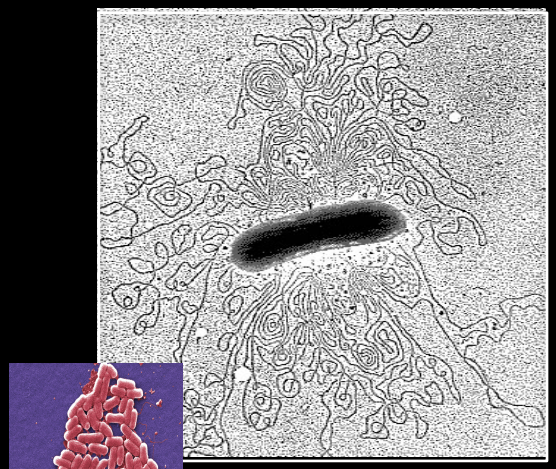
PSEUDOGENE DERIVED lncRNAs

Reason 1:
The non-coding genome (r)evolution

E.coli



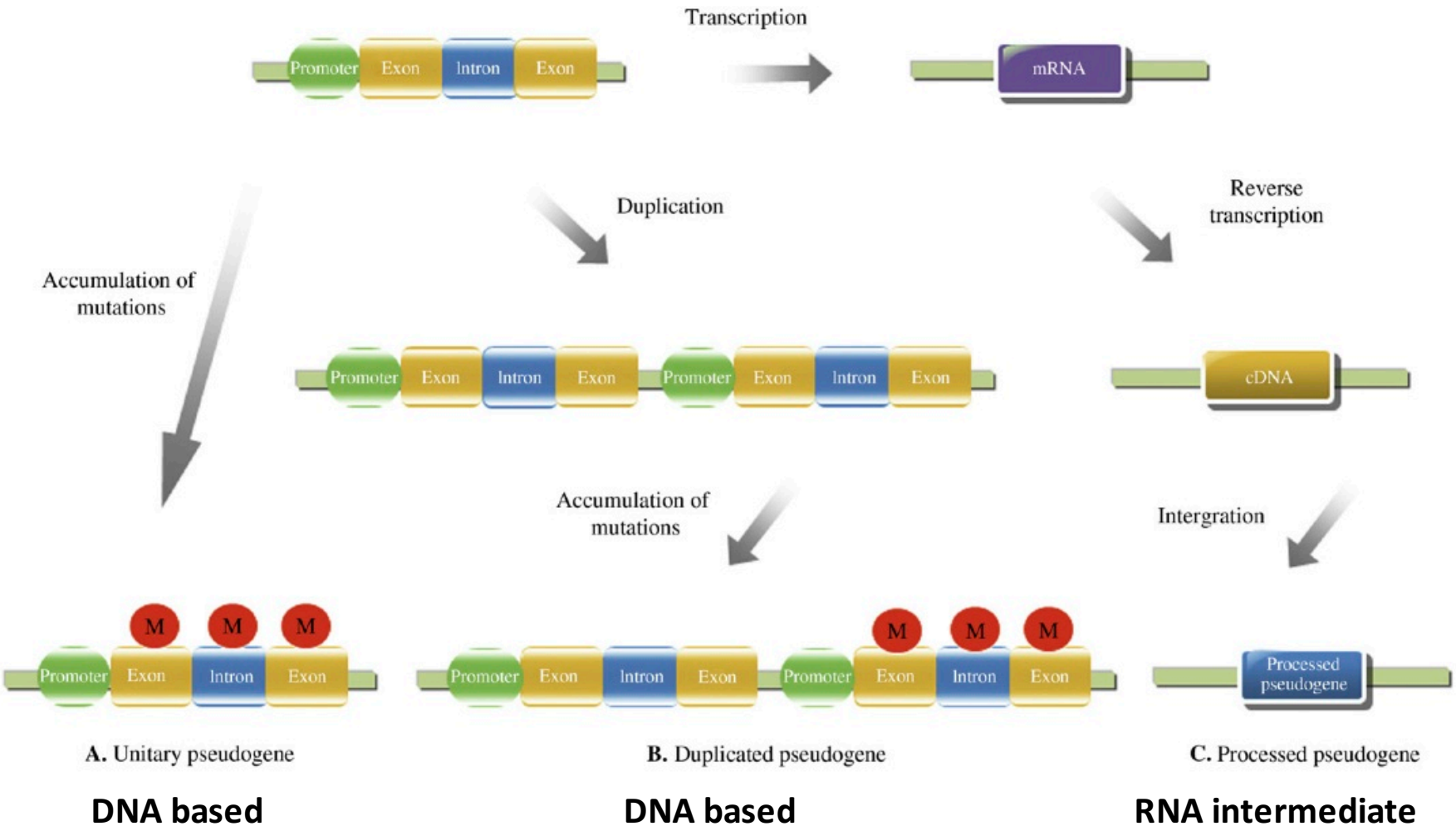
C. elegans

H. sapiens

Genome	$5x10^6$ bp	$1x10^8$ bp	$3x10^9$ bp
Chromosomes	1	6	23
Coding genes	6692	20541	21995
ncDNA	5%	60%	**98%**
non-coding RNA genes	15	23136	ca. 40000
miRNAs	0	224	4274
pseudogenes	21	1522	10616

ENSEMBL 11/2014

Protein coding genes give rise to pseudogenes



DNA based

DNA based

RNA intermediate

Transposition of Retrotransposons

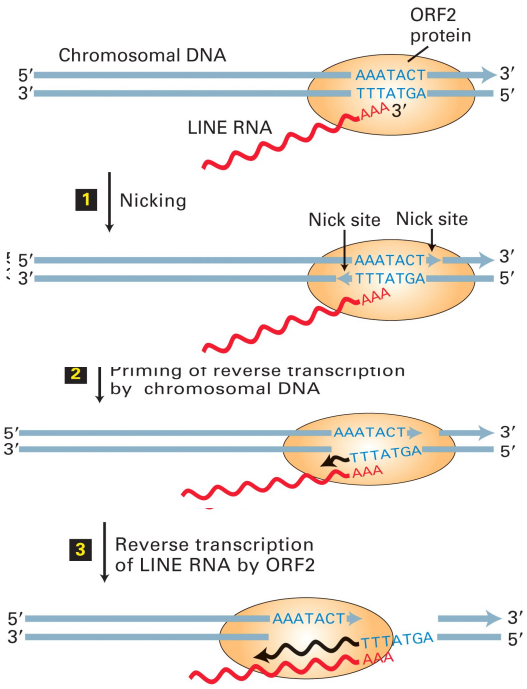
LINE: Long interspersed nuclear elements

LINE elements (L1,L2,L3)

(21% of genome; 800.000 copies)

ORF1: RNA binding protein

ORF2: Endonucelase, Reverse transcriptase

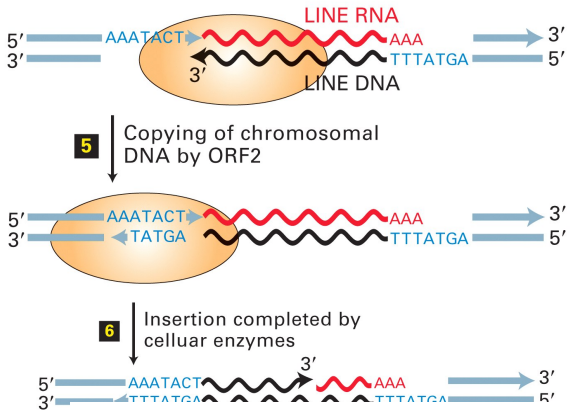


ORF1 protein binds LINE RNA; localizes to TTT rich sequences

ORF2 enonuclease activity nicks target sequence

priming

ORF2 reverse transcriptase activity → makes reverse transcription

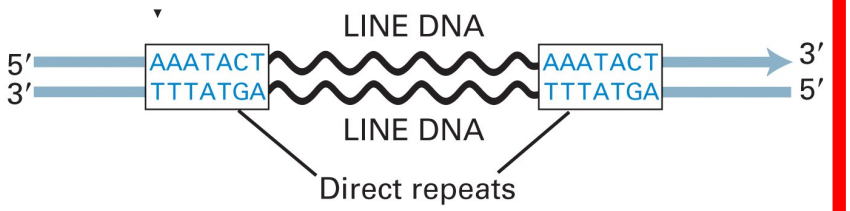


ORF2 reverse transcriptase activity → makes reverse transcription

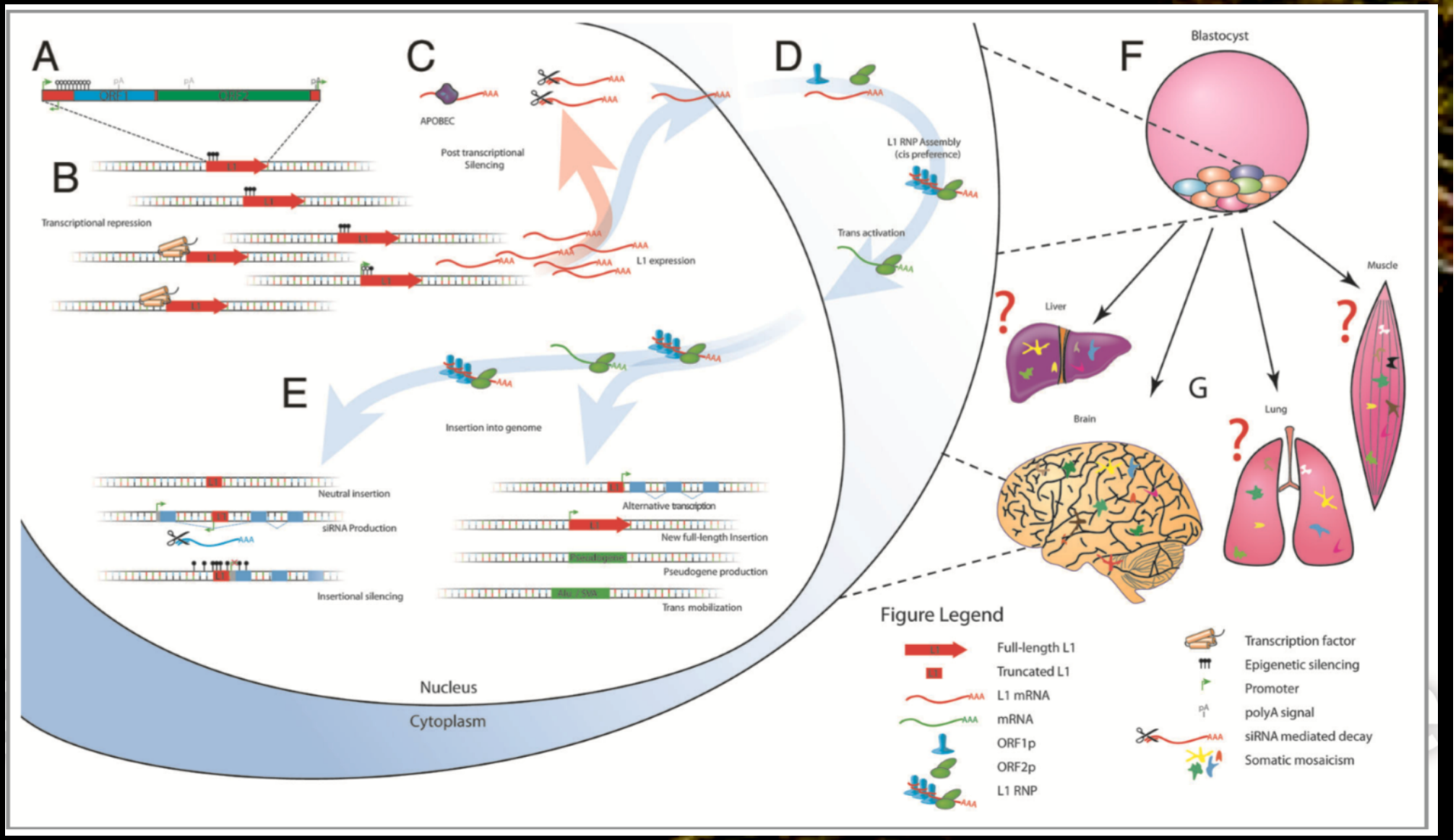
Generation of direct repeats in target site

DNA repair by host cell

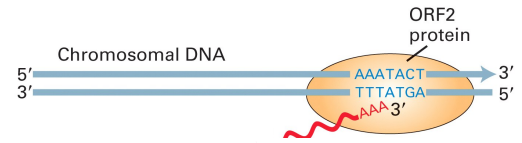
FINAL PRODUCT



Retrotransposons can change genetic context

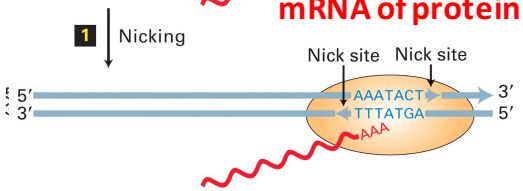


Retro-transposition machinery hijacks endogenous mRNAs

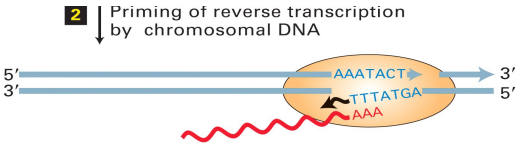


ORF1 protein binds LINE RNA; localizes to TTT rich sequences

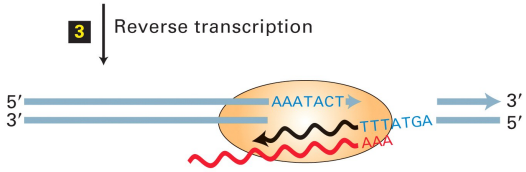
mRNA of protein coding gene - spliced



ORF2 enonuclease activity nicks target sequence

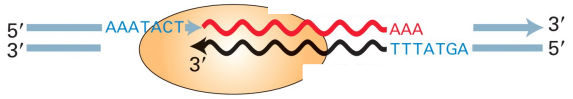


priming

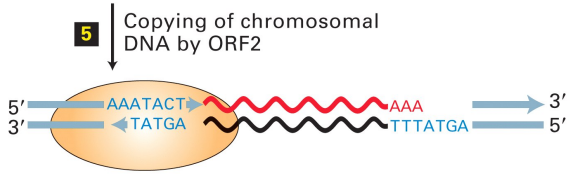


ORF2 reverse transcriptase activity→ makes reverse transcription

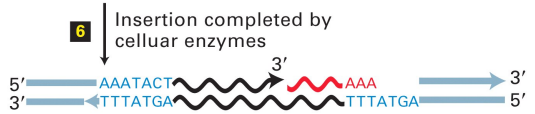
mRNA protein coding gene - spliced



ORF2 reverse transcriptase activity→ makes reverse transcription



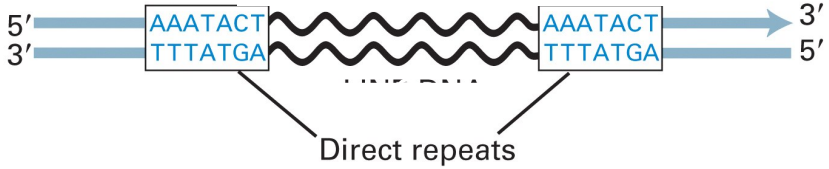
Generation of direct repeats in target site



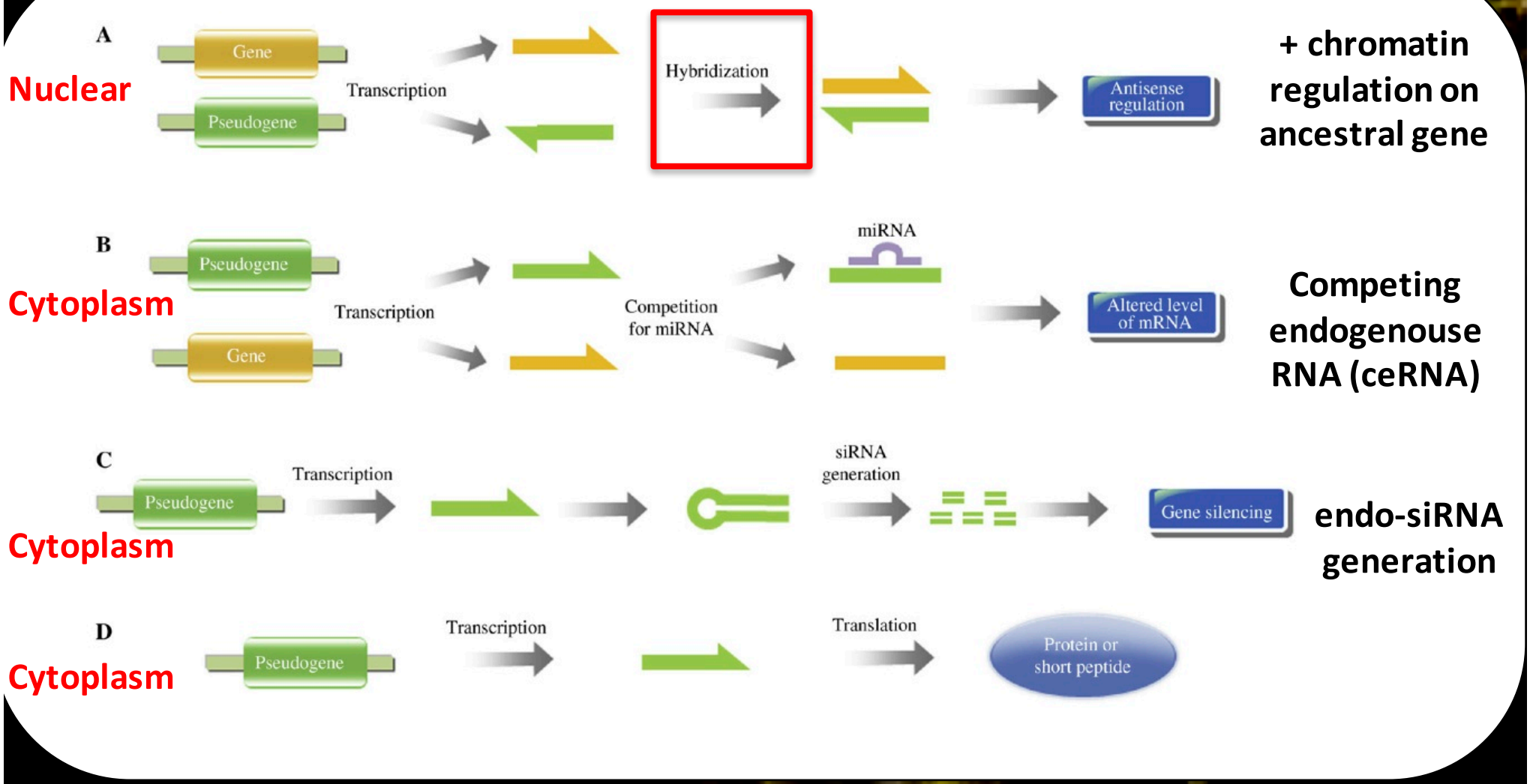
DNA repair by host cell

FINAL PRODUCT: PROCESSED PSEUDOGENE

mRNA protein coding gene – spliced (fragment)



Pseudogene derived RNAs can acquire new functions



PSEUDOGENE BIOTYPES

Table 2 Pseudogene biotypes

Biotype	Definition
Processed pseudogene	Pseudogene created via retrotransposition of the mRNA of a functional protein-coding parent gene followed by accumulation of disabling mutations
Duplicated pseudogene	Pseudogene created via genomic duplication of a functional protein-coding parent gene followed by accumulation of disabling mutations
Unitary pseudogene	Pseudogene for which the ortholog in a reference species (mouse) is coding but the human locus has accumulated fixed disabling mutations
Polymorphic pseudogene	Locus known to be coding in some individuals but with disabling mutations in the reference genome
IG pseudogene	Immunoglobulin gene segment with disabling mutations
TR pseudogene	T-cell receptor gene segment with disabling mutations

Duplicated/Unitary pseudogenes: can bring regulatory sequences, often spliced

Processed pseudogenes: hitch hike on regulatory elements dispersed throughout throughout the genome

PSEUDOGENE BIOTYPES

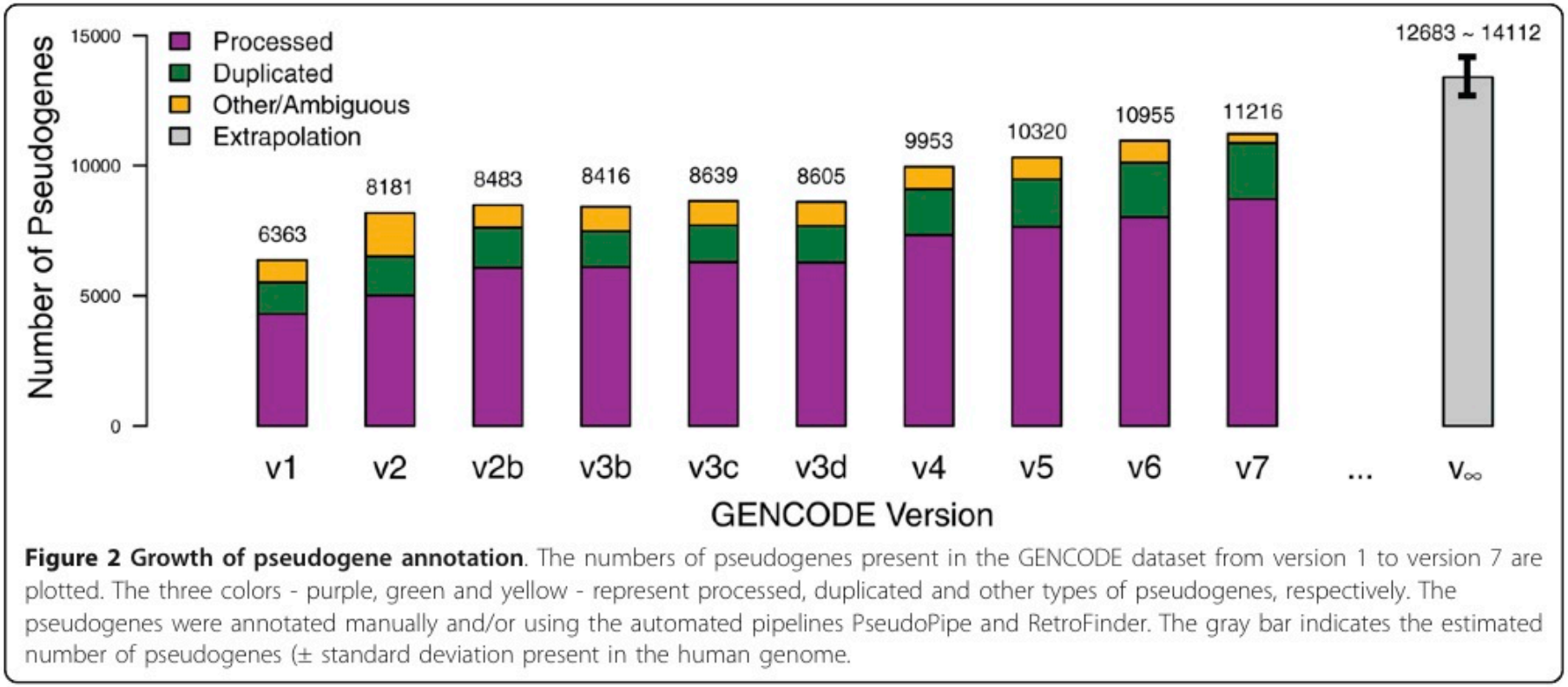
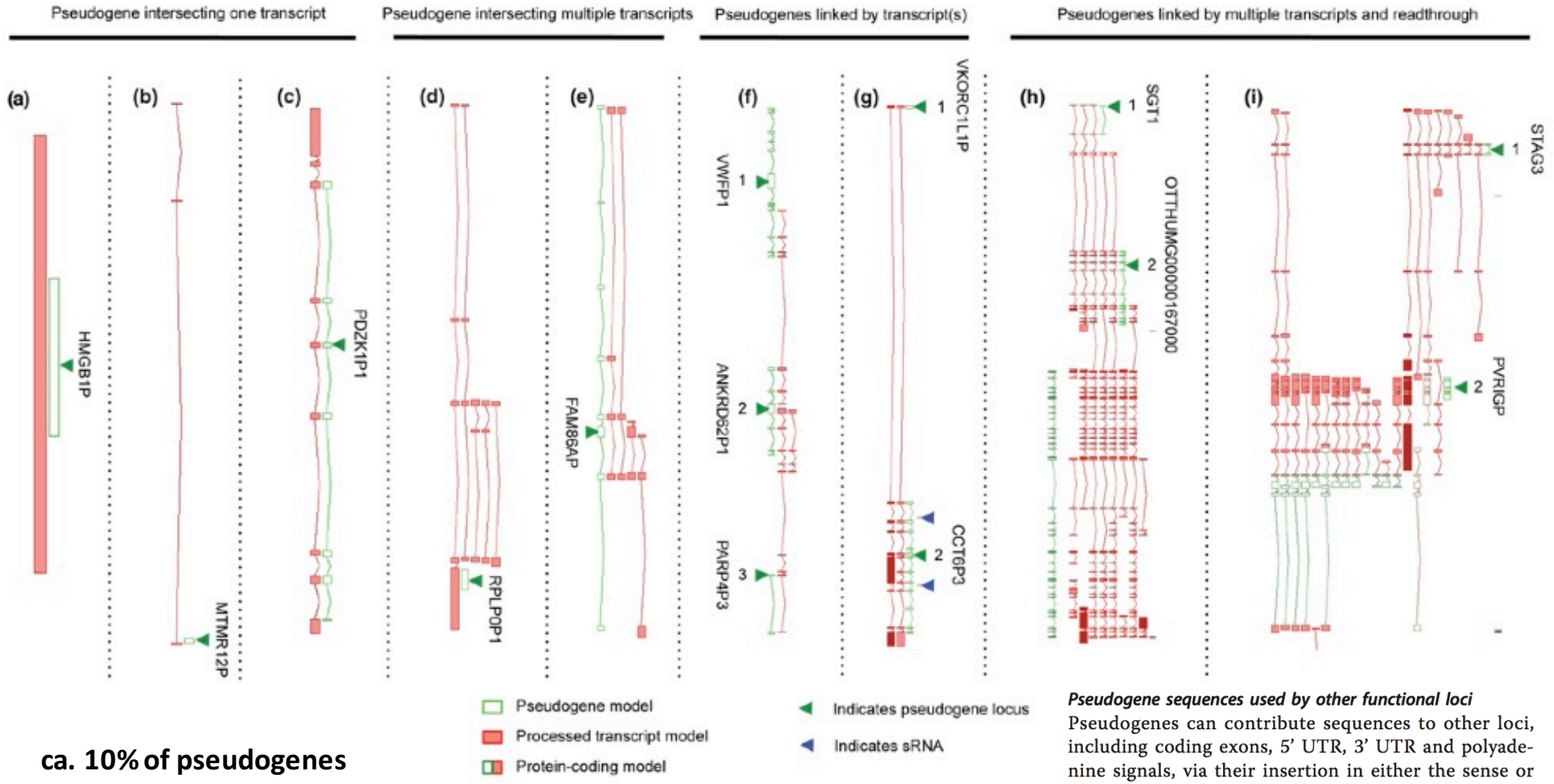


Figure 2 Growth of pseudogene annotation. The numbers of pseudogenes present in the GENCODE dataset from version 1 to version 7 are plotted. The three colors - purple, green and yellow - represent processed, duplicated and other types of pseudogenes, respectively. The pseudogenes were annotated manually and/or using the automated pipelines PseudoPipe and RetroFinder. The gray bar indicates the estimated number of pseudogenes (± standard deviation present in the human genome.

The majority of pseudogenes are processed pseudogenes:
Burst of retro-transposition events in recent phase of evolution

Total No of Genes	60498
Protein-coding genes	19797
Long non-coding RNA genes	15931
Small non-coding RNA genes	9882
Pseudogenes	14477
- processed pseudogenes:	10727
- unprocessed pseudogenes:	3271
- unitary pseudogenes:	172
- polymorphic pseudogenes:	59

PSEUDOGENE LOCI (duplicated/processed) CAN BE USED BY OTHER FUNCTIONAL LOCI



ca. 10% of pseudogenes

→ Pseudogenes contribute to the evolution of protein coding genes

Pseudogene sequences used by other functional loci

Pseudogenes can contribute sequences to other loci, including coding exons, 5' UTR, 3' UTR and polyadenine signals, via their insertion in either the sense or antisense orientation. Such loci range in complexity from simple cases where a single pseudogene is overlapped by one transcript to instances of greater complexity where multiple pseudogenes are overlapped by multiple transcripts, and transcriptional read-through from proximal protein-coding and lncRNA loci (Figure 3). For example, *MST1P9* (Figure S1 in Additional file 1), whose translation is supported by mass spectrometry data, is a potential 'resurrected' pseudogene that has gained a novel function and therefore has been re-annotated as a new protein-coding locus [29]. Another exam-

Figure 3 Complexity of transcribed pseudogenes. Screenshots of pseudogene annotation are taken from the Zmap annotation interface. The pseudogenes are represented as open green boxes and indicated by dark green arrowheads, exons of associated transcript models are represented as filled red boxes and connections are shown by red lines. The coding exons of protein-coding models are represented by dark green boxes and UTR exons as filled red boxes; protein-coding models are also indicated by red arrowheads. **(a-c)** Single pseudogene models intersecting with single transcript models. (a) The processed pseudogene High mobility group box 1 pseudogene (*HMGB1P*; HAVANA gene ID: OTTHUMG00000172132 and its associated unspliced (that is, single exon) transcript. (b) The processed pseudogene Myotubularin related protein 12 pseudogene (*MTMR12P*; HAVANA gene ID: OTTHUMG00000167532) and a spliced transcript model with three exons. (c) A duplicated pseudogene PDZ domain containing 1 pseudogene 1 (*PDZK1P1*; HAVANA gene ID: OTTHUMG00000013746) and a spliced transcript model with nine exons. **(d,e)** Single pseudogene models intersecting with multiple transcripts. (d) The processed pseudogene Ribosomal protein, large, P0 pseudogene 1 (*RPLP0P1*; HAVANA gene ID: OTTHUMG00000158396) and five spliced transcripts. (e) The duplicated pseudogene Family with sequence similarity 86, member A pseudogene (*FAM86AP*; HAVANA gene ID: OTTHUMG00000159782) and four spliced transcripts. **(f,g)** Groups of multiple pseudogenes that are connected by overlapping transcripts. (f) Three pseudogenes with single connecting transcripts: 1 is the duplicated pseudogene von Willebrand factor pseudogene 1 (*VWFP1*; HAVANA gene ID: OTTHUMG00000143725); 2 is a duplicated pseudogene ankyrin repeat domain 62 pseudogene 1 (*ANKRD62P1*; HAVANA gene ID: OTTHUMG00000149993); 3 is the duplicated pseudogene poly (ADP-ribose) polymerase family, member 4 pseudogene 3 (*PARP4P3*; HAVANA gene ID: OTTHUMG00000142831). Pseudogene 1 and 2 are connected by a seven exon transcript, pseudogenes 2 and 3 are connected by a nine exon transcript and there is a third transcript that shares two of its four exons with pseudogene 2. (g) Two pseudogenes with multiple connecting transcripts: 1 is the processed pseudogene vitamin K epoxide reductase complex, subunit 1-like 1 pseudogene (*VKORC1L1P*; HAVANA gene ID: OTTHUMG00000156633); 2 is the duplicated pseudogene chaperonin containing TCP1, subunit 6 (zeta) pseudogene 3 (*CCT6P3*; HAVANA gene ID: OTTHUMG00000156630). The two pseudogenes are connected by two transcripts that initiate at the upstream pseudogene and utilize a splice donor site within the single exon, which is also a splice donor site in the pseudogene's parent locus. Interestingly, the downstream locus hosts two small nucleolar RNAs (snoRNAs) that are present in the parent locus and another paralog. **(h)** A very complex case where multiple pseudogenes, connected by multiple transcripts, read through into an adjacent protein-coding locus: 1 is the duplicated pseudogene suppressor of G2 allele of SKP1 (*S. cerevisiae*) pseudogene (*SGT1P*; HAVANA gene ID: OTTHUMG00000020323); 2 is a novel duplicated pseudogene (OTTHUMG00000167000); and the protein-coding gene is *C9orf174*, chromosome 9 open reading frame 174 (OTTHUMG00000167001). **(i)** A similarly complex case where multiple pseudogenes, connected by multiple transcripts, read through into an adjacent protein-coding locus: 1 is a duplicated pseudogene stromal antigen 3 pseudogene (*STAGP3*; HAVANA gene ID: OTTHUMG00000156884); 2 is a duplicated pseudogene poliovirus receptor related immunoglobulin domain containing pseudogene (*PVRIGP*; HAVANA gene ID: OTTHUMG00000156886); and the protein-coding gene is *PILRB*, paired immunoglobin-like type 2 receptor beta (OTTHUMG00000155363). sRNA, small RNA.

GENOMICS STRATEGIES TO IDENTIFY AND CLASSIFY PSEUDOGENES

Table 3 Fields for pseudogene features in the psiDR annotation file Pseudogene decoration resource

Field	Explanation	psiDR value
Transcript ID	Pseudogene ID from GENCODE annotation. Used for cross-referencing	
Parent	Protein ID, Gene ID, chromosome, start, end and strand. Detailed in section *'Parents of pseudogenes'*	
Sequence similarity	The percentage of pseudogene sequence preserved from parent	
Transcription	Evidence for pseudogene transcription and validation results. May be tagged as EST, BodyMap, RT-PCR or None, which represent pseudogene expression evidence from corresponding data sources. Multiple tags are separated by commas. Detailed in section *'Transcription of pseudogenes'*	1, transcription; 0, otherwise
DNaseI hypersensitivity	A categorical result indicating whether the pseudogene has easily accessible chromatin, predicted by a model integrating DNaseI hypersensitivity values within 4 kb genomic regions upstream and downstream of the 5' end of pseudogenes. Detailed in section *'Chromatin signatures of pseudogenes'*	1, has Dnase hypersensitivity in upstream; 0, otherwise
Chromatin state	Whether a pseudogene maintains an active chromatin state, as predicted by a model using Segway segmentation. Detailed in section *'Chromatin signatures of pseudogenes'*	1, active chromatin; 0, otherwise
Active Pol2* binding	Whether Pol2 binds to the upstream region of a pseudegene. Detailed in section *'Upstream regulatory elements'*	1, active binding site; 0, otherwise
Active promoter region	Whether there are active promoter regions in the upstream of pseudogenes. Detailed in section *'Upstream regulatory elements'*	1, active binding site; 0, otherwise
Conservation	Conservation of pseudogenes is derived from the divergence between human, chimp and mouse DNA sequences. Detailed in section *'Evolutionary constraint on pseudogenes'*	1, conserved; 0, otherwise

*Pol2, RNA polymerase II.

- Parent gene/ancestral gene = functional gene with greatest sequence similarity
- Ancestral gene can be identified for ca. 90% of pseduogenes
- 10% of pseudogenes are highly degraded and is derived from a parent gene with highly similar paralogs
Or parent gene contains a commonly found functional domain
-NOTE: most parental genes have only 1 pseudogene
-NOTE: some parental genes – mainly housekeeping genes - have MANY pseudogenes:
-Robosomal protein L21: 143 pseudogenes
-Gapdh: 68 pseudogenes

Evolutionary constraint on pseudogenes

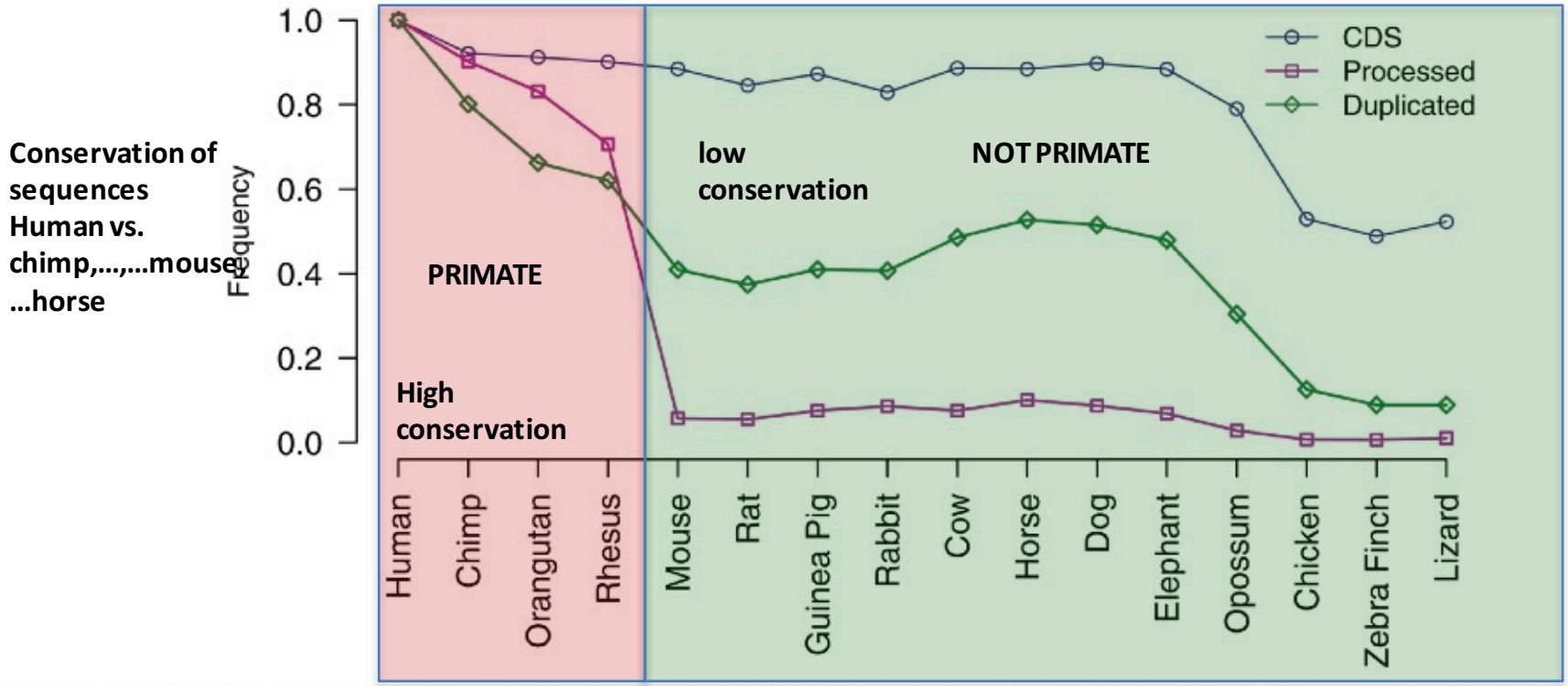
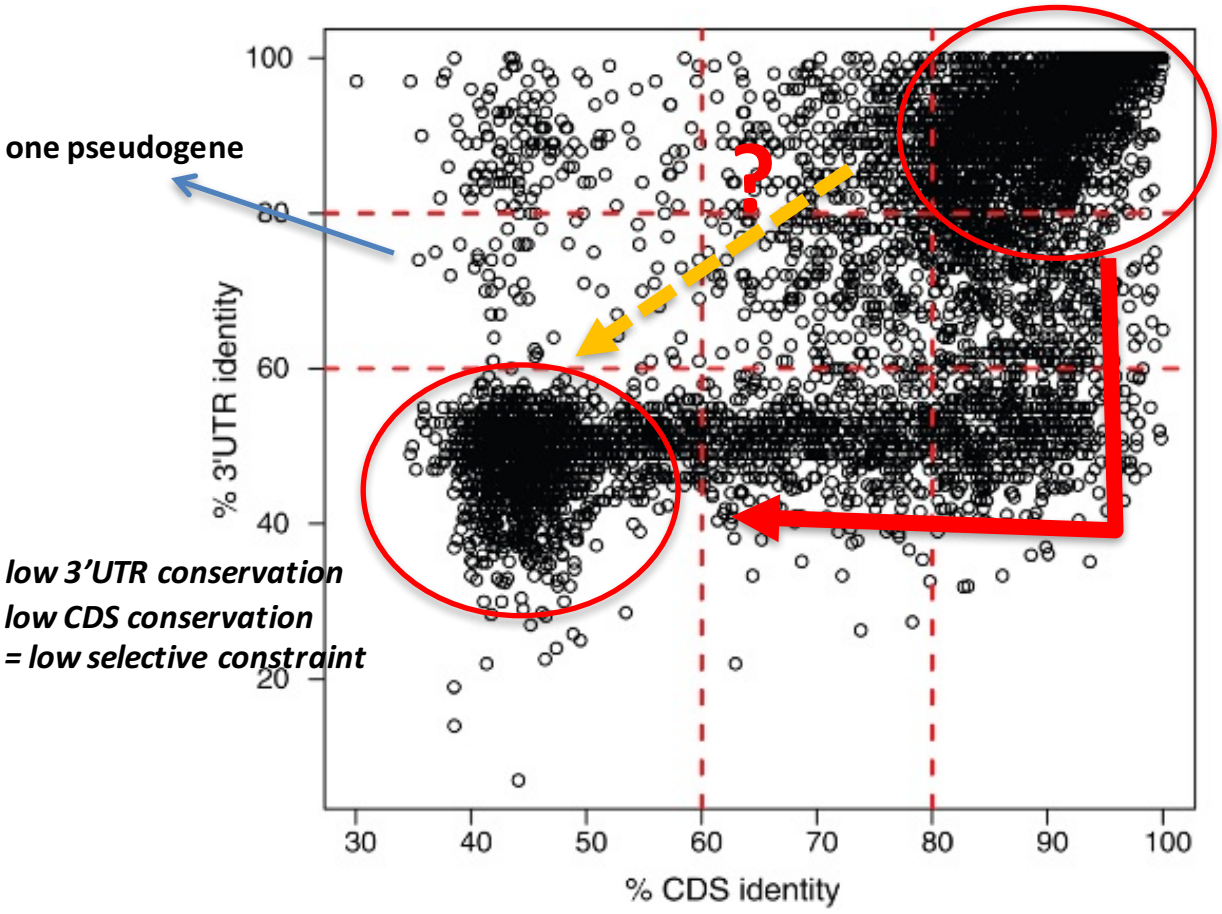


Figure 6 Preservation of human coding sequences, processed pseudogenes and duplicated pseudogenes. Sequences orthologous to human genomic regions from different species were studied. The sequence preservation rate was calculated as the percentage of sequences aligned to human sequence from each species. The calculation was based on a MultiZ multiple genome sequence alignment.

dogenes. While the preservation of duplicated pseudogenes decreases gradually with the increase of evolutionary distance of the species from human, the preservation of processed pseudogenes exhibits an abrupt decrease from macaque to mouse and remains low within the species more divergent than mouse.

These results are in agreement with previous findings showing that most processed pseudogenes in humans and mice are lineage-specific, arising from distinct retrotransposition bursts happening in the two organisms after they diverged [13,41].

Sequence identity between parental and pseudogenes with focus on coding sequence (CDS) and 3'UTR



High CDS conservation

High 3'UTR conservation

=high selective constraint

low 3'UTR conservation

low CDS conservation

= low selective constraint

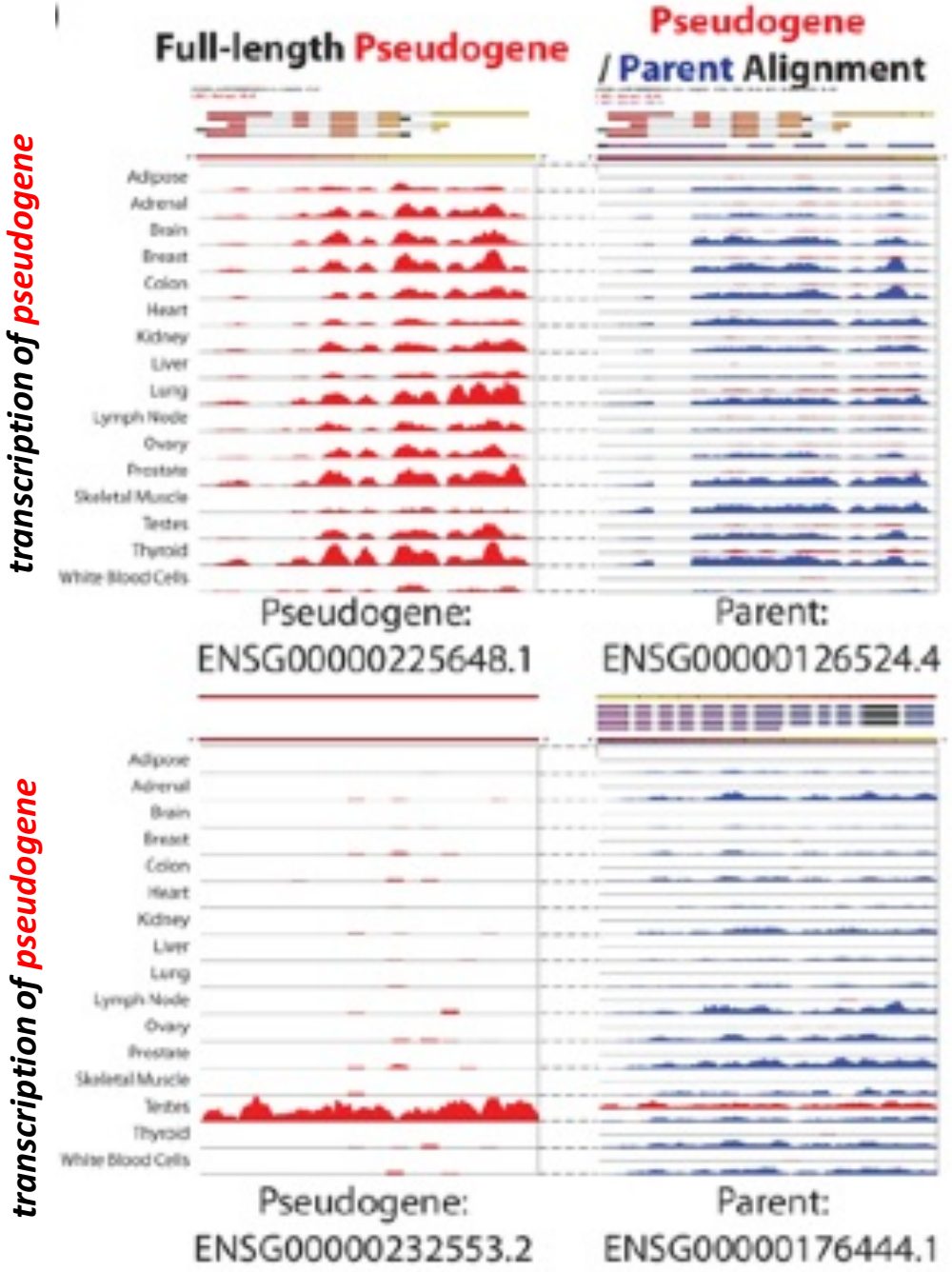
Most pseudogenes have Similar sequence identity between CDS and UTR (high: >80%; low: <60%)

Features of transcribed pseudogenes

Problem: precise analysis of RNA-seq/array data: high sequence similarity pseudogene – parental gene

2012: ca 9000 pseudogenes: 873 are transcribed according to STRINGENT psiDR parameters (real number is higher)

tissue specific expression



differential expression parental/pseudogene

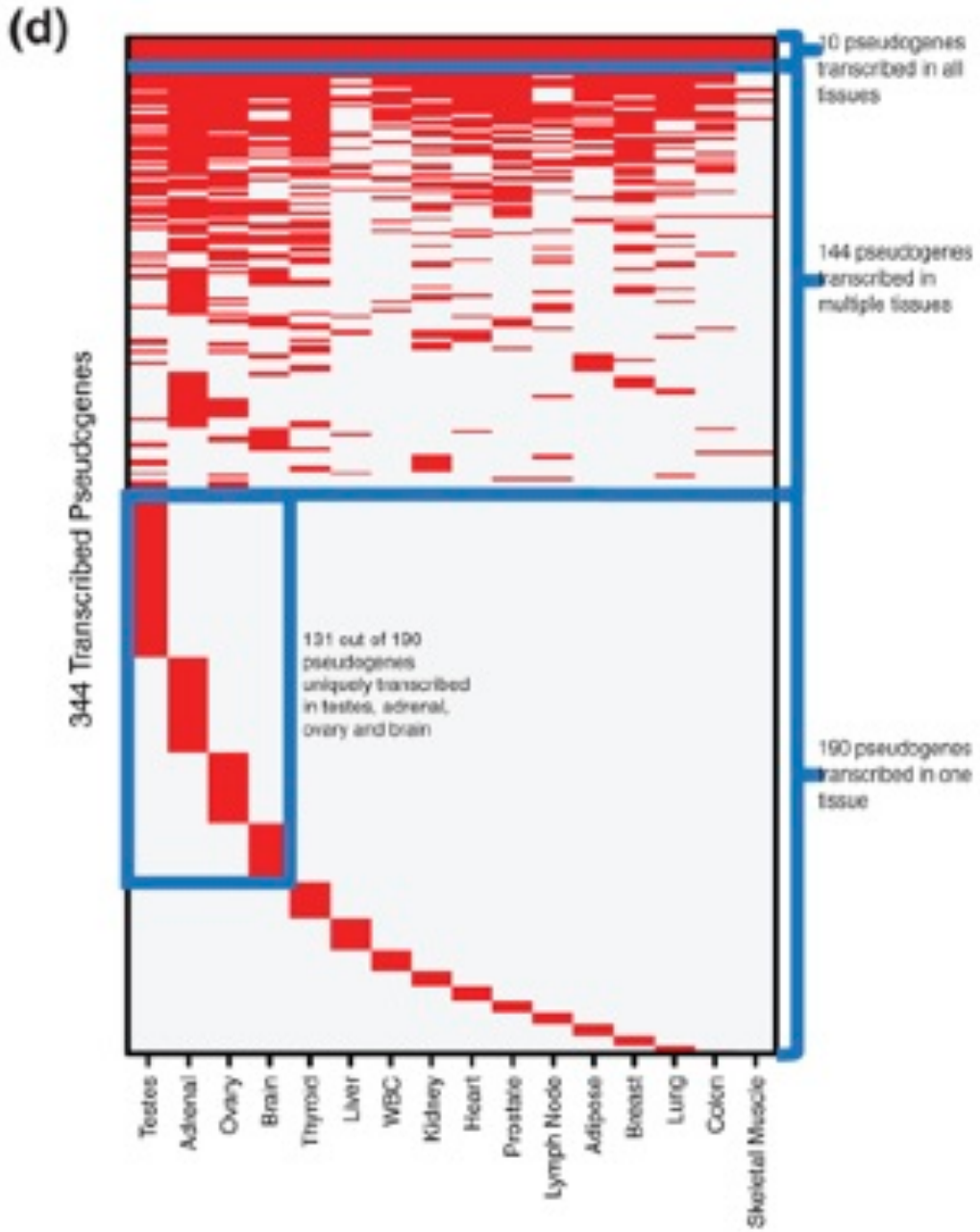
Peudogenes with differential expression compared To parental genes are considered as "expressed"

Pseudogene expression levels are LOWER than coding gene expression

tissue specific expression

differential expression parental/pseudogene

The majority of pseudogenes show tissue specific expression



Categories:

-Expressed in all tissues (10 out of 344 tested pseudogenes)

-144/344 pseudogenes expressed in more then 1 tissue

-190/344 pseudogenes exclusively expressed in 1 tissue

duplicated/processed pseudogenes have specific regulatory elements!!

Chromatin at transcriptional start sited of transcribed pseudogenes is similar to coding genes

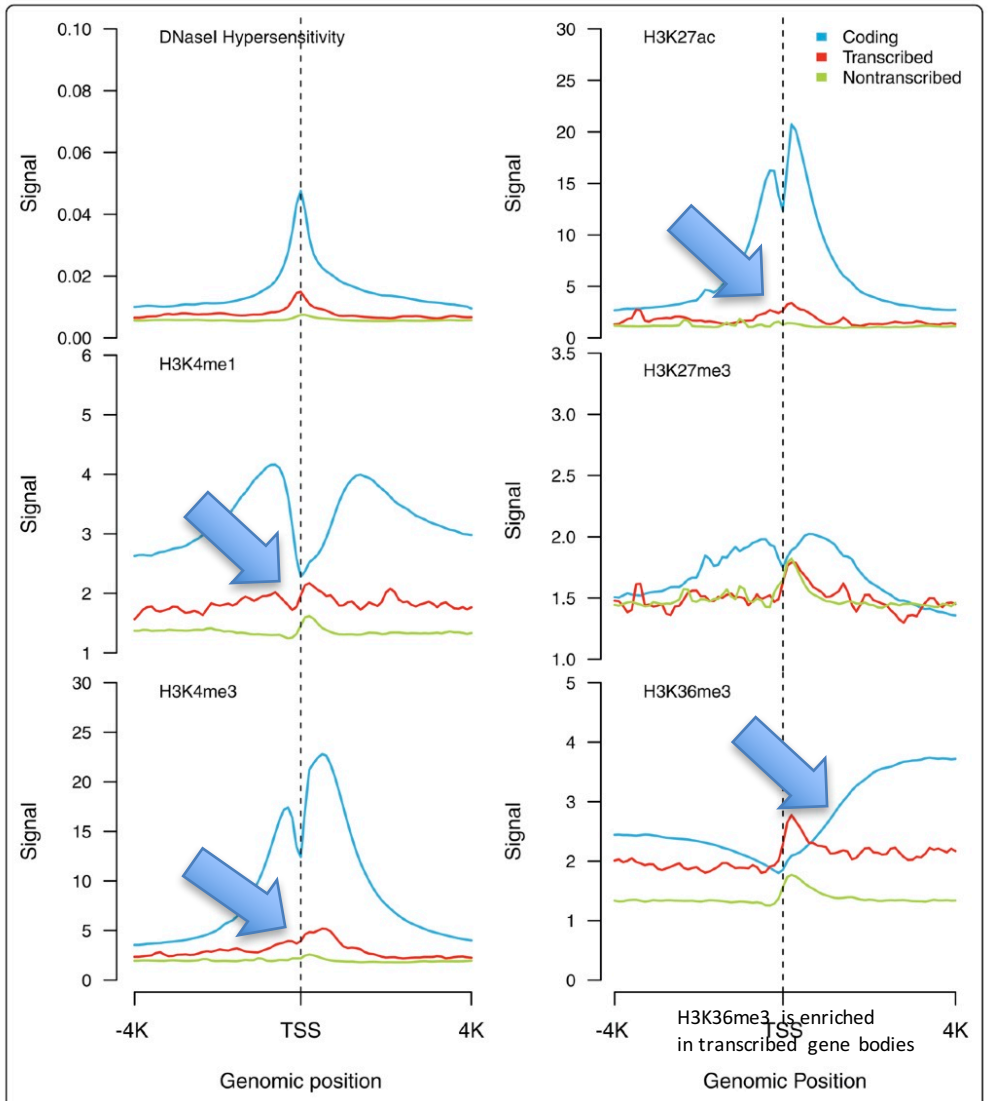
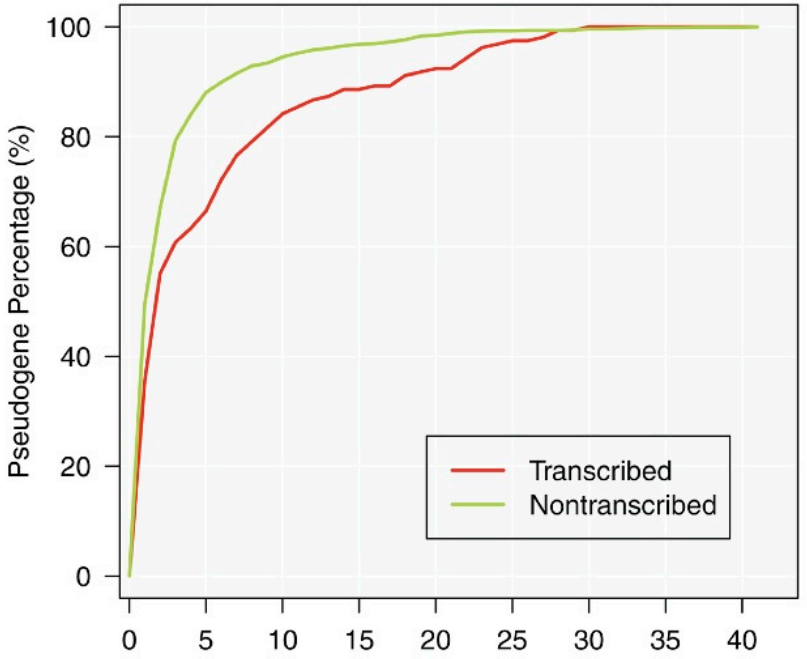


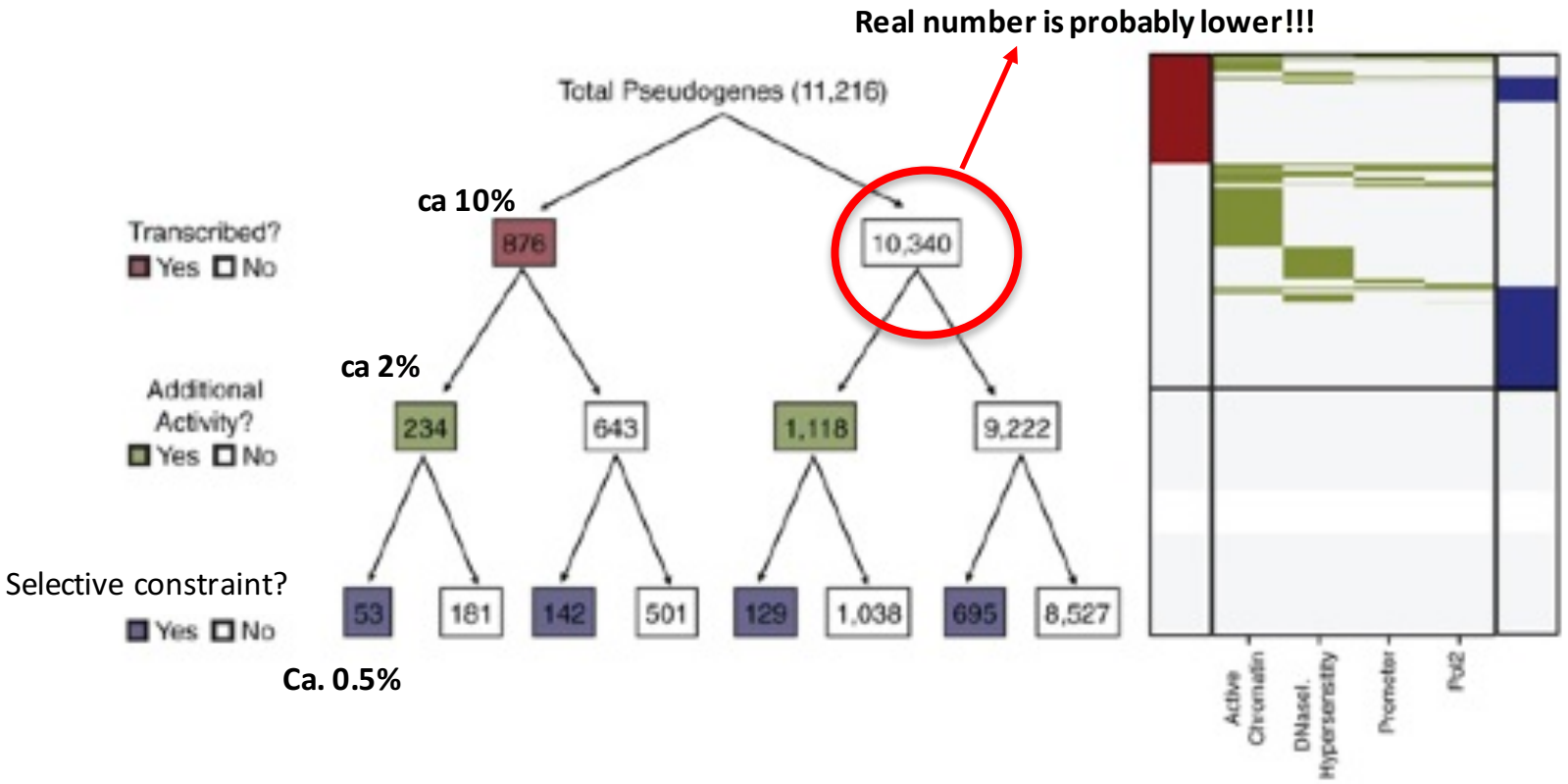
Figure 8 Chromatin signatures: DNaseI hypersensitivity and histone modification. Average chromatin accessibility profiles and various histone modifications surrounding the TSS for coding genes, transcribed pseudogenes, and non-transcribed pseudogenes. The coding gene histone modification profiles around the TSS follow known patterns - for example, enrichment of H3K4me1 around 1 kb upstream of the TSS and the H3K4me3 peaks close to the TSS [63]. Transcribed pseudogenes also show stronger H3K4 signals than non-transcribed pseudogenes. H3K27me3, a marker commonly associated with gene repression [64], showed depletion around the TSS for the coding gene and a distinctive peak in the same region for the pseudogenes. H3K36me3 also shows a similar pattern as H3K27me3 at TSSs, which may relate to nucleosome depletion.



Frequency of transcription factor binding sites enriched in transcribed Pseudogenes vs non-transcribed pseudogenes

Transcribed pseudogenes resemble coding genes; however:
Peaks are not as clear defined = average chromatin marks are less concentrated:
Reason:
→lower expression
→ expressed pseudogenes do not show marks in an uniform manner

Pseudogenes are a diversified group of genetic elements



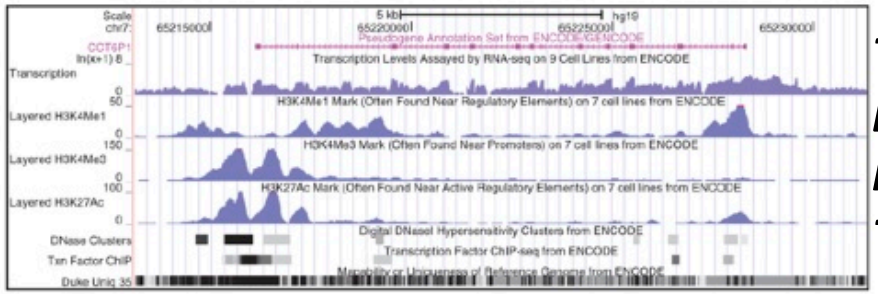
→ few pseudogenes show consistently active signals across all biological features that describe gene activity

→ many pseudogenes show little or no activity

Figure 12 Summary of pseudogene annotation and case studies. **(a)** A heatmap showing the annotation for transcribed pseudogenes including active chromatin segmentation, DNaseI hypersensitivity, active promoter, active Pol2, and conserved sequences. Raw data were from the K562 cell line. **(b)** A transcribed duplicated pseudogene (Ensembl gene ID: ENST00000434500.1; genomic location, chr7: 65216129-65228323)

Pseudogenes are a diversified group of genetic elements

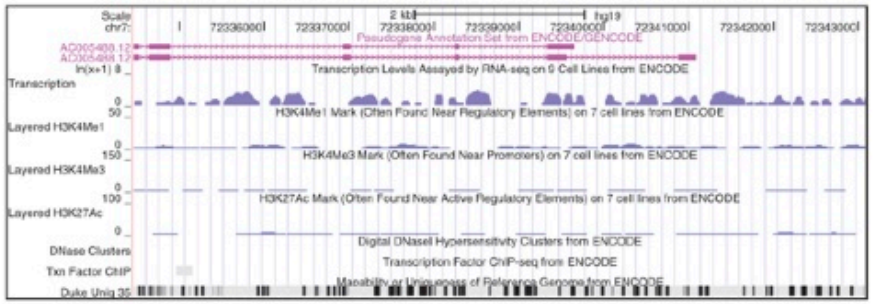
(b)



Transcribed
DNase hypersensitive sites
Histonemarks
Transcription factor

Pseudogene under selective constraint → maintained

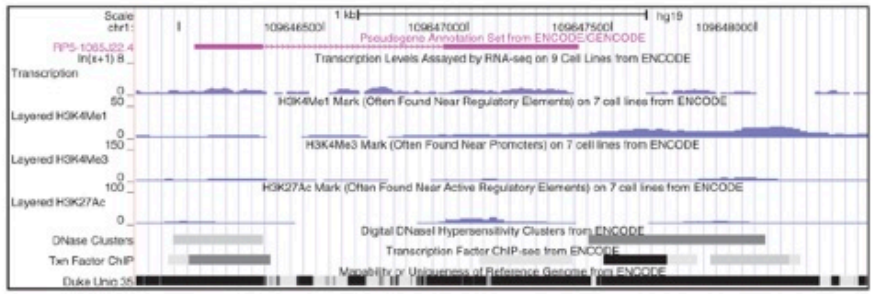
(c)



Transcribed
DNase hypersensitive sites
Histonemarks
Transcription factor

Pseudogenes under low selective constraints →This stage also involves acquisition of new splice sites – resembles a stage of testing new mutations for evolutionary advantage. Result: A. dying pseudogene or B. acquisition of critical feature leading to the resurrection to become a functional pseudogene

(d)



Transcribed
DNase hypersensitive sites
Histonemarks
Transcription factor

Figure 12 Summary of pseudogene annotation and case studies. **(a)** A heatmap showing the annotation for transcribed pseudogenes including active chromatin segmentation, DNaseI hypersensitivity, active promoter, active PolI2, and conserved sequences. Raw data were from the K562 cell line. **(b)** A transcribed duplicated pseudogene (Ensembl gene ID: ENST00000434500.1; genomic location, chr7: 65216129-65228323) showing consistent active chromatin accessibility, histone marks, and TFBSs in its upstream sequences. **(c)** A transcribed processed pseudogene (Ensembl gene ID: ENST00000355920.3; genomic location, chr7: 72333321-72339656) with no active chromatin features or conserved sequences. **(d)** A non-transcribed duplicated pseudogene showing partial activity patterns (Ensembl gene ID: ENST00000429752.2; genomic location, chr1: 109646053-109647388). **(e)** Examples of partially active pseudogenes. E1 and E2 are examples of duplicated pseudogenes. E1 shows *UGT1A2P*

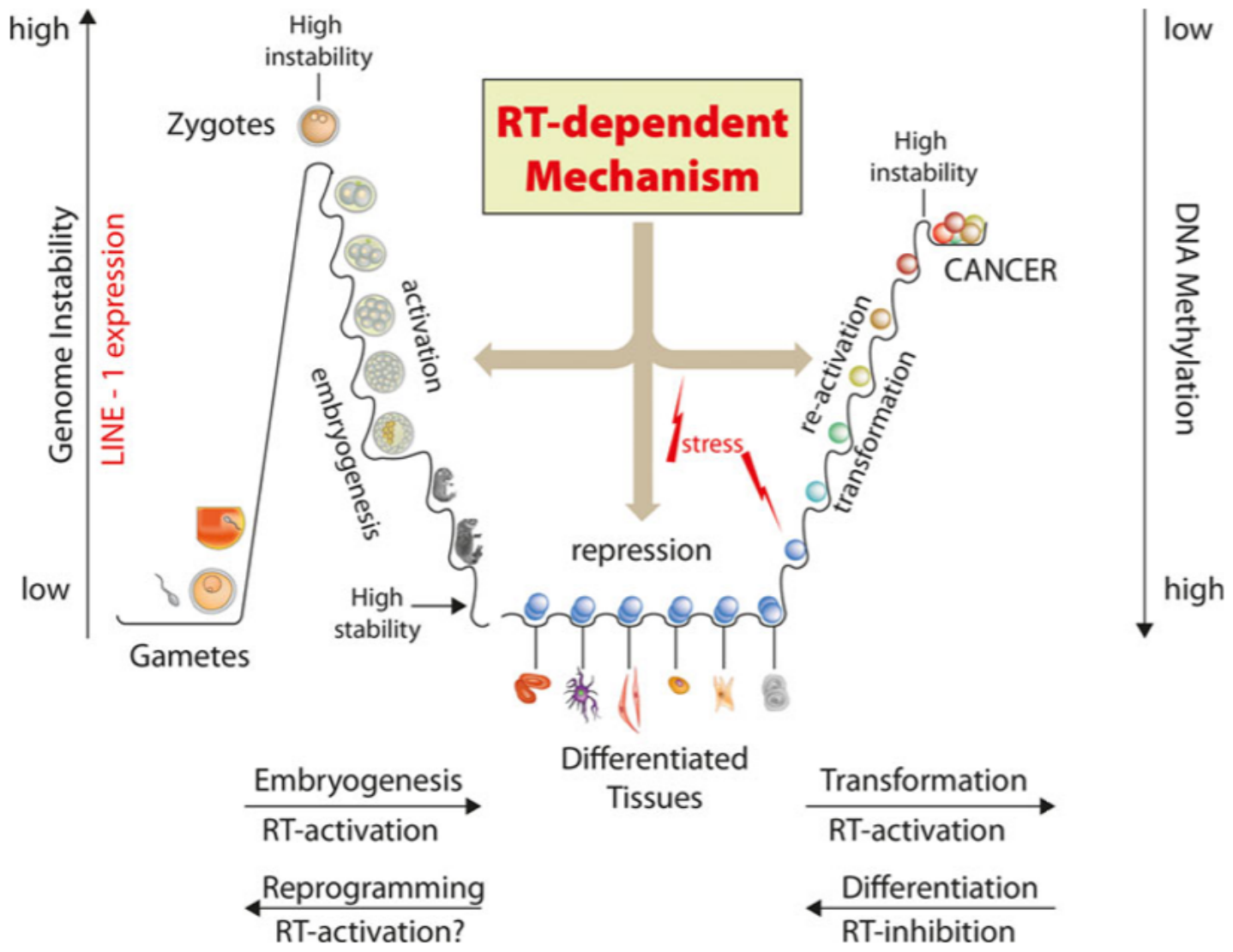
In light of these examples, we believe that the partial activity patterns are reflective of the pseudogene evolutionary process, where a pseudogene may be in the process of either resurrection as a ncRNA or gradually losing its functionality. Understanding why pseudogenes show partial activity may shed light on pseudogene evolution and function.

PSEUDOGENE lncRNAs EXAMPLE 1

Pseudogene lncRNA that controls embryonic stem cell Self-renewal

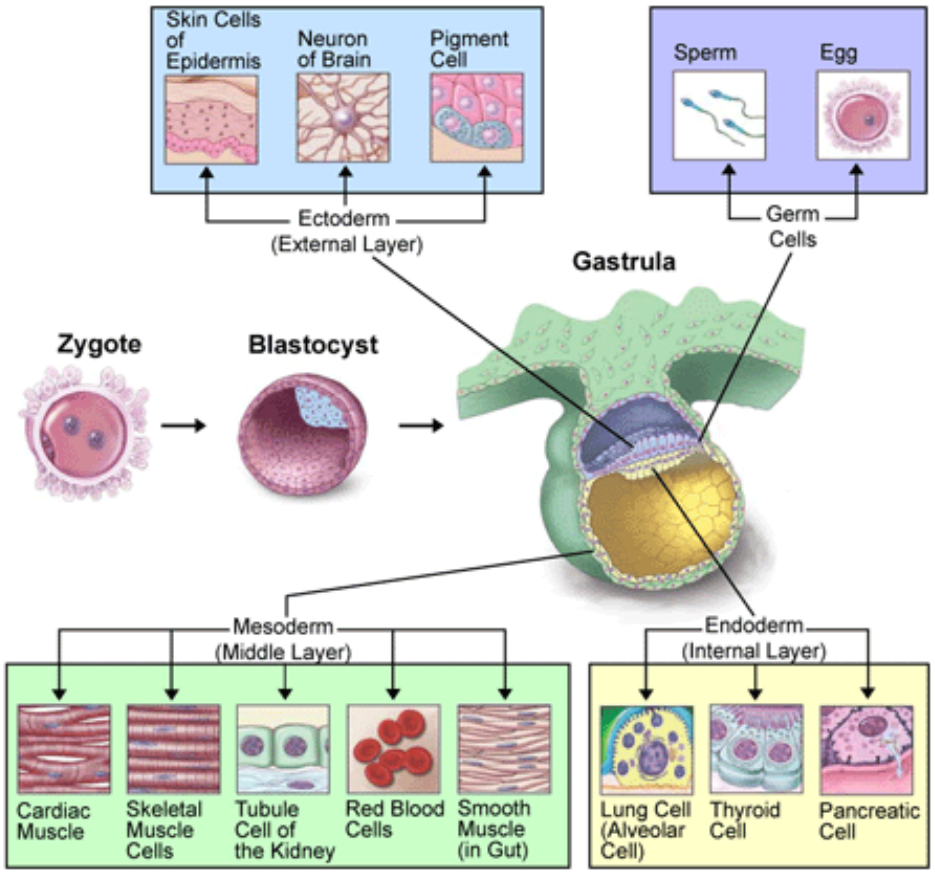
A Oct4P4 pseudogene derived lncRNA silences the ancestral Oct4 gene in trans

Retrotransposon activity during development

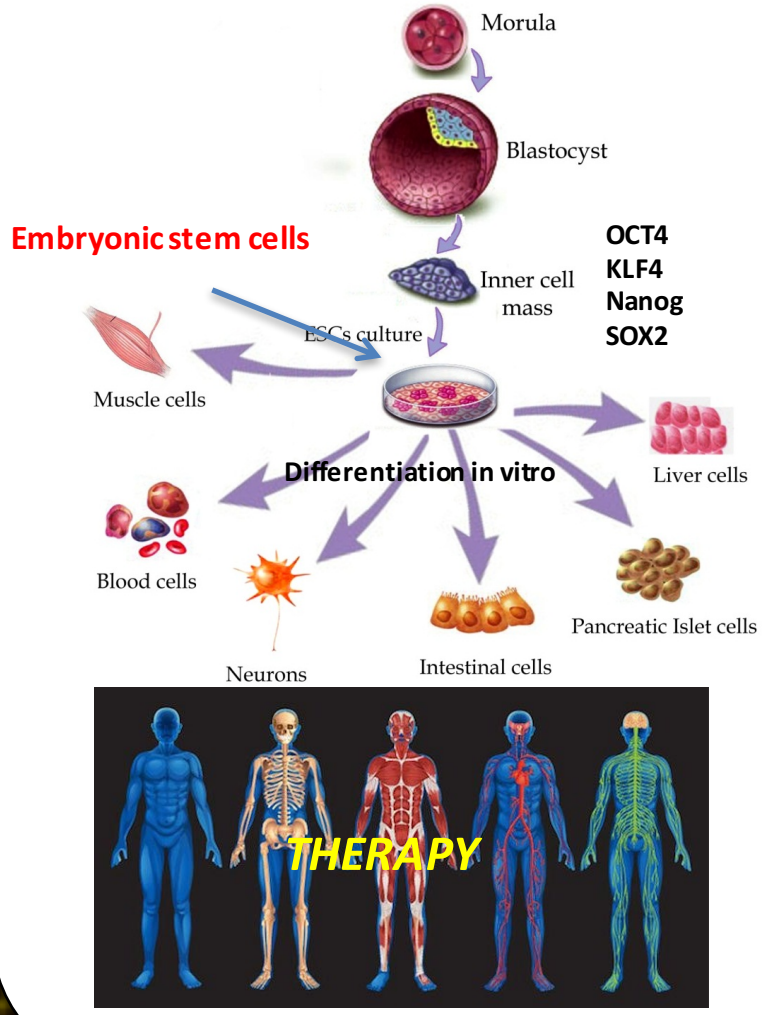


The inner cell mass of the blastocyst are the source of pluripotent embryonic stem cells

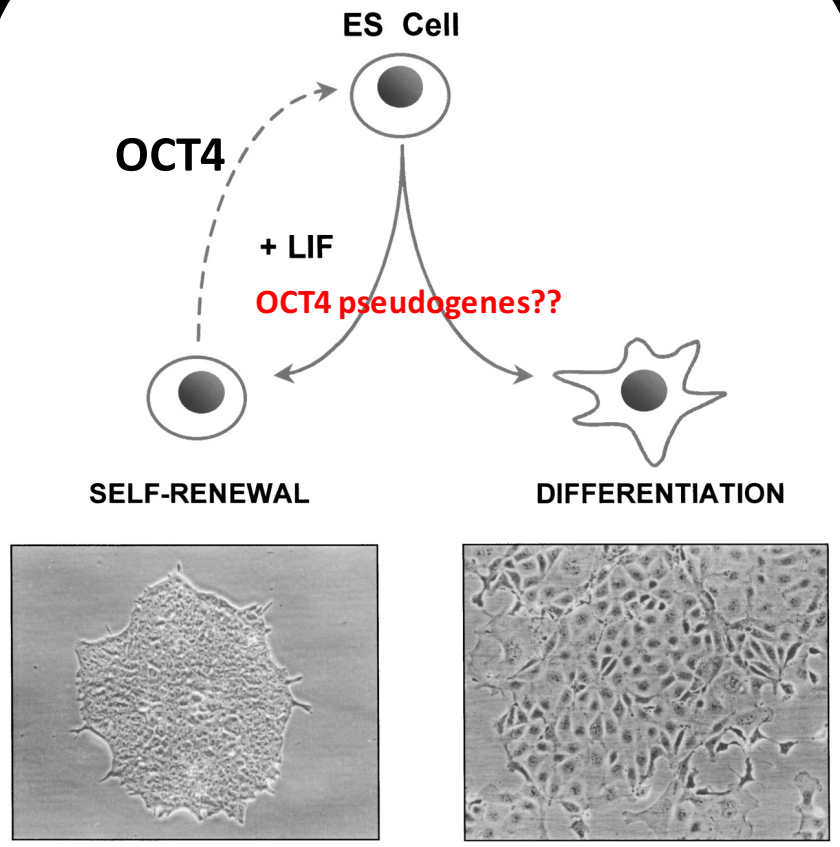
Blastocyst cells give rise to all organs and cell types

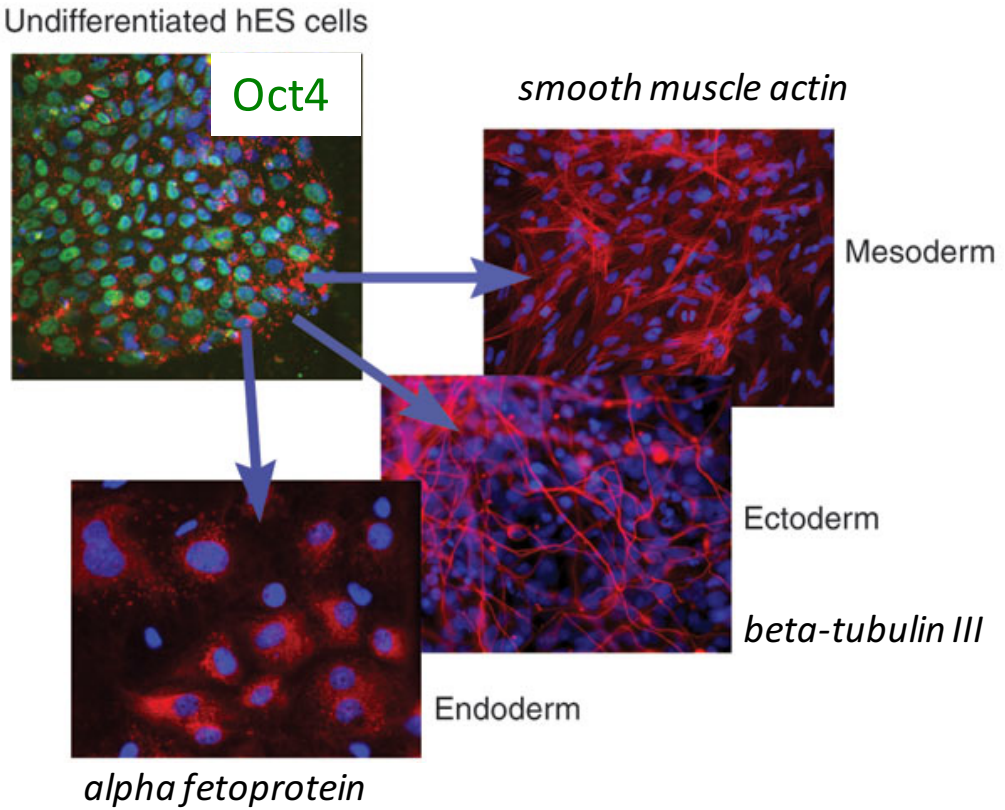


Blastocyst cells can be Isolated and cultivated

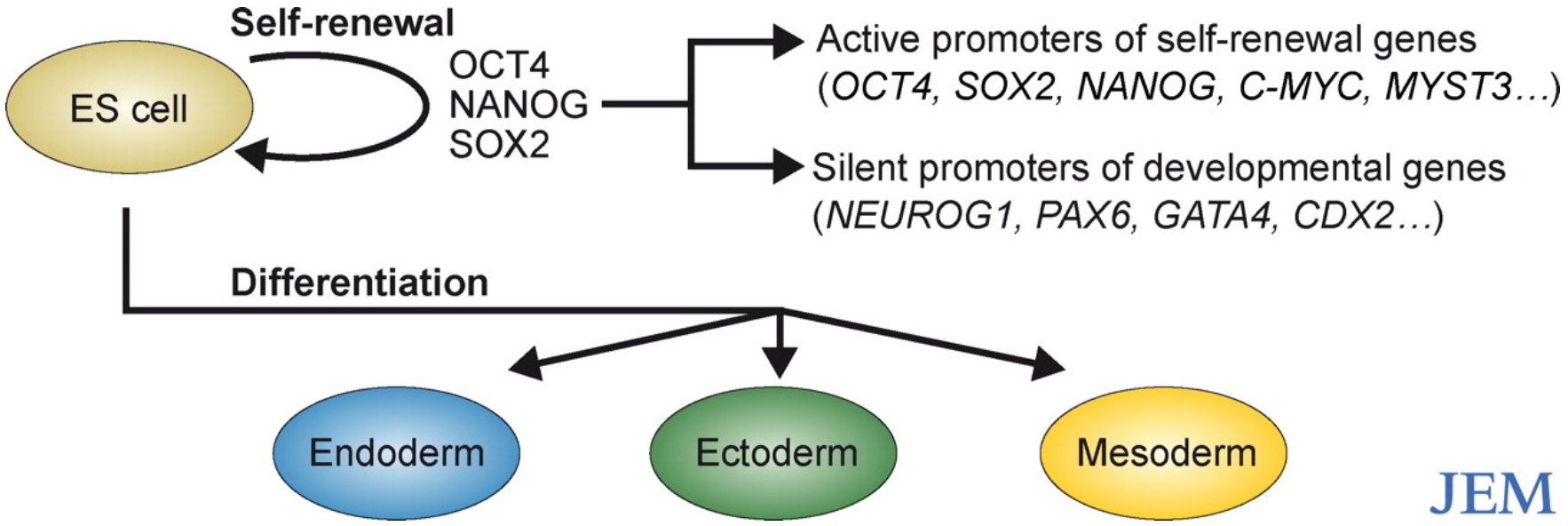


OCT4 expressing ES cells have self-renewing and differentiation potential in vitro



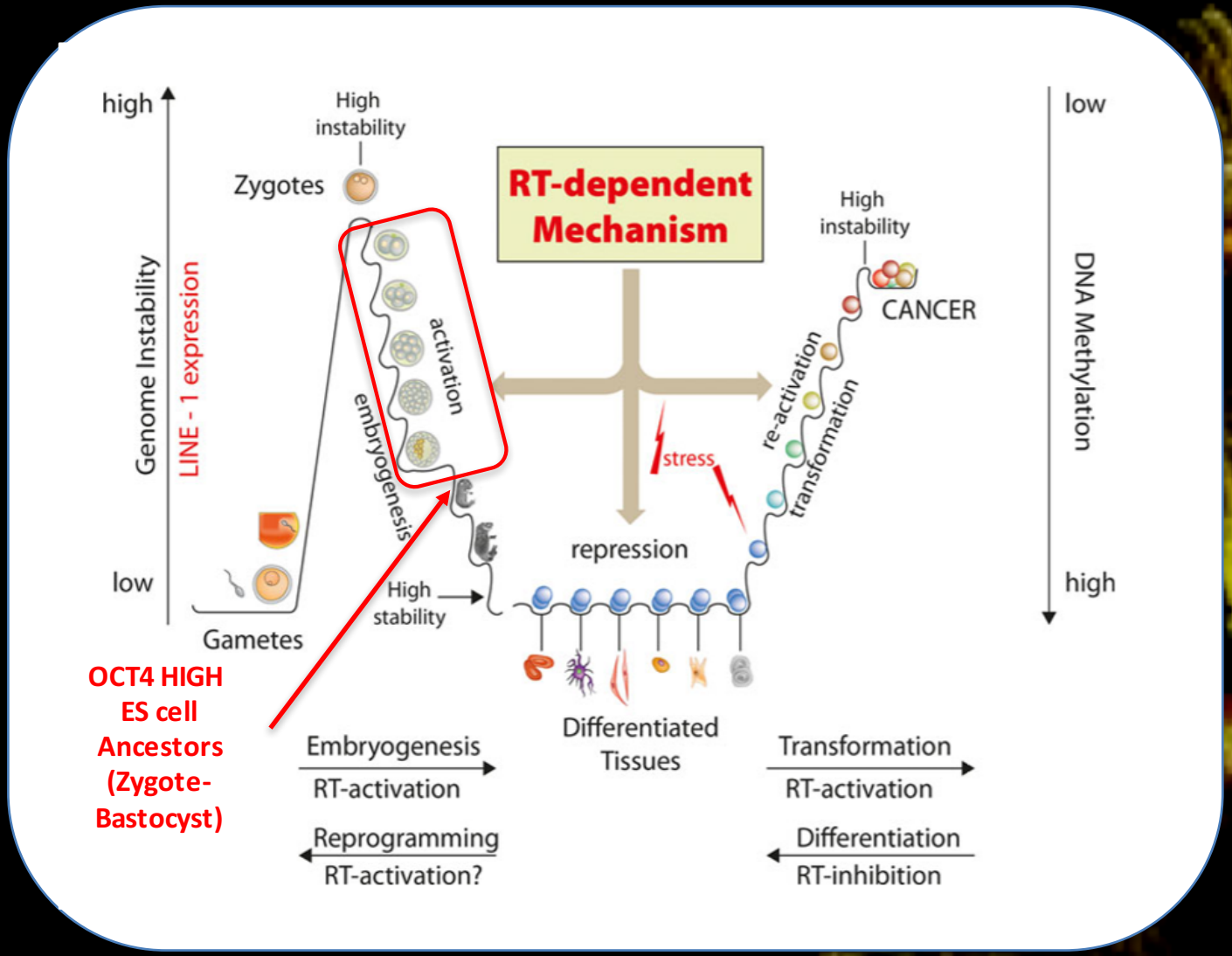


The self-renewal transcription factor Oct4 is essential for embryonic stem cell self-renewal



Nicolaj Strøyer Christophersen, and Kristian Helin J Exp Med 2010;207:2287-2295

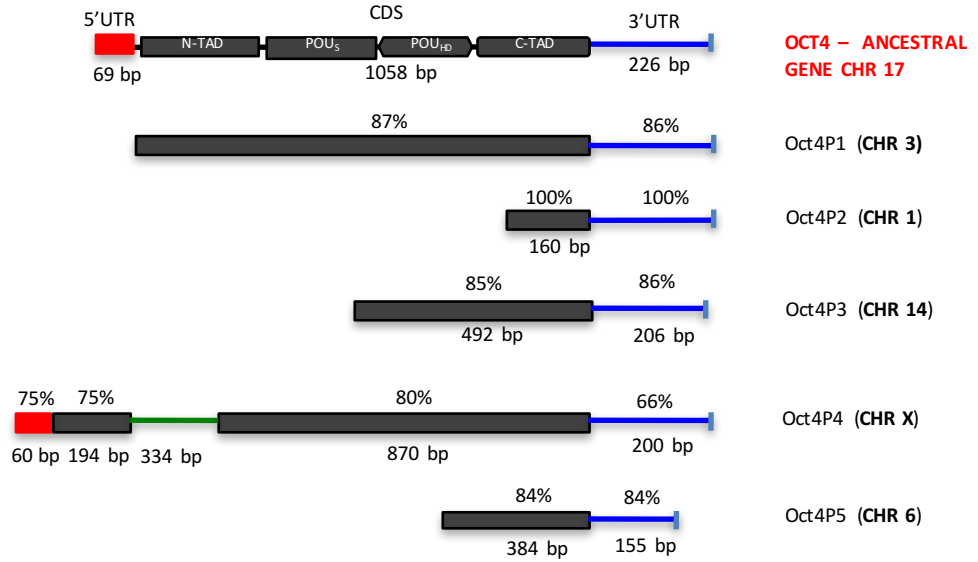
Mouse and human contain several processed OCT4 pseudogenes

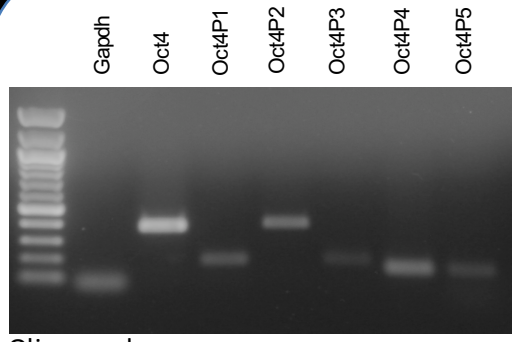


Human:
1 ancestral OCT4
7 processed OCT4 pseudogenes

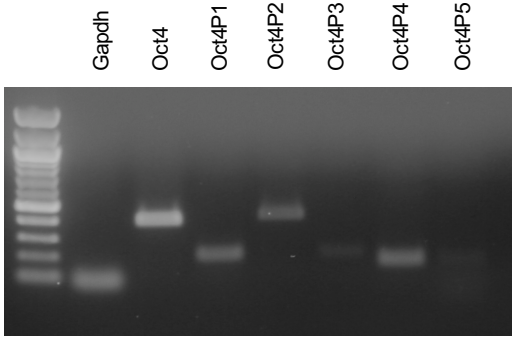
Mouse:
1 nacestral Oct4
5 processed OCT4 pseudogenes

Ancestral OCT4 gave rise to 5 processed pseudogenes that are expressed in mESCs



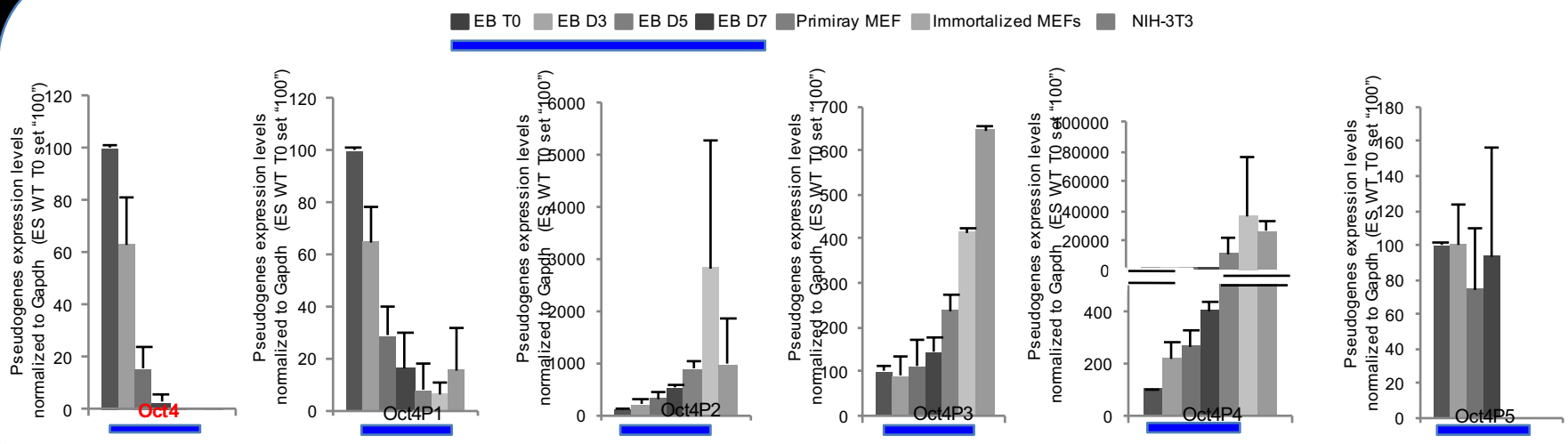


Oligo random

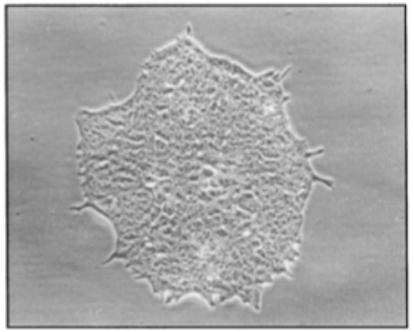


Oligo dT

Oct4 pseudogenes are tightly controlled during the differentiation of mESCs

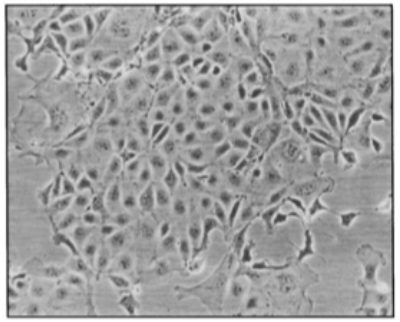


SELF-RENEWAL



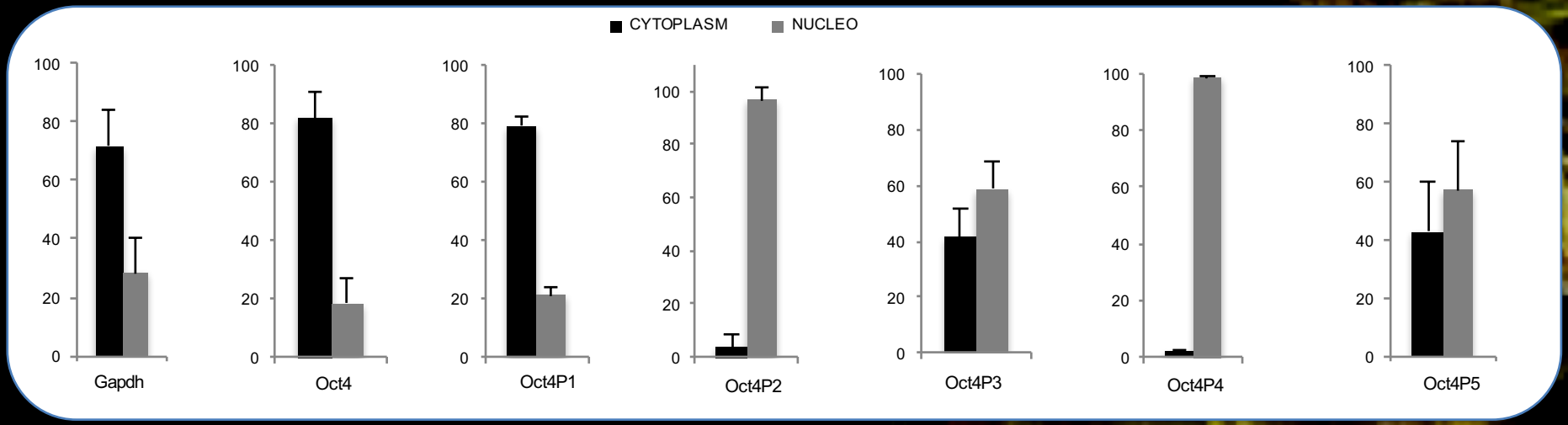
Oct4
Oct4P1 (-10X)

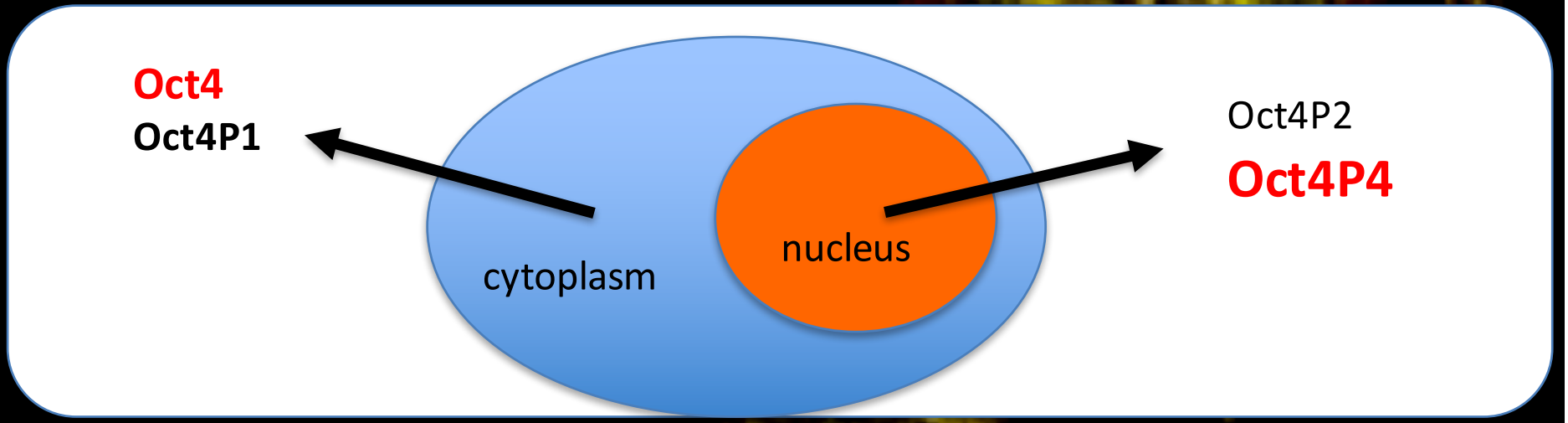
DIFFERENTIATION



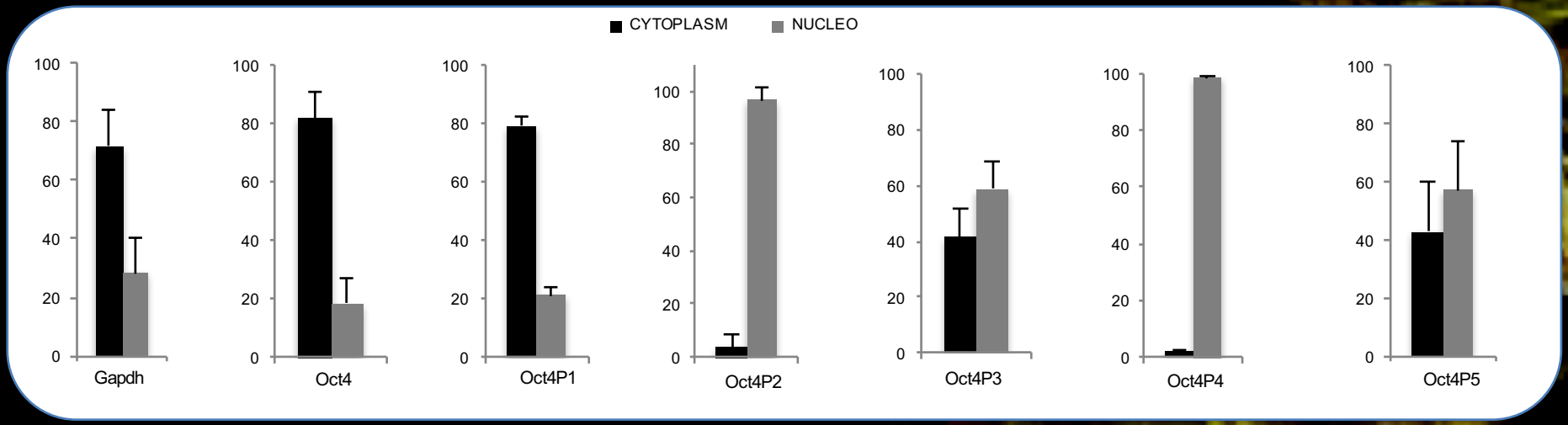
Oct4P2 (+9x)
Oct4P3 (+2x)
Oct4P4 (+4x; Fiborbl. +200x)

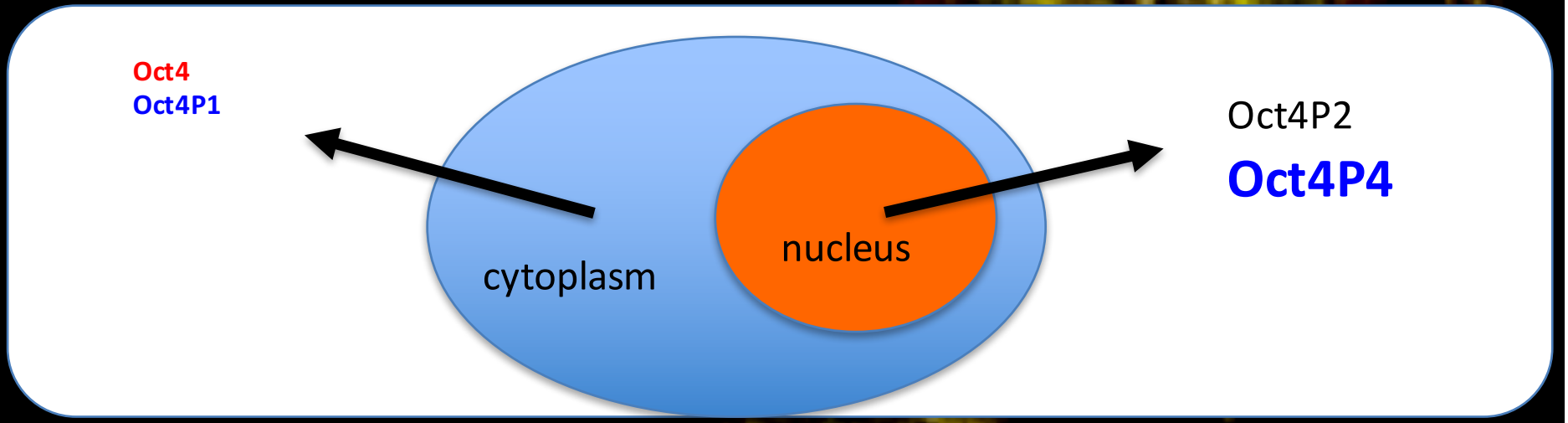
OCT4 pseudogenes are localized to nuceloplasm or cytoplasm



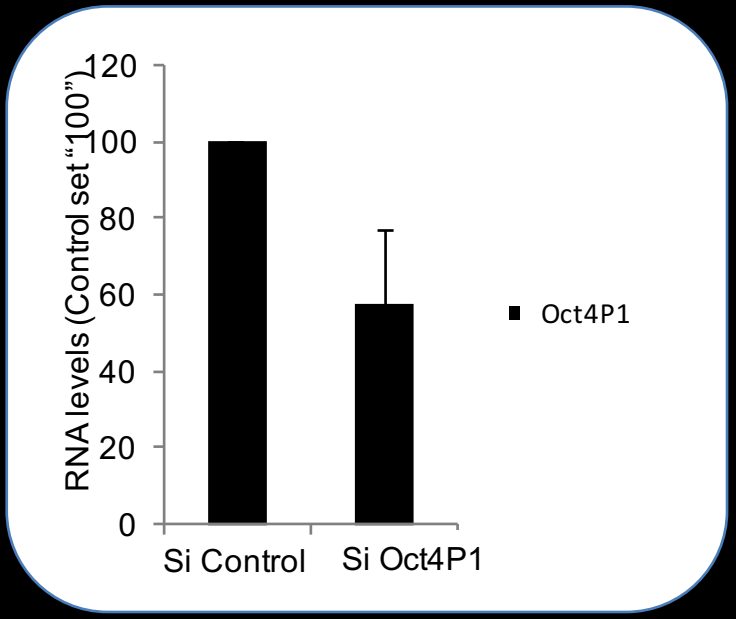


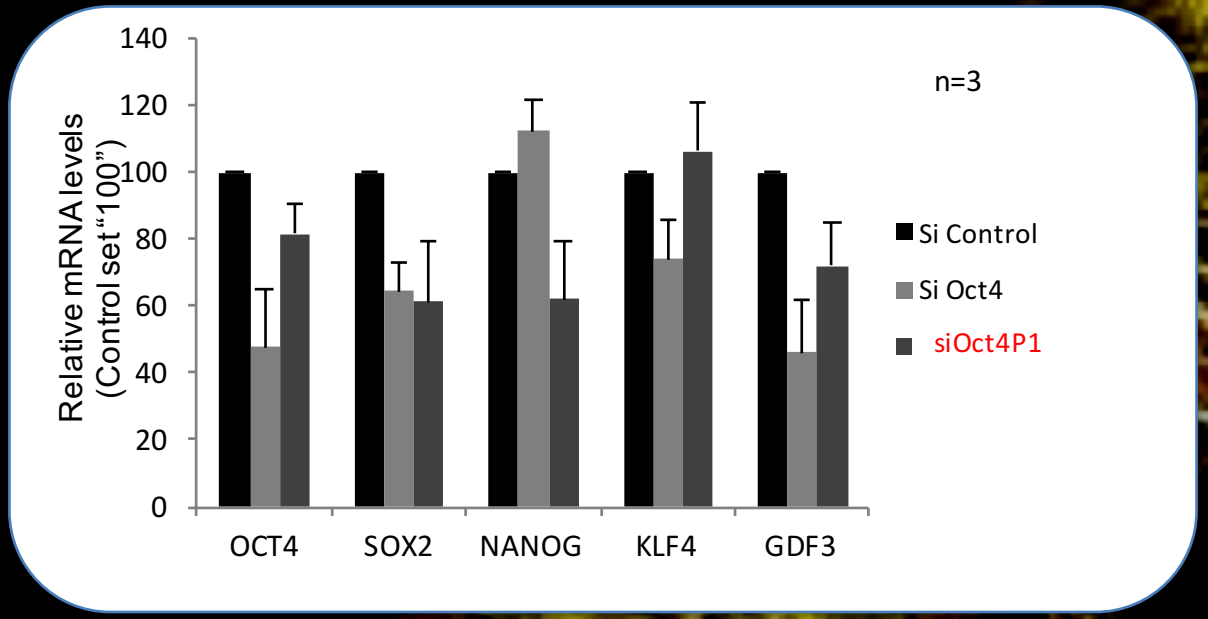
OCT4 pseudogenes are localized to nuceloplasm or cytoplasm

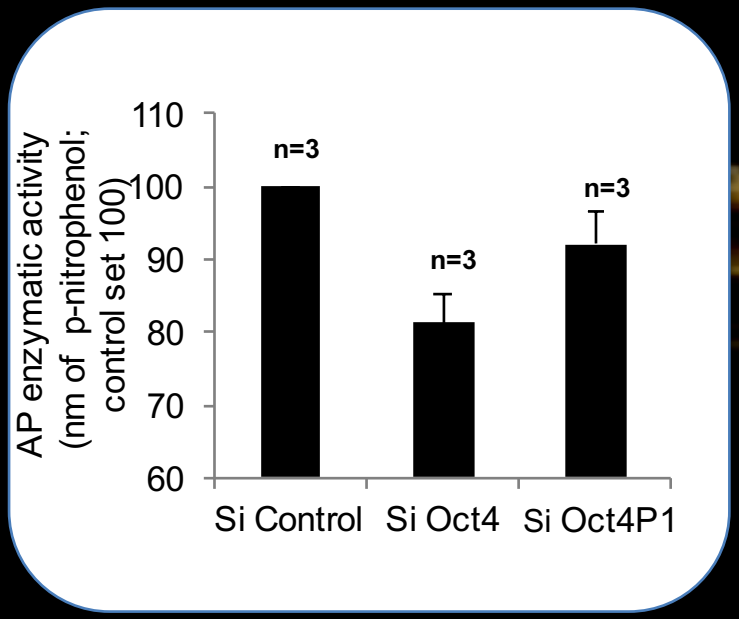


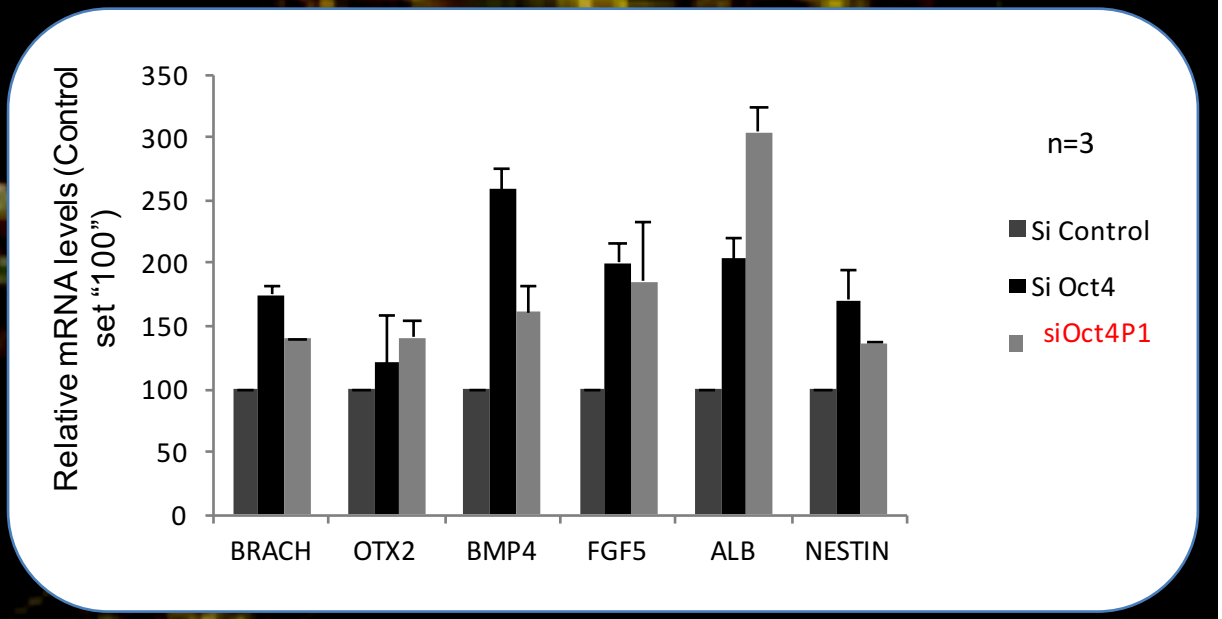


Cytoplasmatic OCT4P1 promotes mESC self-renewal

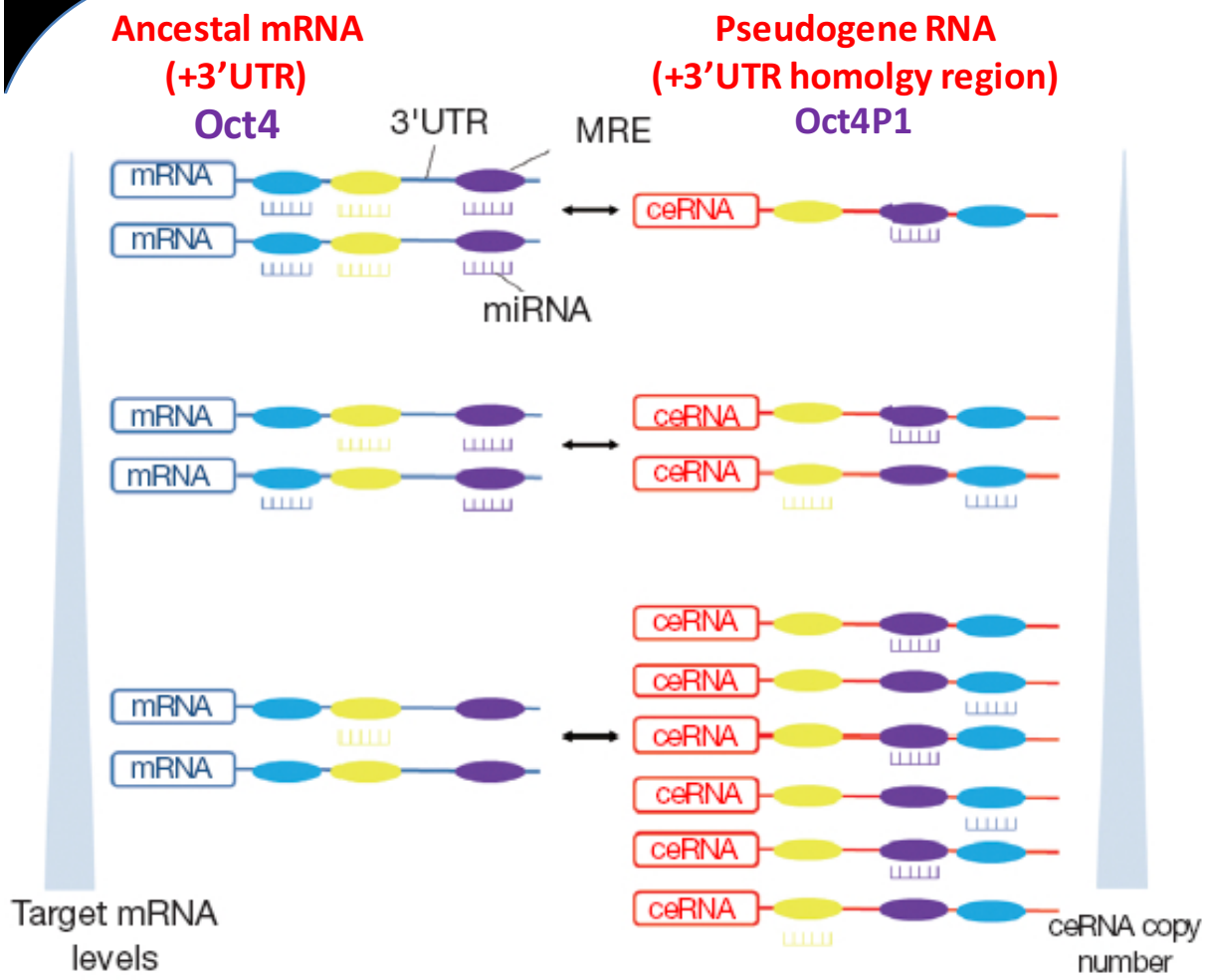








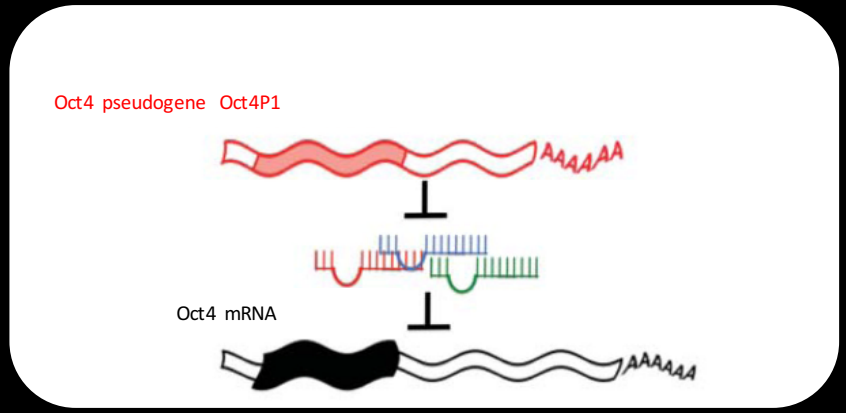
Pseudogene sponge miRNAs that target the ancestral gene

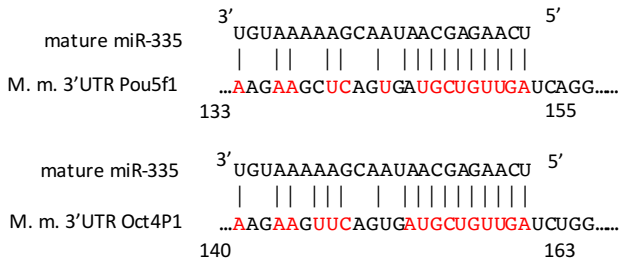


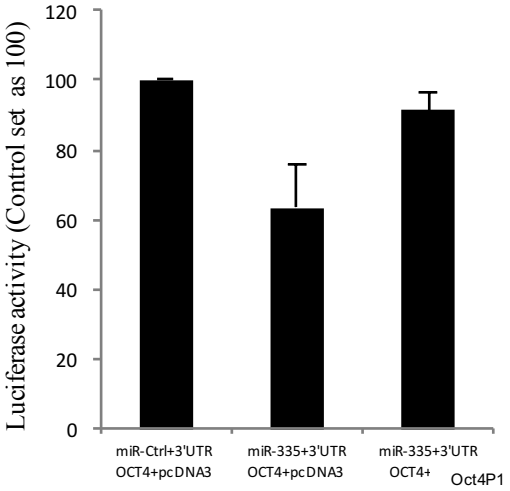
miRNA that targets Oct4: i.e. miR-335
MRE: miRNA response element

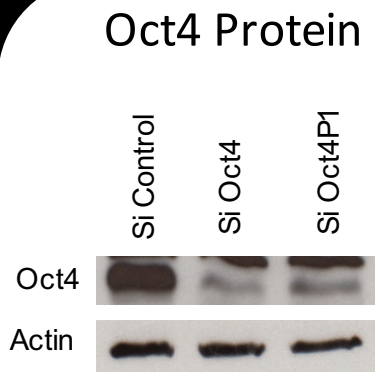
Evolution of pseudogenes to fine-tune the expression of ancestral genes

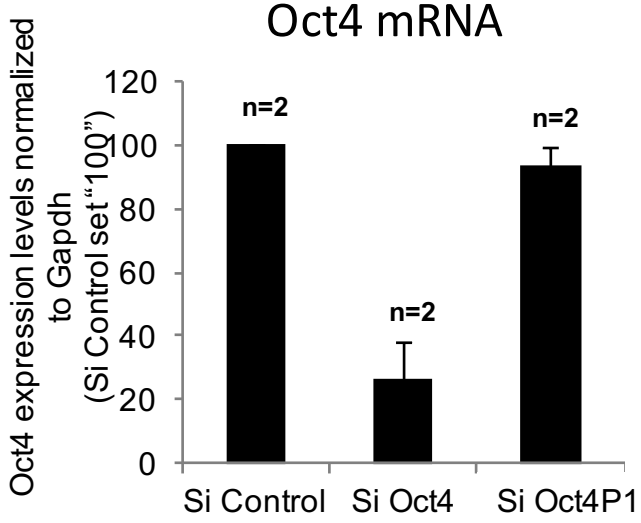
Cytoplasmatic OCT4P1 acts as Oct4/Rb1 ceRNA







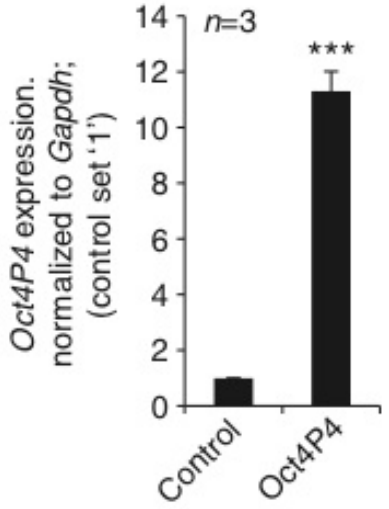


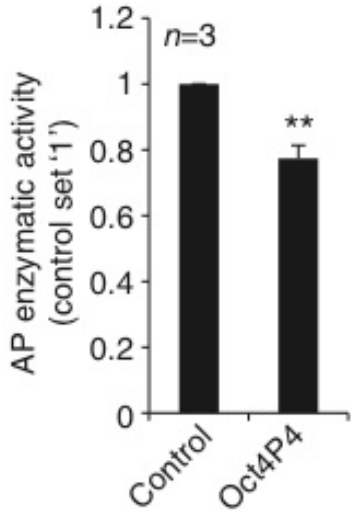


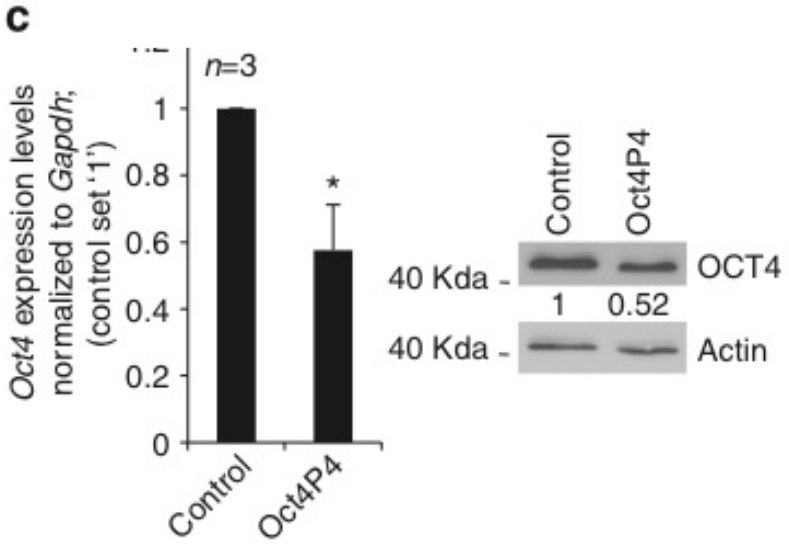
OCT4P1 promotes self-renewal by sponging miRNAs that target Oct4

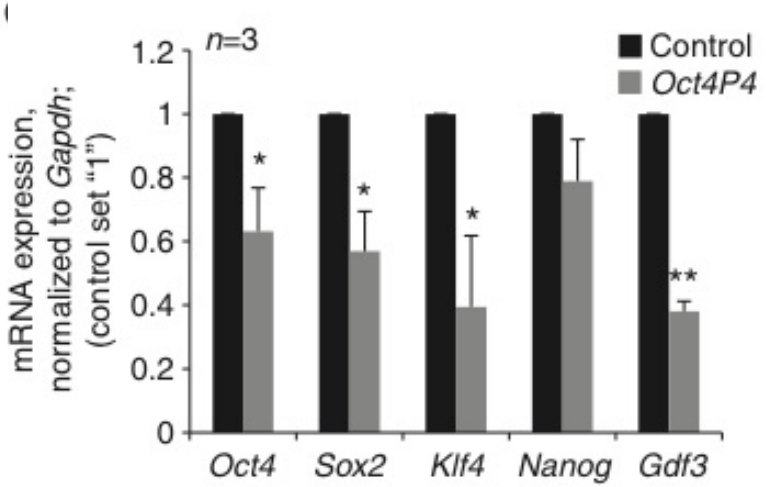
Nuclear OCT4P4 promotes mESC differentiation

Oct4P4 ⊣ Oct4

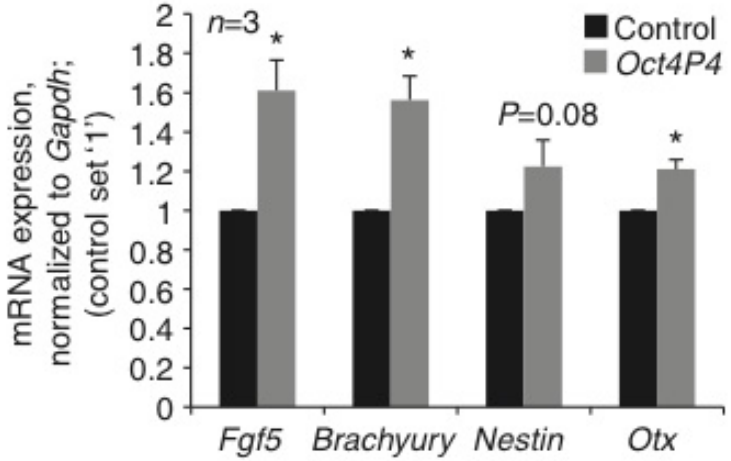






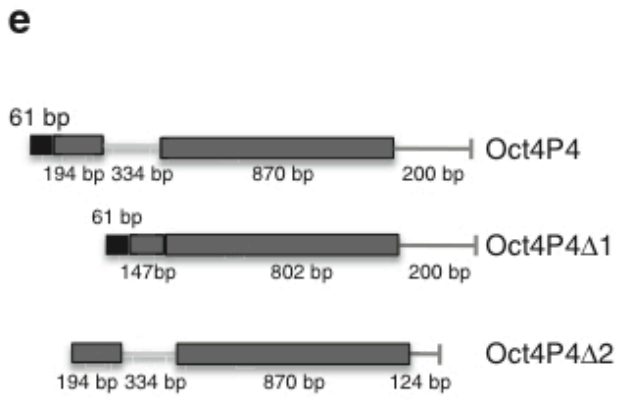


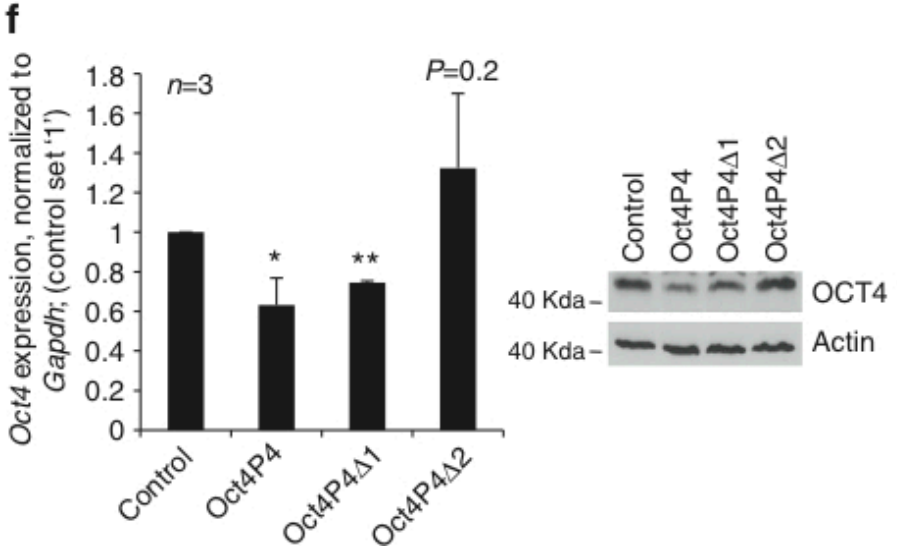
Self-renewal genes

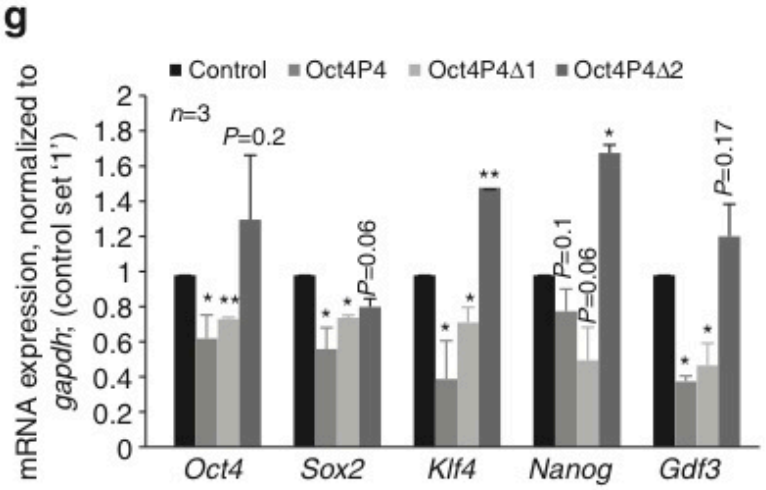


Differentiation genes

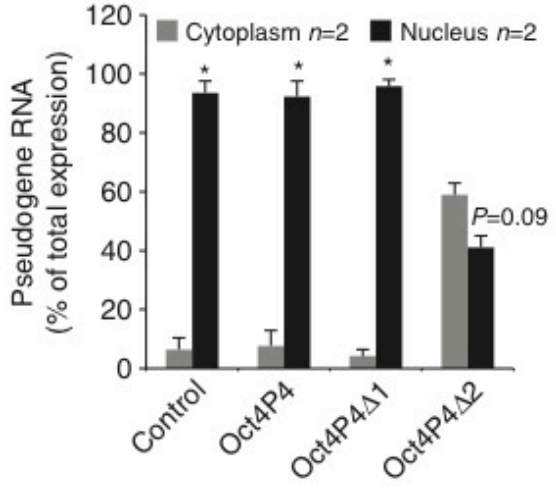
5′ and 3′ UTR homolgy domains are required to repress self-renewal marker genes



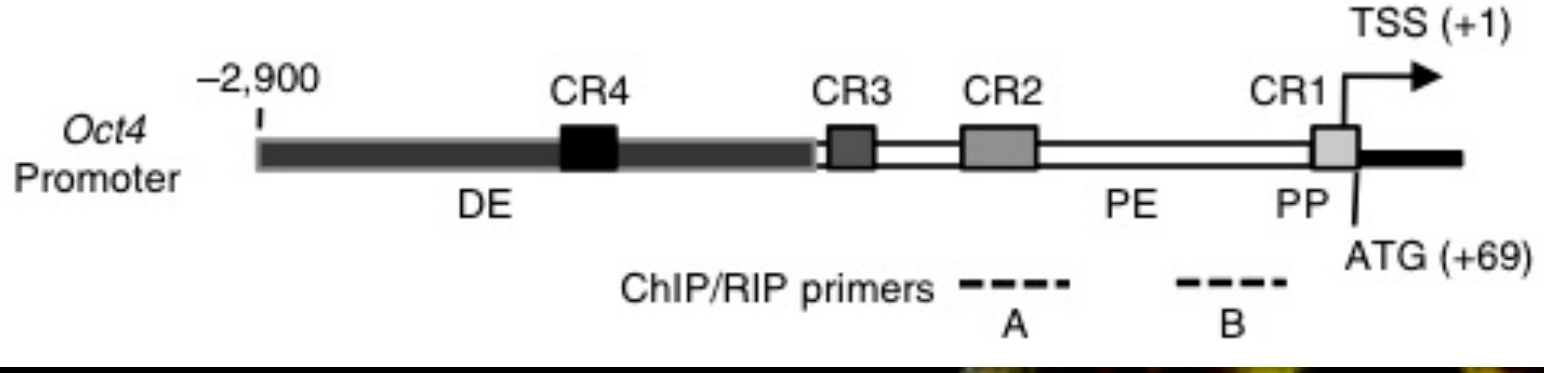


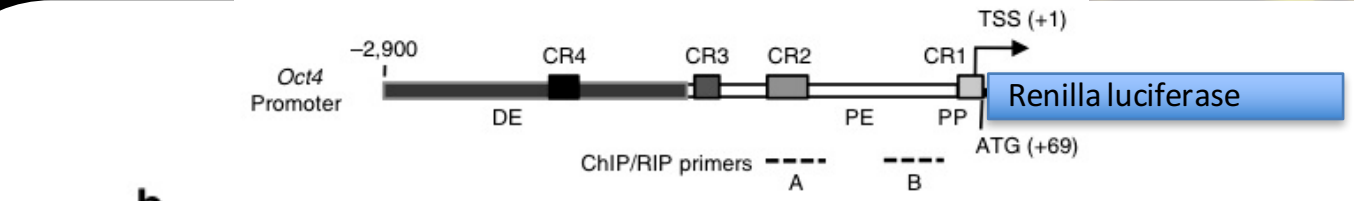


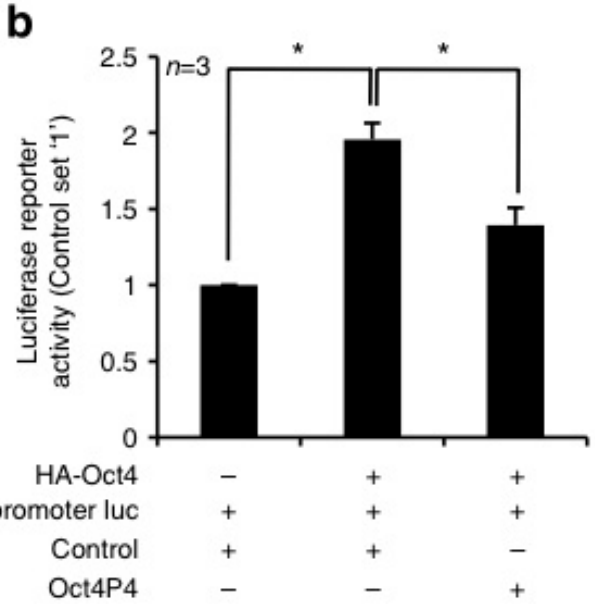
Self-renewal genes

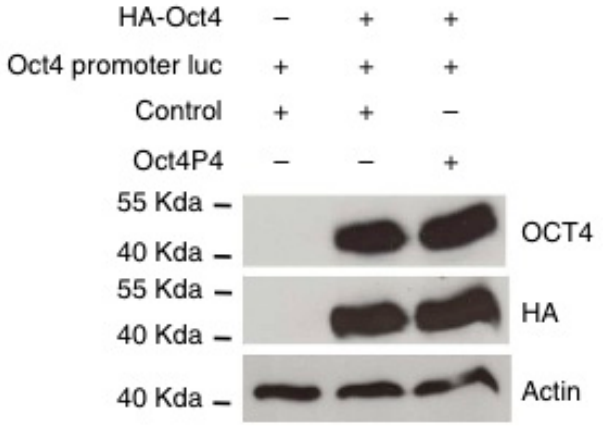


Oct4P4 interferes with the ancestral Oct4 promoter

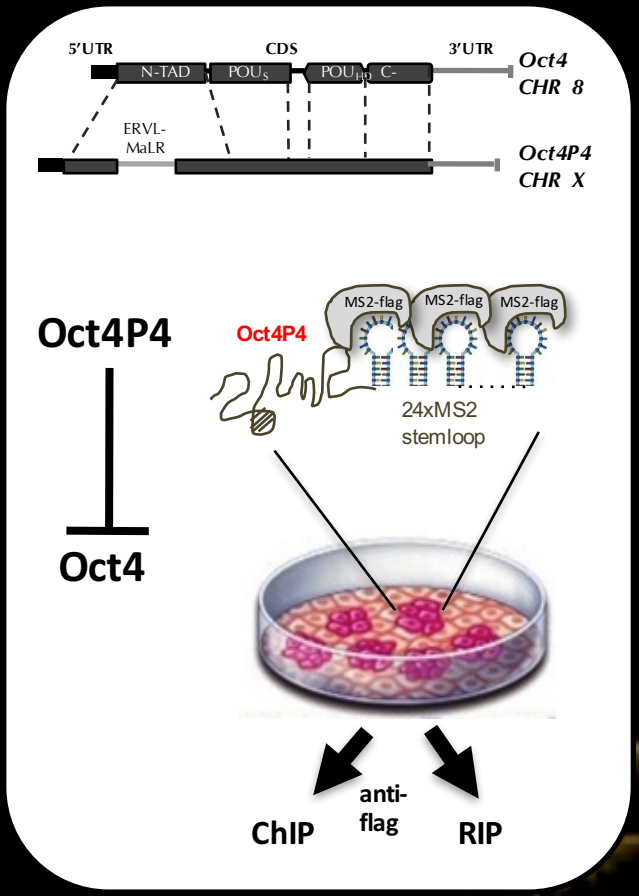




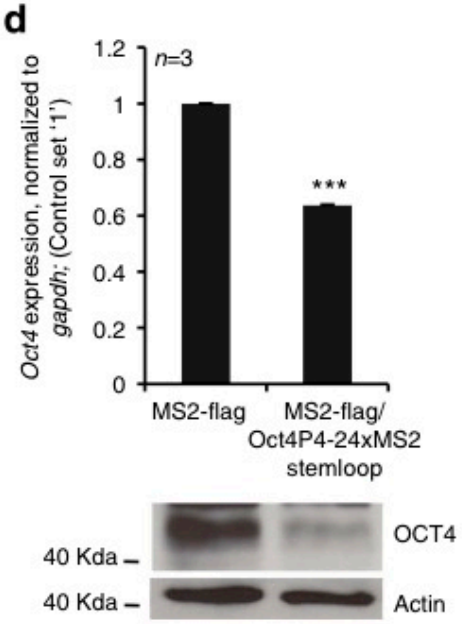


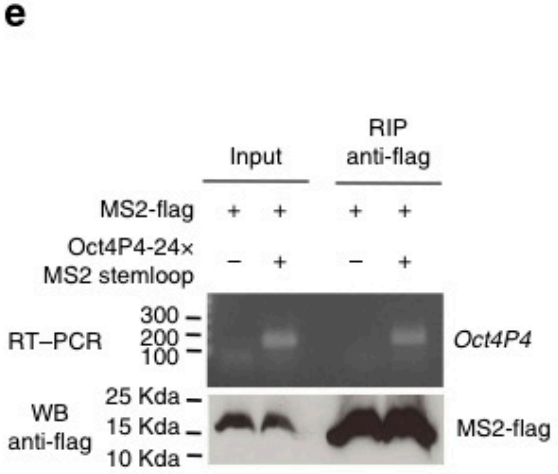


A model system to study Oct4P4 lncRNA localization



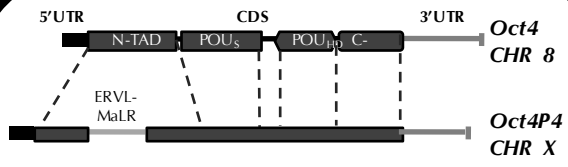
RNA immunoprecipitation anti-flag then RT-PCR for Oct4P4 lncRNA

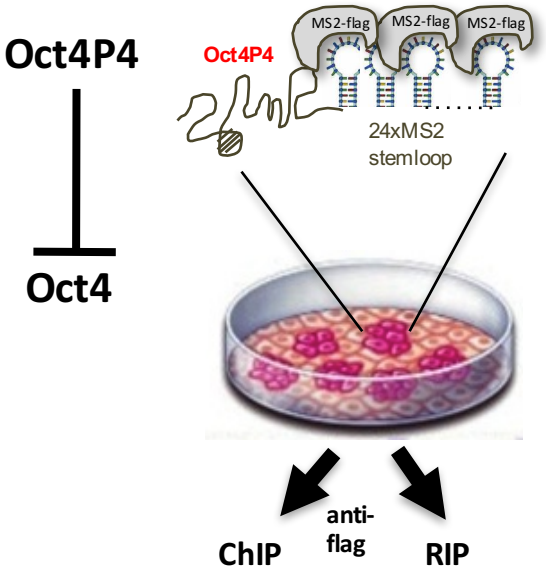




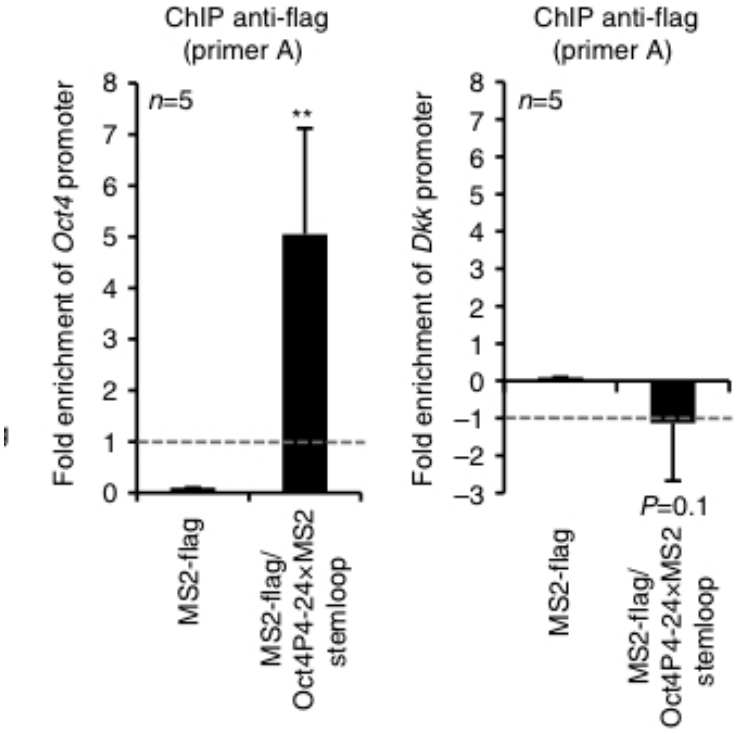
MS2 stem loop tagged Oct4P4 co-expressed with flag-MS2

A model system to study Oct4P4 lncRNA localization





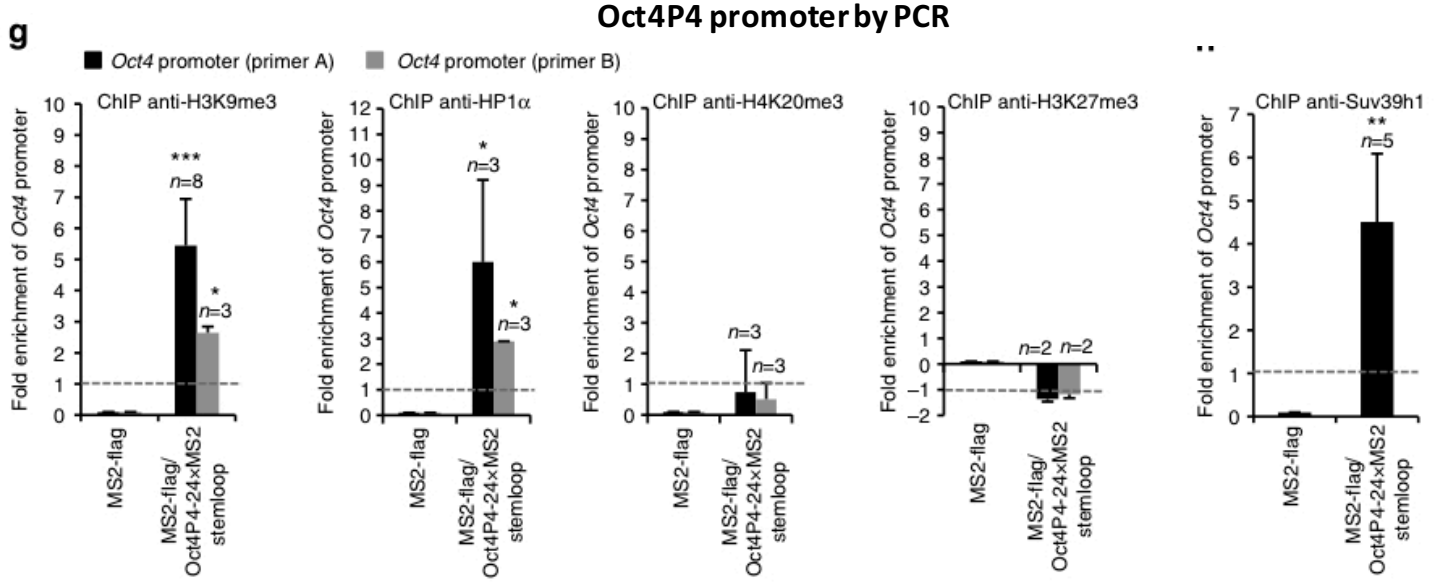
ChIP using anti-flag then use the immuno-precipitate to detect the Oct4P4 promoter by PCR

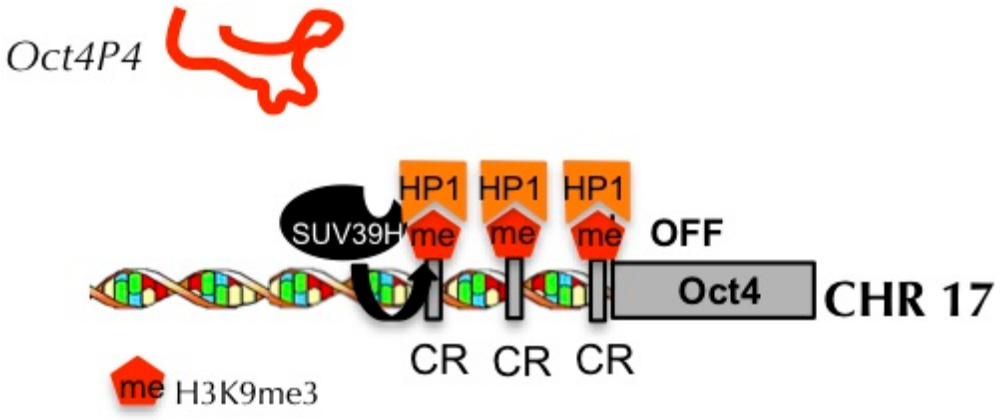


Oct4P4-MS2 lncRNA localizes to Oct4 promoter

Oct4P4-MS2 directs Suv39h1 to Oct4 promoter

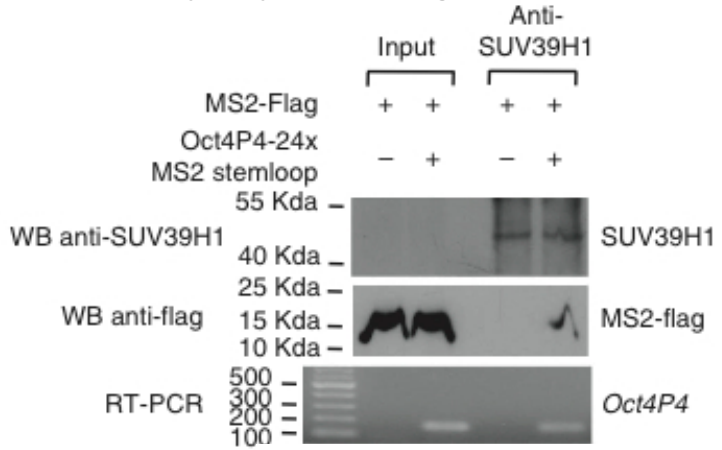
ChIP using specific antibodies then use the immuno-precipitate to detect the Oct4P4 promoter by PCR



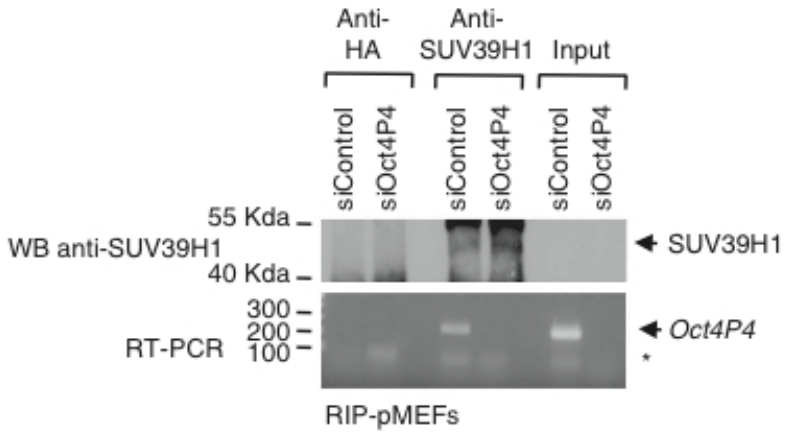


Oct4P4-MS2 directly interacts with Suv39h1

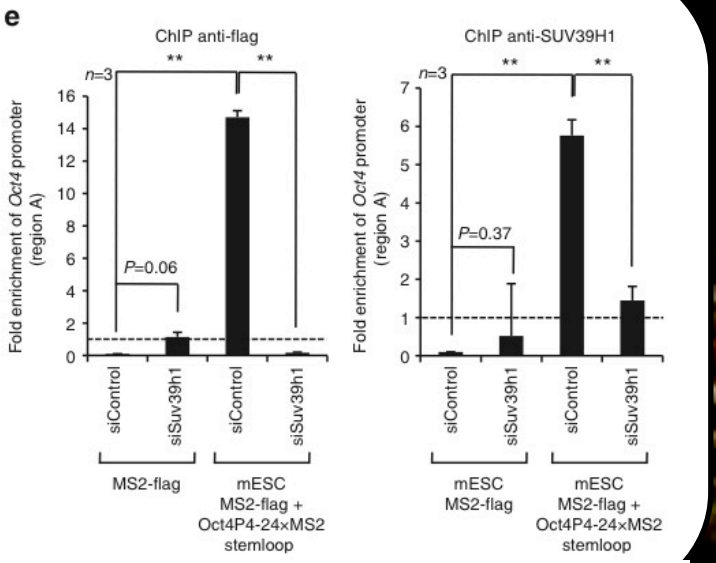
RNA immunoprecipitation using anti-SUV39h1;



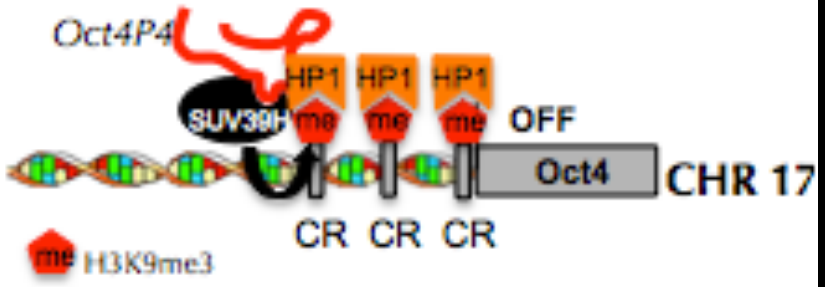
Western for MS2-flag



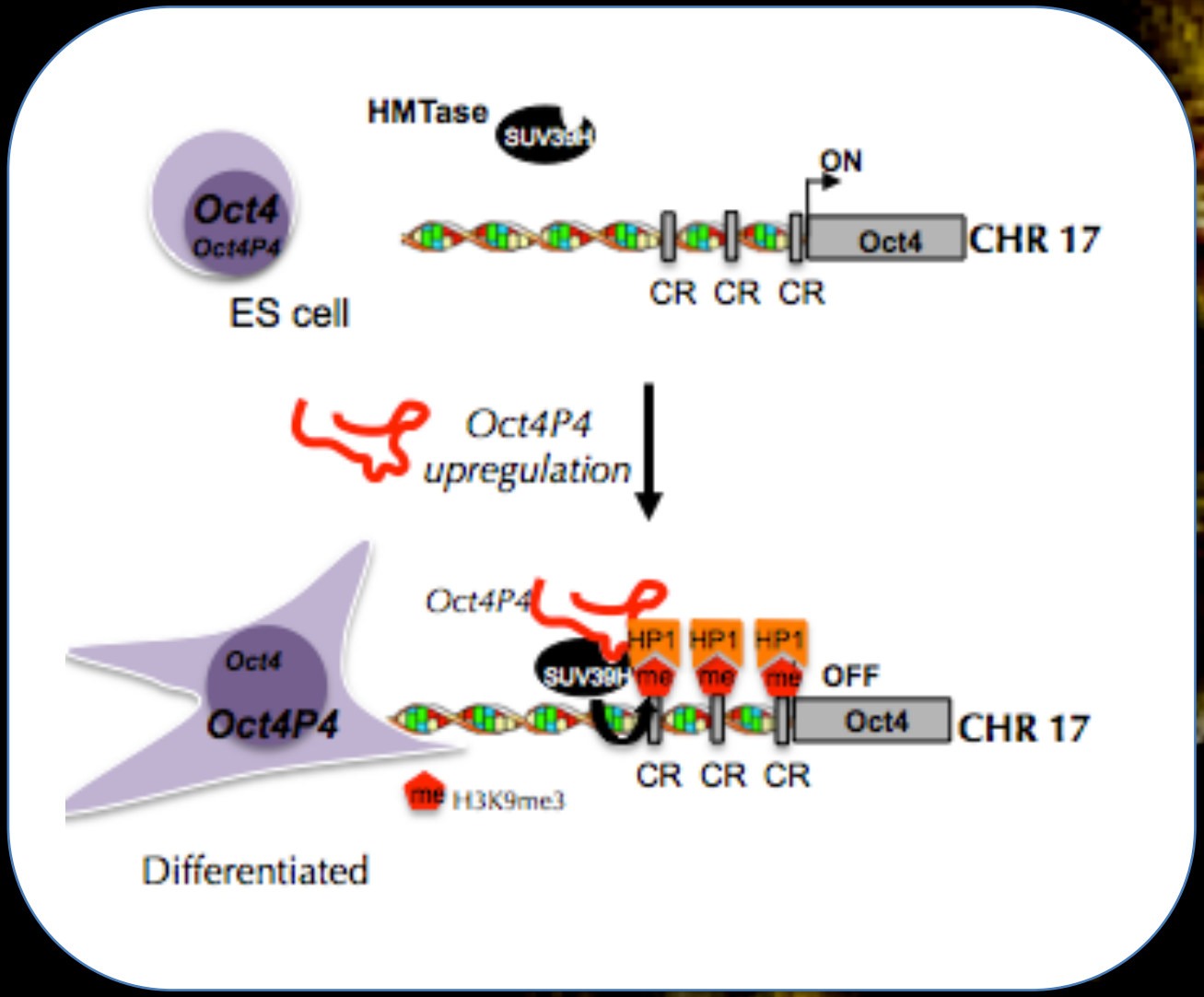
RT-PCR for Oct4P4 lncRNA



ChIP using specific antibodies then use the immuno-precipitate to detect the Oct4P4 promoter by PCR



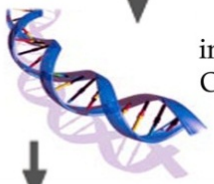
Oct4P4-MS2 recruits Suv39h1 To direct silencing of the Oct4 promter



REVERSIBILITY???

INDUCING PLURIPOTENCY IN ADULT CELLS

Adult cells

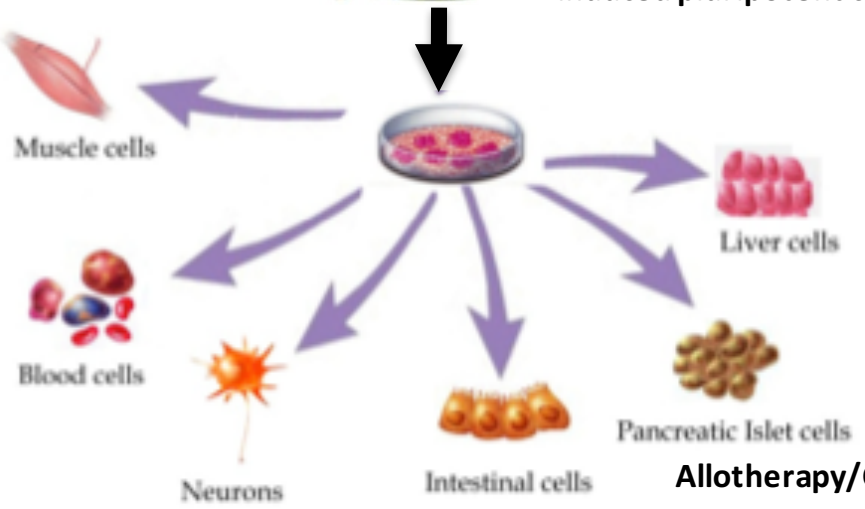


Genes inserted to
induce reprogramming
Oct4, Klf4, Sox2, c-Myc

Reprogram into
ES like-cells

iPS cells

=induced pluripotent cells



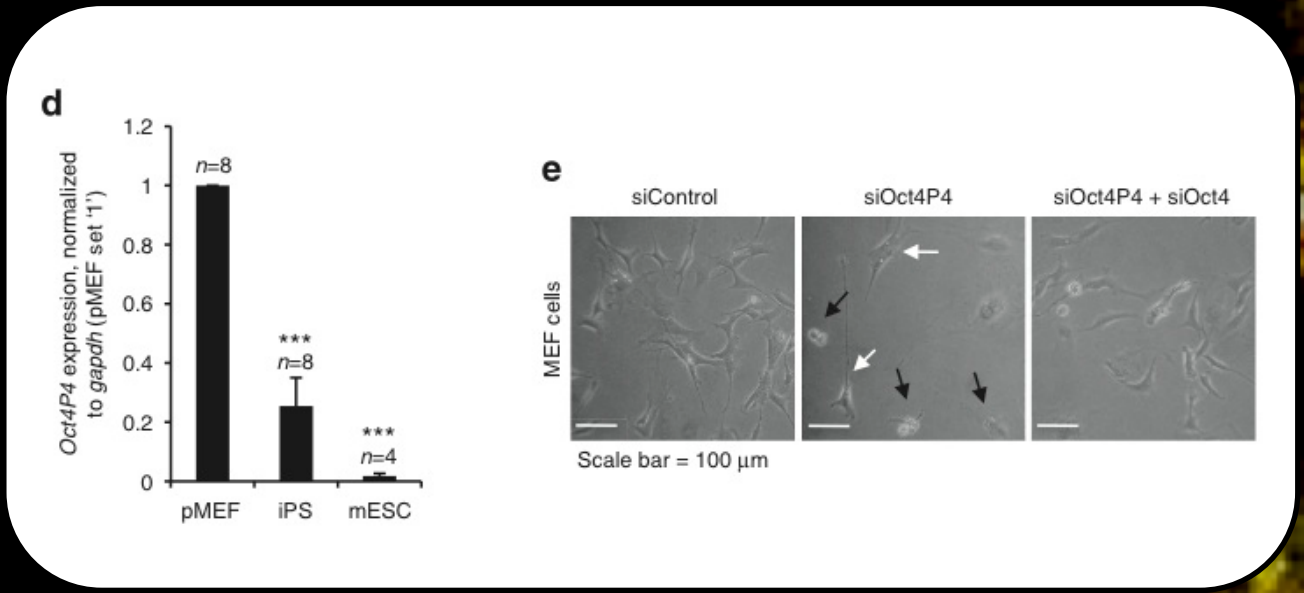
Allotherapy/Cell therapy

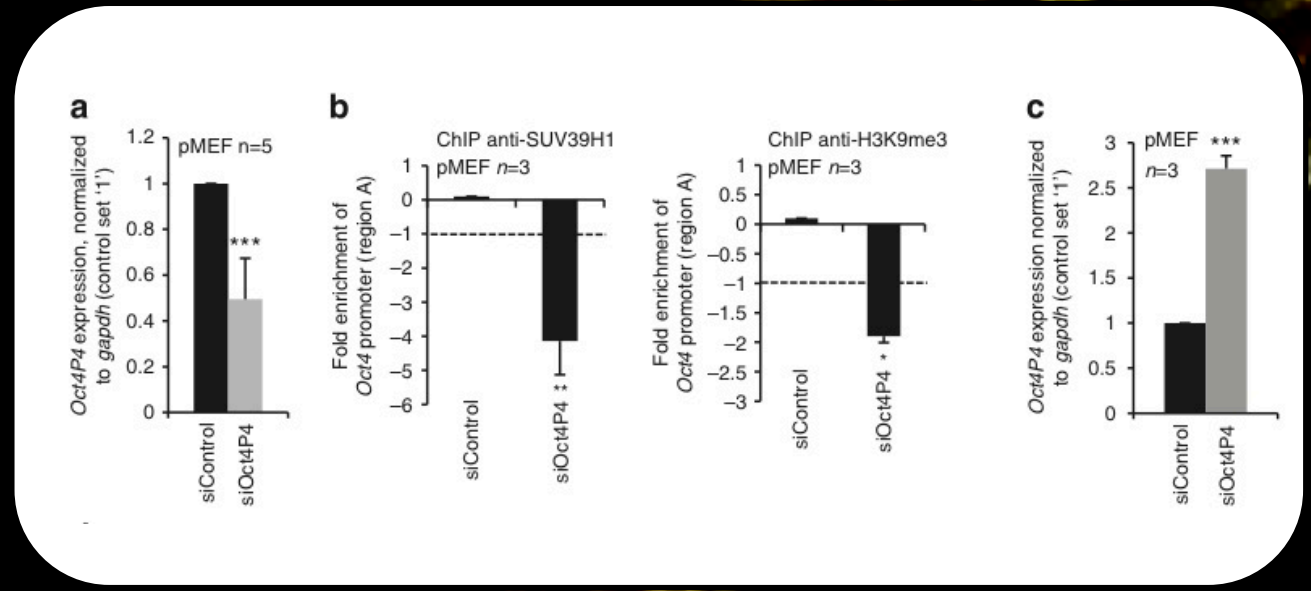
DIFFERENTIATED

UNDIFFERENTIATED
(SELF-RENEWAL)

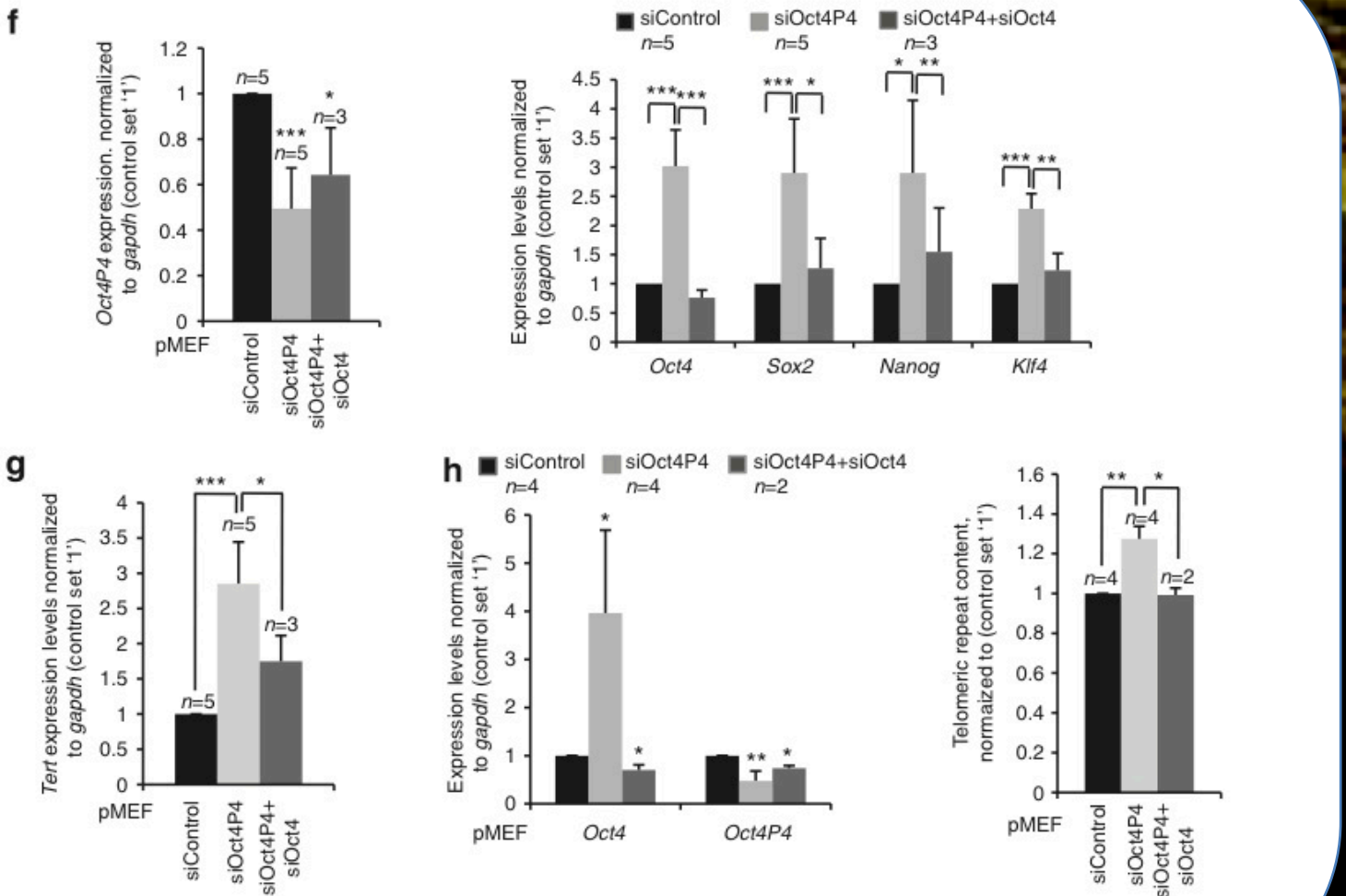
INDUCE CELL
LINES SPECIFIC
DIFFERENTIATION

Oct4P4 depletion in pMEFs causes the re-acquisition of self-renewal features

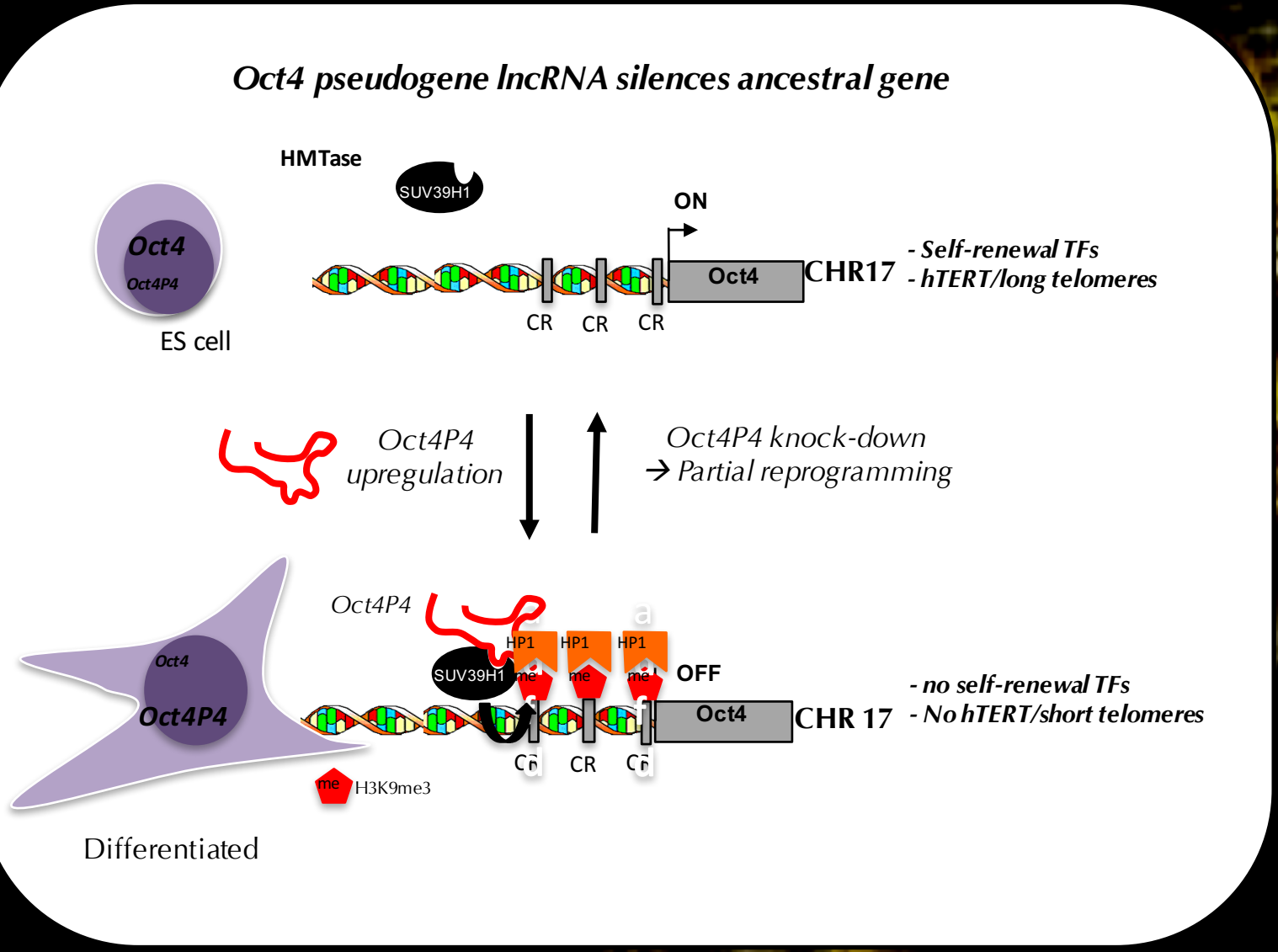
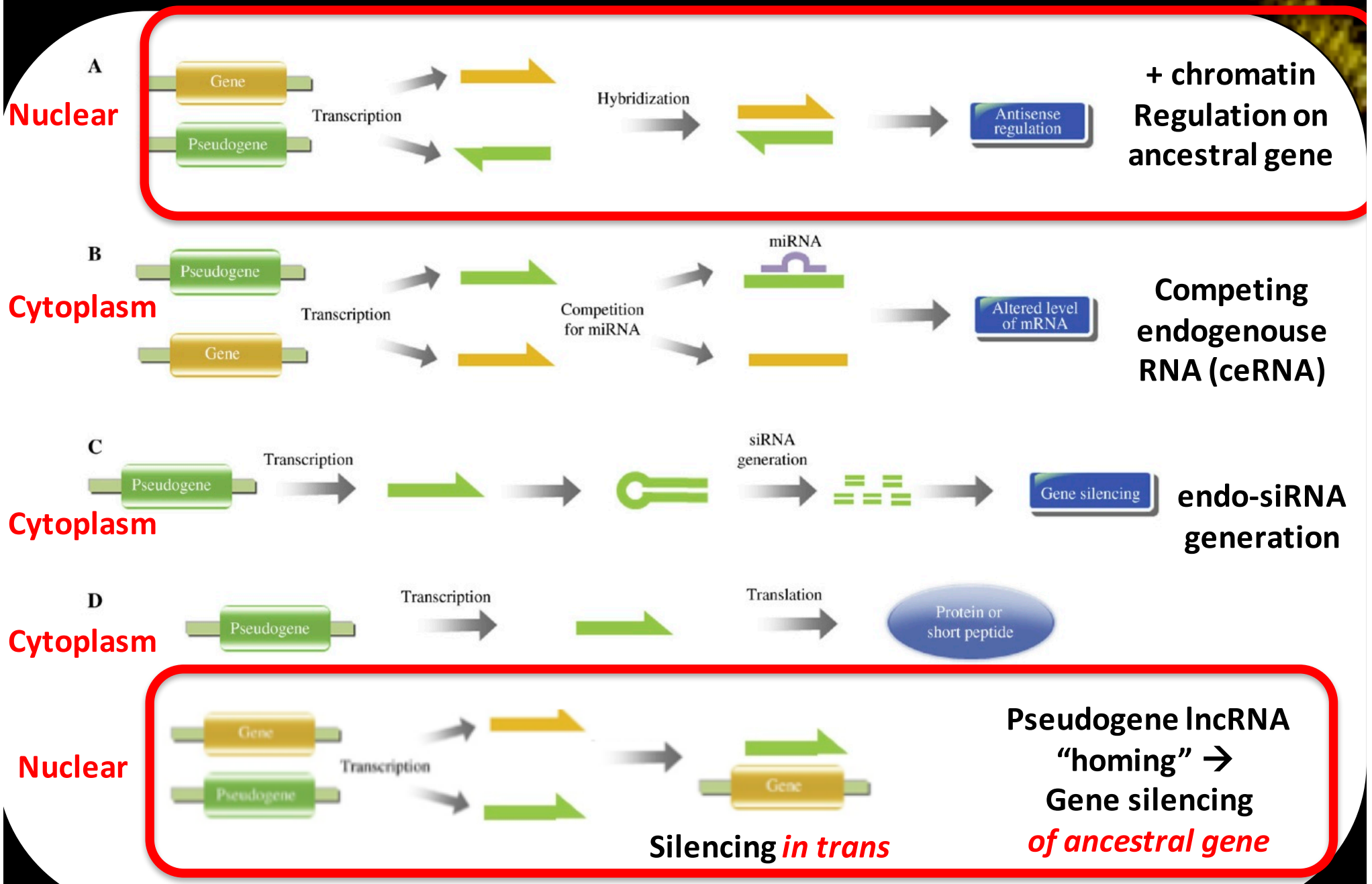




Oct4P4 depletion in pMEFs causes the re-acquisition of self-renewal features



Pseudogenes control the epigenetic status of ancestral genes



seudogenes are powerful regulators of gene expressio