The human PRD-like homeobox gene LEUTX has a central role in embryo genome activation

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ABSTRACT
Leucine twenty homeobox (LEUTX) is a paired (PRD)-like homeobox gene that is expressed almost exclusively in human embryos during preimplantation development. We previously identified a novel transcription start site for the predicted human LEUTX gene based on the transcriptional analysis of human preimplantation embryos. The novel variant encodes a protein with a complete homeodomain. Here, we provide a detailed description of the molecular cloning of the complete homeodomain-containing LEUTX. Using a human embryonic stem cell overexpression model we show that the complete homeodomain isoform is functional and sufficient to activate the transcription of a large proportion of the genes that are upregulated in human embryo genome activation (EGA), whereas the previously predicted partial homeodomain isoform is largely inactive. Another PRD-like transcription factor, DPRX, is then upregulated as a powerful repressor of transcription. We propose a two-stage model of human EGA in which LEUTX acts as a transcriptional activator at the 4-cell stage, and DPRX as a balancing repressor at the 8-cell stage. We conclude that LEUTX is a candidate regulator of human EGA.

KEY WORDS: Embryo genome activation, Homeobox gene, Preimplantation development, Embryonic stem cells

INTRODUCTION
Homebox genes encode homeodomain-containing transcription factors, which often regulate developmental processes and cellular differentiation (Holland, 2013; Monteiro et al., 2006; Mallo and Alonso, 2013; Seifert et al., 2015). An analysis of human embryo genome activation (EGA), focusing on transcription start sites (TSSs) in oocytes, zygotes and isolated single blastomeres from 4-cell and 8-cell embryos (Töhönen et al., 2015), revealed the expression of many paired (PRD)-like homeobox genes. One of the previously unannotated TSSs implicated in human EGA marks the PRD-like homeobox gene LEUTX.

The LEUTX gene is characterized by a highly conserved PRD class homeodomain, except for a leucine at position 20, and a lack of paired domain, similar to the other PRD-like transcription factors (Galliot et al., 1999; Bürglin, 2011). Evolutionarily, neither Leutx nor three other PRD-like gene families (Dprx, Argfx and Tprx) are present in invertebrates (Holland, 2013). Closely related sequences for human LEUTX have been detected in other primates and highly divergent orthologs are present in several other placental mammals (Zhong and Holland, 2011). LEUTX is deduced to have arisen by tandem duplication and divergence from the Otx family gene CRX during the early radiation of placental mammals, and it has been subsequently lost from rodents (Holland, 2013; Zhong and Holland, 2011).

The RefSeq model for LEUTX (NM_001143832.1) was predicted in silico from human genomic sequence (Holland et al., 2007). The annotation was based on computationally predicted mRNA (XM_001129035.1; LOC342900) supported by partial 5′ cDNA sequence and predicted stop codon location. One partial cDNA clone of human LEUTX was isolated from a placenta cDNA library in 1995 (IMAGE clone ID: 150840) and the existence of LEUTX mRNA was further confirmed in 2005 by PCR from pooled mRNA sources (clone ID: MGC10SS.1.L1.L1.E01).

Our recent study on human preimplantation development indicated expression from a novel TSS in the first intron of the RefSeq LEUTX sequence (Töhönen et al., 2015). Here, we provide a full description of the molecular cloning of the embryonically expressed human LEUTX encoding a complete homeodomain (hereafter LEUTX.n). We show experimental evidence of LEUTX expression in human embryonic stem cells (hESCs) and 8-cell stage embryos and a survey of publicly available expression profiles. By overexpression of LEUTX.n in hESCs we identify its target genes. We also suggest that the LEUTX.n isoform can act via the 36 bp DNA motif that is found enriched among upregulated genes during human EGA (Töhönen et al., 2015). We observed that ~25% of the genes upregulated in 8-cell embryos represent experimentally validated target genes for the LEUTX.n isoform. DPRX acts as a suppressor of a large number of overlapping target genes. Our findings suggest that human LEUTX might act as a main regulator of EGA.

RESULTS
Cloning of a complete homeodomain LEUTX isoform from human preimplantation embryos
Our TSS-focused RNA sequencing data on human preimplantation development (Töhönen et al., 2015) suggested the expression of a variant of LEUTX from a previously unannotated TSS within the
first intron of the predicted gene, and we verified the full sequence by cloning from a single 8-cell stage embryo library. In the current study, we validate the cloning using cDNA libraries from three whole 8-cell embryos.

In order to clone the putative new LEUTX transcript, we designed a forward primer at the observed TSS at position chr19:40269483 (GRCh37/hg19) and the reverse primer at the predicted 3′ UTR of LEUTX (Fig. 1A). PCR yielded a single amplicon (Fig. S1), which was sequenced and found to include an unannotated 5′ exon spliced into exons 2 and 3 of LEUTX.

An analysis of the cDNA sequence revealed an open reading frame (ORF) with a complete homeodomain in the novel LEUTX isoform (LEUTX.n), whereas the RefSeq LEUTX (hereafter LEUTX.R) would encode a protein with a partial homeodomain (Fig. 1B). The new first exon contains a translation initiation codon (ATG), adding 30 amino acids upstream of the previously known start codon. The experimentally confirmed new cDNA and the translated amino acid sequence are shown in Fig. 1C. The single-cell resolution TSS expression data (Töönen et al., 2015) indicated a peak in LEUTX.n expression in 4-cell stage blastomeres and continued expression at the 8-cell stage (Fig. 1D).

**LEUTX is not conserved in mouse**

To explore the conservation of LEUTX and its function in mouse, we performed a blastp search against the latest NCBI non-redundant database using our new human LEUTX amino acid sequence as a query. The most similar sequence was XP_006544220.2 (‘PREDICTED: leucine-twenty homeobox isoform X1’; E=6×10^-15; 36% identity in the 60-190 amino acid range of human LEUTX).

By UCSC Blat aligner, the corresponding mRNA sequence XM_006544157.2 aligned to chr7:28243022-28243753 of mouse reference genome sequence (mm10) without exon splicing. The ORF begins with ten copies of MPVS(E/G)(A/S)(S/L)(S/I)N(Q/P) A repeats, but it lacks the cognate homeodomain. With a lower amino acid sequence identity, other similar sequences were NP_031796.1 and NP_001106801.1 (cone-rod homeobox protein isoform 1 and 2; E=2×10^-9 and 3×10^-9, respectively; 32% identity in the 4-16 amino acid range of human LEUTX), but these were more similar to mouse Otx1 and Otx2; therefore, the alternative name of this cone-rod homeobox protein is Otx3 (also known as Crx) (Chen et al., 1997). There were no alignments of human LEUTX amino acid sequences against mm10 by UCSC Blat aligner with translation. Thus, we confirm the earlier results of Zhong and Holland (2011) that mouse lacks the orthologous gene for human LEUTX.

**High-level expression of LEUTX is restricted to early embryos**

In order to confirm the expression level of LEUTX protein in human preimplantation embryos, we studied three human 8-cell embryos by immunostaining. The results showed the presence of LEUTX in all apparently normal blastomeres, with prominent nuclear staining (Fig. 2A). The specificity of the staining was confirmed by overexpressing GFP-conjugated LEUTX.n in hESCs and human embryonic kidney cells (HEK-293), and labeling the cells with the same LEUTX antibody with and without competing peptide (Fig. S2).

To study LEUTX expression in pluripotent cells, we subjected single cells from two different hESC lines and 8-cell embryos to single-cell tagged reverse transcription (STRT) sequencing (Islam et al., 2012). STRT is an RNA-sequencing method that can be applied to single cells or low amounts of RNA. The method detects the very 5′-end of the poly(A) + transcripts and allows simultaneous analysis of 48 or 96 multiplexed samples. Our sequencing library comprised 15 cells from the inner cell mass-derived hESC line HS980, 15 cells from a single 8-cell blastomere-derived cell line HS983a (Rodin et al., 2014) and 14 individual 8-cell blastomeres from two different embryos. LEUTX was detectable in 6/15 HS983a cells and one of the HS980 cells (Fig. 2B) and in all 8-cell blastomeres. Using qPCR, we detected low-level LEUTX expression in the hESC lines HS980, HS401 and H9 (Fig. 2C), whereas the expression level of LEUTX in an 8-cell embryo was significantly higher.

To further confirm LEUTX expression in human preimplantation embryos, we extracted LEUTX expression patterns from three previously published RNA-seq datasets (Yan et al., 2013; Xue et al., 2013; Petropoulos et al., 2016). These results are in agreement with our data showing that LEUTX is expressed at the 4- and 8-cell stages and is reduced again in the morula and at the latest blastocyst stage (Fig. 2D-F). Owing to methodological differences, the highest peak is detected either at the 4-cell or 8-cell stage (Fig. S3).

The expression of the LEUTX.n isoform across different tissues and cell culture models was assessed in the functional annotation of the mammalian genome (FANTOM5) database (Forrest et al., 2014; Lizio et al., 2015). FANTOM5 facilitates systematic investigation of the gene expression profiles in all human cell types, including TSS data from 1829 biological samples. The FANTOM5 data showed a total of seven normalized reads aligned to the LEUTX.n promoter and no reads corresponding to the RefSeq TSS (Fig. S4). All the reads were found in six human induced pluripotent stem cell (iPSC) line-derived samples, with an average of 1.17 reads per sample (Fig. 2G). The number of reads for LEUTX was too low to be recognized as a TSS in the FANTOM5 database. In comparison, the FANTOM5 database shows more than 10,000 normalized reads for OTX2, a previously characterized PRD-like gene. FANTOM5 data are thus consistent with our data for both the TSS location and barely detectable expression in iPSCs only.

The expression of LEUTX in pluripotent stem cells was assessed further in two GEO datasets, GSE20750 (Saito et al., 2011) and GSE13834 (Cao et al., 2008), representing iPSC derivation from human amniotic mesoderm fibroblasts and hESC differentiation into cardiomyocytes, respectively. Both datasets showed higher expression of LEUTX (third exon) in iPSCs or hESCs compared with other samples as assessed by expression microarray (Fig. 2H-I).

The data cannot distinguish between the two TSSs.

Very low expression in placenta, but not in other tissues, has also been shown by others using RT-PCR (Chinen et al., 2014) and RNA-seq (Fig. S5A) (Metsalu et al., 2014). This is in concordance with our failure to detect LEUTX in different human tissues by western blot (Fig. S6). The observation is strengthened by the fact that LEUTX is not detected at the RNA or protein level in any tissue in the Human Protein Atlas (Uhlén et al., 2015). However, Yao et al. (2014) showed that LEUTX is upregulated following DUX4 overexpression in human muscle cells and is expressed in muscle biopsies from facioscapulohumeral dystrophy (FSHD) patients. Interestingly, they also detected the expression of the novel first exon of LEUTX.

We then assessed LEUTX expression in the publicly available data on 675 human cancer cell lines (Klijn et al., 2015). The data showed LEUTX expression levels in three cell lines (KMS.11, KLE and SU.86.86) at very low levels (Fig. S5B). The highest RPKM value (∼20) was detected in SU.86.86, which expressed a suggested
fusion transcript of PAK4 and LEUTX. In comparison, the housekeeping gene GAPDH showed RPKM values >2000 in all samples. To conclude, both our own data and publicly available datasets suggested that high-level LEUTX expression is restricted to early embryos. The databases investigated in this study are listed in Table S1.

The LEUTX.n isoform activates genes in hESCs
In order to study the transcription factor profile and target genes of LEUTX, we overexpressed both the LEUTX.n and LEUTX.R isoforms in hESCs. The LEUTX.R vector was engineered from our LEUTX.n clone to correspond to the RefSeq prediction. The experiment is outlined in Fig. 3A. The genes were cloned into a
modified bicistronic pFastBac vector co-expressing eGFP marker, transfected into the hESC line HS401 and sorted in three to four replicates by fluorescence-activated cell sorting (FACS) based on the GFP expression originating from the same vector as the overexpressed gene of interest (Fig. S7). Sorted cells were analyzed using STRT RNA-seq (Islam et al., 2011; Krjütskov et al., 2016) data, all supporting LEUTX expression in 4- and 8-cell embryos and downregulation in the morula/blastocyst. (F) Day 3 to day 7 refer to embryos from the 8-cell stage to late blastocyst. (G) FANTOM5 data for LEUTX (n=1829). Only six iPSC samples contained tag clusters in the LEUTX region. The average expression refers to the FANTOM5 CAGE phase 1 and 2 samples in which expression was detected. (H) Human somatic fibroblasts from amniotic mesoderm (n=3) were used to generate human iPSCs (hiPS, n=15) (GEO dataset GSE20750, probe 42402). LEUTX shows higher expression in human iPSCs. (I) hESCs (n=4) were differentiated into cardiomyocytes (n=4) and analyzed by RNA expression array (GEO dataset GSE13834, probe 42402). LEUTX was expressed in hESCs, but not in differentiated cardiomyocytes. LEUTX is detected by Agilent 014850 Whole Human Genome Microarray 4x44K G4112F (H,I). Error bars indicate mean±s.e.m.; all comparisons for G-I are statistically significant (Student’s t-test, P<0.05).
with three sets of controls: GFP-positive samples from mCherry control, GFP-negative samples from both mCherry and the gene of interest, and GFP-negative samples from the whole library (each separately with q<0.1) (Fig. 3C). For robustness, the intersection of these three gene lists was used for further analyses. The number of upregulated and downregulated TFEs by their genomic locations is shown in Fig. 3D. More than 2500 TFEs were upregulated and 500 downregulated by LEUTX.n. The number of differentially expressed TFEs by either LEUTX.R or OTX2 suggested more modest effects with higher repressor than inducer activity (Fig. 3D). The overexpression of DPRX was followed by a massive downregulation of TFEs, suggesting that it acts as a repressor rather than an inducer. In all comparisons, most of the TFEs mapped to the 5′UTR of coding exons of coding genes. However, the results also showed a number of TFEs without previous annotation, suggesting novel transcripts from previously unannotated TSSs. These novel TSSs might be developmental stage specific and/or encode novel transcript variants. Because of the lack of functional information for novel TFEs, we focused further analyses on the TFEs located at the 5′UTR of the annotated, coding genes. The TFEs, their corresponding genes and genomic annotations are given in Table S2. The top 20 genes upregulated by LEUTX.n are shown in Table 1. The expression of the experimental target genes of LEUTX.n in human preimplantation embryos was confirmed using the Yan et al. (2013) dataset (Fig. S9). The LEUTX.n targets included the pluripotency-associated genes DPPA3 (also called STELLA), HESX1 and KAT7, whereas the LEUTX.R isoform had no effect on these genes (Table S2). We then applied the GOrilla tool (Eden et al., 2009) to analyze Gene Ontology (GO) category enrichment among the genes upregulated by LEUTX.n.

All three categories – biological processes, molecular function and cellular component – revealed enrichment related to fundamental functions of the cell: RNA binding, protein localization, translation, gene expression and ribosomal unit (Table 2, Fig. S10).

**LEUTX.n and DPRX affect genes that are activated in early embryos**

The detection of LEUTX.n in human preimplantation embryos but not in adult tissues suggested its importance during early development. DPRX, on the other hand, was upregulated at the 8-cell stage, thus being a potential target gene of LEUTX, but it

<table>
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<tr>
<th>Table 1. Top 20 genes upregulated by LEUTX.n</th>
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<tr>
<td>Gene</td>
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<tr>
<td>DPPA3</td>
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<tr>
<td>HESX1</td>
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<tr>
<td>VPREB3</td>
</tr>
<tr>
<td>LEUTX*</td>
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<tr>
<td>UBE2T</td>
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<tr>
<td>SGK1</td>
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<tr>
<td>ARL4D</td>
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<tr>
<td>SAT1</td>
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<tr>
<td>ZMYM5</td>
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<tr>
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<td>DDI3</td>
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<tr>
<td>GDF15</td>
</tr>
<tr>
<td>ZNF737</td>
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<tr>
<td>MSH2</td>
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<td>PRR11</td>
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Fold change (FC) is based on comparison between LEUTX.n-overexpressing versus GFP-negative samples from the whole library (n=22). Q<0.0005 for all genes. Score refers to the differential expression score, which is the average Wilcoxon statistics with multiple Poisson resampling, TFE, transcript far 5′-end. *LEUTX reads originating from the overexpression vector are indistinguishable from the endogenous LEUTX expression.
Table 2. Significantly overrepresented GO categories for molecular function among upregulated LEUTX.n targets

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
<th>P-value</th>
<th>FDR q-value</th>
<th>Enrichment</th>
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<tbody>
<tr>
<td>GO:0003735</td>
<td>Structural constituent of ribosome</td>
<td>7.01×10^{-12}</td>
<td>2.45×10^{-6}</td>
<td>3.28</td>
</tr>
<tr>
<td>GO:0044822</td>
<td>poly(A) RNA binding</td>
<td>4.88×10^{-11}</td>
<td>8.52×10^{-6}</td>
<td>1.68</td>
</tr>
<tr>
<td>GO:0003723</td>
<td>RNA binding</td>
<td>3.35×10^{-10}</td>
<td>3.9×10^{-7}</td>
<td>1.56</td>
</tr>
<tr>
<td>GO:0005198</td>
<td>Structural molecule activity</td>
<td>2.32×10^{-7}</td>
<td>2.02×10^{-4}</td>
<td>1.96</td>
</tr>
</tbody>
</table>

P-values determined by hypergeometric distribution as implemented in GOseq (Eden et al., 2009).

LEUTX and DPRX both target the same DNA motif

A de novo predicted 36 bp DNA motif was found enriched among the promoters of early activated genes and suggested to play a role in EGA (Töhönen et al., 2015). In order to test whether this motif is enriched in the promoter region of experimentally regulated genes, we applied the motif enrichment analysis (MAST) (Bailey and Gribskov, 1998) to the LEUTX.n and DPRX target genes promoters at genomic positions −2000 to +500 bp around the regulated TFEs. An equal number of non-regulated TFEs or random start sites from FANTOM5 were used as control datasets. The analysis showed that the 36 bp motif was indeed enriched among the promoter regions of the experimentally regulated genes (Fig. 5A,B).

To experimentally confirm that LEUTX and DPRX can mediate their effects through the 36 bp motif sequence, we constructed luciferase reporter vectors with one copy or four tandem copies of the 36 bp sequence (Fig. 5C), cotransfected these with the PRD-like homeobox genes into HEK-293 cells, and measured the luciferase signals 24 h after transfection. We first studied the ability of LEUTX.n and LEUTX.R isoforms to act on the motif and induce luciferase expression. The results show that cotransfection with the LEUTX.n isoform induced the luciferase signal up to 10-fold, whereas LEUTX.R did not have any effect (Fig. 5D), further supporting LEUTX.n as the functional LEUTX isoform. We transfected OTX2 as a reference gene with a previously shown binding site (GGATTA) (Briata et al., 1999; Hoch et al., 2015), which is also part of the 36 bp motif, and it showed up to 15-fold induction of luciferase expression. In order to study whether LEUTX.R can act as dominant-negative isoform, we performed a similar luciferase assay by cotransfecting LEUTX.R with LEUTX.n or OTX2. Cotransfection only mildly attenuated the luciferase signals, thus suggesting that LEUTX.R does not act as a dominant negative (Fig. S11). We then studied whether LEUTX.n binds directly to the motif by mutating the LEUTX.n expression construct at a single amino acid at a central position of the homeodomain (K57A), similar to previous studies on bicaudal Drosophila melanogaster (Hanes and Brent, 1989). The mutation abolished LEUTX.n function in the luciferase assay (Fig. 5E). Furthermore, electrophoretic mobility shift assay (EMSA) revealed binding of LEUTX.n, but not LEUTX.R or mutated LEUTX.n, to the 36 bp motif (Fig. 5F, Fig. S12).

Interestingly, also the DPRX binding sites (NRGATTACKYN and NNRGATTADN) predicted using systematic evolution of ligands by exponential enrichment (SELEX) overlap with the 36 bp motif (Eden et al., 2009). We then performed the luciferase reporter assay to study the effect of LEUTX.n, DPRX and OTX2 alone and in combination. The data showed that both LEUTX.n and OTX2 induced luciferase expression compared with the empty vector, whereas DPRX suppressed the expression (Fig. 5G). DPRX also reduced luciferase expression when cotransfected with either LEUTX.n or OTX2. Similar results as those in 24 h transfections were obtained at the 10 h time point, suggesting that both LEUTX.n and DPRX act directly on the 36 bp motif (Fig. S13). This finding suggested that DPRX might be a direct competitor for the DNA binding motif and that it might counteract the effect of LEUTX through binding to the same 36 bp motif.

DISCUSSION

Human EGA is poorly understood. Knowledge concerning what governs differentiation and pluripotency might be highly useful for stem cell research and regenerative medicine. In our recent study, we used improved single-cell promoter-tagging RNA sequencing methods and transcript quantification on external normalization rather than the commonly used relative proportion methods. We previously identified putative new transcription factor genes and implicated the PRD-like homeobox genes as key players in human EGA (Töhönen et al., 2015). Here, we describe the molecular cloning and functional characterization of the novel LEUTX variant that encodes a complete homeodomain (LEUTX.n) as well as the earlier RefSeq annotated isoform with an incomplete homeodomain (LEUTX.R). Comparison of the target gene profile of LEUTX.n and genes upregulated in 4-cell and 8-cell embryos suggested a key role for LEUTX.n in EGA.

LEUTX is one of the first genes expressed in human preimplantation embryos. Here, we confirmed LEUTX expression in human 8-cell blastomeres on the RNA and protein level and, in addition, we show the relatively low-level expression of LEUTX in hESCs. Our data, as well as previously published datasets, support the expression of LEUTX in pluripotent cells but not in any terminally differentiated cells (Forrest et al., 2014; Lizio et al., 2013).
An exception is the LEUTX expression implicated in FSHD, suggesting that dysregulated DUX4 might induce LEUTX expression in diseased muscles, which might disturb cell differentiation (Yao et al., 2014). These findings suggest that LEUTX expression is restricted to human preimplantation development and that LEUTX might play a key role in EGA.

Further support was gained by the profiling of target genes in hESCs overexpressing LEUTX. The gene targets included known pluripotency genes, such as DPPA3 and HESX1. Dppa3 (also known as Stella or Pgc7) was initially observed in mouse primordial germ cells (Sato et al., 2002), characterized as a maternal effect gene in mouse (Payer et al., 2003), and suggested to be a marker for...
pluripotency in both mouse and human (Bowles et al., 2003). Furthermore, Dppa3 has been shown to be a direct downstream target of Tbx3 (Waghray et al., 2015) that is necessary for the maintenance of pluripotency. Although the importance of Dppa3 has been shown, little is known about Dppa3 regulation. Hesx1 has been characterized as encoding a PRD-like homeodomain factor that acts as a repressor and is connected with pituitary organogenesis (Dasen et al., 2001). Furthermore, it is expressed in murine ESCs and has been shown to be essential in maintaining pluripotency (Li et al., 2015; Webb et al., 1993). Interestingly, NANOG is also among the upregulated targets of LEUTX.n.

Direct evidence for the binding and functionality of LEUTX.n but not LEUTX.R or mutated LEUTX.n came from EMSA and luciferase reporter assays using the previously predicted 36 bp EGA-specific DNA binding motif (Töhönen et al., 2015). Further experiments suggested that both LEUTX.n and OTX2 can induce expression through the 36 bp EGA motif, and that DPRX acts as a partial repressor. The computationally tested specificity of LEUTX.n and DPRX binding to target gene promoters indeed indicated that the predicted motif is significantly enriched at positions −2000 to +500 bp from the TSSs. The novel finding of DPRX as a repressor of embryo activated genes may suggest a further regulatory mechanism in EGA involving the selective repression of activated genes after the 8-cell stage.

Early embryo development is still largely uncharacterized in humans. However, in model systems such as Drosophila, a number

Fig. 5. LEUTX.n and DPRX act on a motif predicted to play a central role in early human embryos. (A,B) MAST analysis for (A) genes upregulated by LEUTX.n (n=901) and (B) downregulated by DPRX (n=2090) using the consensus motif visualized in C. The solid red lines show the count of motifs in LEUTX and DPRX target genes, while blue and green dotted lines shows counts on equal numbers of start sites from FANTOM5 database and non-regulated TFEs, respectively. (C) The luciferase vector pGL4.25 containing a minimal promoter and the 36 bp motif. (D,E) Log2 fold changes in luciferase expression between motif-containing and corresponding empty vector with cotransfection of LEUTX.n, LEUTX.R or OTX2 (D) and with cotransfection of non-mutated or mutated (mutK57A) LEUTX.n (E). (F) EMSA showing that LEUTX.n, but not LEUTX.R or mutated LEUTX.n, binds to the 36 bp motif (n=3). Samples are shown in technical duplicates. NTC, non-template control refers to reticulocyte lysate only. (G) Log2 fold changes in luciferase expression between cotransfection of LEUTX.n, DPRX or OTX2 alone or in combination. In D,E,G, gray and black columns represent 1 and 4 repeats of the motif, respectively. Experiments were performed in three biological replicates with two technical replicates each. Error bars indicate mean±s.e.m.
of key observations have been made that might also have relevance to human EGA. For example, similar to the 36 bp motif in human, the so-called TAGTeam motif identified in *Drosophila* has been shown to be enriched in the upstream regions of genes activated around the time of the maternal-to-zygote transition (ten Bosch et al., 2006). Furthermore, key transcription factors, such as Zelda and Stat92E, have been shown to act on the TAGTeam motif and control transcription of the zygotic genome at the very beginning of embryonic development (Tsurumi et al., 2011; Liang et al., 2008).

Interestingly, the 36 bp regulatory motif was identified to frequently overlap with Alu elements (Töhönen et al., 2015), which are primate-specific retrotransposon elements. In mouse, EGA is controlled via murine-specific long terminal repeat (LTR) retrotransposons and murine-specific endogenous retroviruses (Macfarlan et al., 2012; Peaston et al., 2004). However, at EGA in mouse, the murine-specific retroelements were shown to be frequently transcribed as an alternative 5′ exon of host genes, whereas the 36 bp motif in human EGA is usually located far upstream of the TSSs. Although the *LEUTX* promoter itself contains a similar sequence to the 36 bp motif, the human *LEUTX* TSS does not overlap with retroelements. Therefore, it seems that the regulation of not only *LEUTX*, but also EGA, is evolutionarily optimized for each mammalian species.

In conclusion, we propose that the complete homeodomain *LEUTX.n* isoform is the functional *LEUTX* isoform that is suggested to play a key role in early embryo development and pluripotency. *LEUTX* activates the first genes in human preimplantation development by acting on the predicted DNA sequence motif, and some of the early activated genes are then later repressed by DPRX.

**MATERIALS AND METHODS**

**Human preimplantation embryos, hESCs and cDNA generation**

The sample sources and methods for sample preparation have been described (Töhönen et al., 2015). Human preimplantation embryos used for this study were donated by couples who underwent infertility treatment by *in vitro* fertilization (IVF), and they were collected in Sweden (ethics approvals Dnr 2010/937-31/4 and 2012/1765-31/1 of the Regional Ethics Board in Stockholm). Cryopreserved cells not needed for IVF and destined for destruction since the legal storage time had been reached were donated by the Swedish National Board in Stockholm). Cryopreserved cells not needed for IVF and destined for destruction since the legal storage time had been reached were donated by the Swedish National Board in Stockholm.

For cDNA library preparation, human 4-cell stage embryos were thawed using ThawKit™ (ThawKit™ for destruction since the legal storage time had been reached were donated by the Swedish National Board in Stockholm). Cryopreserved cells not needed for IVF and destined for destruction since the legal storage time had been reached were donated by the Swedish National Board in Stockholm).

The sample sources and methods for sample preparation have been described (Töhönen et al., 2015). Human preimplantation embryos used for this study were donated by couples who underwent infertility treatment by *in vitro* fertilization (IVF), and they were collected in Sweden (ethics approvals Dnr 2010/937-31/4 and 2012/1765-31/1 of the Regional Ethics Board in Stockholm). Cryopreserved cells not needed for IVF and destined for destruction since the legal storage time had been reached were donated by the laboratory of O.H. For cDNA library preparation, human 4-cell stage embryos were thawed (ThawKit™ Cleave following the manufacturer’s instructions and cultured overnight on G-1™ medium, allowing them to develop until the 8-cell stage. Embryos were fixed in 4% paraformaldehyde for 15 min, permeabilized in 0.3% Triton X-100 in PBS, and treated with anti-LEUTX antibody (NB1P-90890, Novus Biologicals; 1:100) overnight at 4°C followed by donkey anti-rabbit IgG Alexa Fluor 647 (A31573, Molecular Probes; 1:1000) for 2 h at room temperature and Hoechst 33342 nuclear stain (H3570, Molecular Probes; 1:1000) for 20 min at room temperature. Images were acquired with a Zeiss LSM710-NLO point scanning confocal microscope. Post-acquisition analysis was carried out using Imaris (Bitplane). Control staining to validate antibody specificity was described in the supplementary Materials and Methods.

**STRT RNA-seq of human preimplantation embryos**

The data on human preimplantation embryos and data processing using the STRT RNA-seq method were previously described by Töhönen et al. (2015). The processed STRT reads supporting the results of the present study are available in the European Nucleotide Archive (http://www.ebi.ac.uk/ena/data/view/PRJEB899). TFE for *LEUTX* is FE270433 (chr19:40269482-40269570, +). The normalized expression values used for drawing beeswarm plots are given in Table S3.

**Quantitative PCR (qPCR)**

Total RNA extracted from three hESC lines (H9, HS401 and HS980) in three biological replicates of each was converted to cDNA using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, 11752) according to the manufacturer’s instructions. qPCR assay was performed using 22 ng cDNA from each cell line sample and 10 ng human 8-cell library that was used for cloning of the genes. qPCR was carried out using an ABI PRISM 7500 Fast Real-Time PCR System with FastStart Universal SYBR Green Master Mix (Roche) according to the manufacturer’s instructions. The primer sequences are given in Table S4. To confirm the qPCR amplicon, it was cloned into pCRII-dual promoter TOPO vector using the TOPO TA cloning kit (Invitrogen), and the sequence was verified by Sanger sequencing (Eurofins Genomics).

**Construction of expression vectors**

In order to overexpress *LEUTX.R* (NM_001143832.1), *LEUTX.n*, DPRX and OTX2 (NM_172337) in human cells, the ORFs were cloned into a modified pFastBac expression vector CMvE.FE1.eGF-P-WPRE that was kindly provided by Prof. Shu Wang (Institute of Bioengineering and Nanotechnology, Singapore) (Du et al., 2010). The modifications are described in detail in the supplementary Materials and Methods and by Töhönen et al. (2015).

ORFs for *LEUTX* and DPRX were amplified from the TOPO vectors containing the full-length clone (European Nucleotide Archive accession numbers LN651090 and LN651088, respectively) and OTX2 was amplified from a mixture containing equal amounts of cDNAs originating from human placenta, testis and hESC lines HS401 and H9. The ORF for predicted *LEUTX.R* was amplified from the same TOPO vector as the novel isoform, but using a forward primer 90 bp downstream of novel start site (Fig. 1A). The amplification primers included *AscI* and *Paci* restriction sites (primer sequences are given in Table S5). The PCR products were digested using *AscI* and *Paci* (New England Biolabs) and ligated into pFastBac vector digested with the same enzymes. mCherry fluorescent protein was used as a control and amplified from the standard injection marker construct e2-::GFP for *C.elegans* (a kind gift from Gert Jansen, The Erasmus University Medical Center, Rotterdam, The Netherlands). In order to mutate *LEUTX.n* in the pFastBac vector, the QuikChange II Site-Directed Mutagenesis Kit (Agilent) was used according to the manufacturer’s instructions and the primers described in Table S6.

*LEUTX.n* and *LEUTX.R* were further cloned into the pcDNA3.1/V5-His-TOPO vector (Invitrogen) from the TOPO vector containing the full-length clone LN651090. The primer sequences, including either BanHI or NotI restriction site at their 5′-end, are given in Table S7.

**Indirect immunofluorescence labeling**

Four-cell stage embryos were thawed using ThawKit™ Cleave following the manufacturer’s instructions and cultured overnight on G-1™ medium, allowing them to develop until the 8-cell stage. Embryos were fixed in 4% paraformaldehyde for 15 min, permeabilized in 0.3% Triton X-100 in PBS, and treated with anti-LEUTX antibody (NB1P-90890, Novus Biologicals; 1:100) overnight at 4°C followed by donkey anti-rabbit IgG Alexa Fluor 647 (A31573, Molecular Probes; 1:1000) for 2 h at room temperature and Hoechst 33342 nuclear stain (H3570, Molecular Probes; 1:1000) for 20 min at room temperature. Images were acquired with a Zeiss LSM710-NLO point scanning confocal microscope. Post-acquisition analysis was carried out using Imaris (Bitplane). Control staining to validate antibody specificity is described in the supplementary Materials and Methods.

**Western blot**

Total protein extracts from HEK-293 cells overexpressing *LEUTX.n* or *LEUTX.R* were analyzed by western blot as described in the supplementary Materials and Methods.

**Overexpression of LEUTX in hESCs and FACS for STRT RNA-seq**

Human 4-cell stage embryos were thawed using ThawKit™ Cleave following the manufacturer’s instructions and cultured overnight on G-1™ medium, allowing them to develop until the 8-cell stage. Embryos were fixed in 4% paraformaldehyde for 15 min, permeabilized in 0.3% Triton X-100 in PBS, and treated with anti-LEUTX antibody (NB1P-90890, Novus Biologicals; 1:100) overnight at 4°C followed by donkey anti-rabbit IgG Alexa Fluor 647 (A31573, Molecular Probes; 1:1000) for 2 h at room temperature and Hoechst 33342 nuclear stain (H3570, Molecular Probes; 1:1000) for 20 min at room temperature. Images were acquired with a Zeiss LSM710-NLO point scanning confocal microscope. Post-acquisition analysis was carried out using Imaris (Bitplane). Control staining to validate antibody specificity is described in the supplementary Materials and Methods.
and a fluorescent marker in the same cells. The cell suspension was transferred to a fresh Laminin-521-coated well, the cells were allowed to settle in the transfection solution for 15 min, and the medium was subsequently changed to mTeSR®+.

FACS was performed on trypsin-treated cells 9-11 h after transfection. Cells were sorted by BD FACSAria III using an 85 µm nozzle according to their eGFP expression to positive or negative wells. Clustered cells and debris were excluded by FSC and SSC gates, and dead cells were excluded by propidium iodide viability stain. GFP-positive cells were sorted in triplicate (3×75 cells for novel LEUTX, DPRX and OTX2) or quadruplicate (4×50 cells for RefSeq LEUTX) into 5 µl STRT lysis buffer and two STRT libraries were prepared. Equal numbers of GFP-negative cells per transfection were sorted in triplicate.

STRT RNA-seq library preparation and data analysis
STRT sequencing libraries were prepared for two purposes: (1) single-cell expression profiling in two hESC lines and single 8-cell blastomeres and (2) target gene detection following overexpression experiments. The first library included 16 human single blastomeres from 8-cell stage embryos, 16 human regular hESCs (line HS980), 16 human single 8-cell blastomere-derived hESCs (line HS983a), all picked manually under a microscope into 5 µl lysis/cDNA synthesis buffer. Thawing of human embryos for STRT is described in the supplementary Materials and Methods.

STRT sequencing libraries consisting of 48 samples were prepared for sequencing on an Illumina HiSeq 2000 platform according to a modified STRT protocol (Krutisko et al., 2016). STRT sequencing data analysis is described in the supplementary Materials and Methods and Table S9. Pre-processing of STRT reads and alignments were performed using an established analysis pipeline (Krutisko et al., 2016). The data normalization and differential expression analyses were performed using the R package SAMStr (Katayama et al., 2013). The R package SAMStr (Katayama et al., 2013). STRT reads supporting the results of the present study are available in the European Nucleotide Archive (http://www.ebi.ac.uk/ena/data/view/PRJEB12467 and http://www.ebi.ac.uk/ena/data/view/PRJEB12453). The normalized reads and TFE locations for the overexpression experiment can be visualized as a ‘my hub’ on the UCSC genome browser (http://jke.bioci. ki.se/hubs/Li345ub/leutx.txt).

Construction of luciferase reporter vector and the luciferase assay
In order to study the recently discovered DNA motif – referred to as the 36 bp de novo motif (Töhönen et al., 2015) – a 216 bp synthetic construct containing four motifs in tandem with intervening restriction sites was constructed (Eurofins). The construct sequence is given in the supplementary Materials and Methods. The construct containing the 4+36 bp de novo motif was restricted with SfiI and ligated into restriction digested pGL4.25 [luc2Cp/minP] luciferase reporter vector (Promega). The pGL4.25 luciferase reporter vector contains a minimal promoter. To obtain a construct with only one repeat of the 36 bp de novo motif, the construct in pGL4.25 was subjected to three cycles of digestion, purification and religation using EcoRV, Nhel and SacI (all from New England Biolabs) in that order. Construct sequences were verified by Sanger sequencing (Eurofins Genomics). The luciferase reporter assays were performed as described by Töhönen et al. (2015).

EMSA
EMSA was performed using proteins synthesized with the TNT T7 Quick for PCR DNA (Promega) transcription/translation system according to the manufacturer’s protocol. Primer sequences are given in Table S8. For details, see the supplementary Materials and Methods.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
E.M., E.-M.J., L.V., R.M., S.P., A.P.R. and K.K. performed the experiments; E.M., E.-M.J., L.V. and J.K. wrote the manuscript; E.M., E.-M.J., T.B. and S.K. analyzed the data; V.T., S.L., F.L. and O.H. provided the materials and methods to perform the study; all authors contributed to the planning of the experiments, interpretation of the data, critical review of the manuscript text, and approved the final version of the manuscript.

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Data availability
The complete STRT RNA-seq data are available at the European Nucleotide Archive (ENA) under accession numbers PRJEB12453 and PRJEB12467.

Supplementary information
Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.134510.supplemental

References


Chikazawa, E., Fukutawate, Y., Miyagawa, Y. et al. (2011). Possible linkages between the inner and outer cellular states of human induced pluripotent stem cells. BMC Syst. Biol. 5 Suppl. 1, S17.
