persikov and Singh 2014).

Acknowledgements
We thank Lars von Oerthel for his technical assistance with the FACS experiments.
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Supplemental figures and tables

**Supplemental Figure 1. Multiple sequence alignment of ZNF611 protein sequence derived from different primate lineages.**

Adifferent domains of ZNF611 are indicated: KRAB-A and KRAB-B (purple) connected with spacer region (light purple) and zinc finger domains (pink). Residues essential for zinc finger formation (zinc coordinating cysteines and histidine in purple) and recognized DNA code (-1,2,3,6 of alpha helix,yellow) are highlighted. An online prediction tool (http://zf.princeton.edu/index.php) was used to predict if zinc finger domains are likely to be functional.
Supplemental Figure 2. Chimeric SVA-gene transcripts in ZNF91 ko hESCs
RNA seq coverage tracks showing SVA-F driven HORMAD1 transcript in ZNF91 ko hESCs.

Supplemental Figure 3. ZNF91 and ZNF611 promoter binding
A) Promoter binding motifs of ZNF91 and ZNF611 aligned with SVA binding motifs. Promoter motifs were obtained with peak summits extended 50bp to each side localizing to 1kb up- and downstream of TSS of UCSC genes. For ZNF91 the top200 peaks were included and for ZNF611 the top100. B) Pie charts showing the number of gene promoters that show KAP1 binding at the same location as ZNF91 or ZNF611 peaks in hESCs (data from Jacobs et al 2014). C) Boxplots showing Log2 Fold Change of ZNF91 ko (left) and ZNF611 ko (right) of KAP1 positive ZNF91 (green, 107) and ZNF611 (yellow, 38) bound gene promoters compared to a size matched random set of genes. Only expressed (baseMean>10) were included. For statistical testing an unpaired wilcoxon rank sum test was used.
Supplemental Figure 4. KZNF gene regulation in ZNF91 and ZNF611 ko cells independent of evolutionary age.
A) Principal component analysis of gene expression in hESC lines with indicated genotype. B) Pie chart showing number of KZNF genes not bound (grey), ZNF611 bound (yellow), ZNF611 and ZNF91 bound (light green), and ZNF91 bound (green). C) Boxplots showing Log2Fold-change of ko versus wildtype of KZNF genes residing in clusters on chromosome 19 and solo. ZNF91 ko in green and ZNF611 in yellow. D) Boxplots showing Log2Foldchange of KZNF gene expression and a similar number of random genes in ZNF91/ZNF611 dko versus ZNF91 ko hESCs. E) Boxplots showing log2Foldchange of KZNF genes grouped by evolutionary age left ZNF91 ko, right ZNF91/ZNF611 dko. In all analyses Only expressed (baseMean>10) genes were included. For statistic testing an unpaired Wilcoxon rank sum test was used followed by an FDR correction if multiple groups were compared (*P<0.05).
### Suppl. Table 1. Oligos used for the generation of ZNF91 and ZNF611 ko hESCs

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<th>Oligo name</th>
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<td>ZNF611</td>
<td>qPCR primer reverse</td>
<td>CATCCAGAGGACAGCCCCTCA</td>
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### Suppl. Table 2. Clonal KZN9F wt and ko hESC lines used for RNA sequencing.

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<th>Clonal line</th>
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