Journal Club:

Epigenetic Reprogramming to Reverse Aging in Mice

Yang et al., 2023, Cell (Laboratory of Prof David Sinclair, Harvard)

The importance of the epigenome*

EPIGENOME = everything that regulates gene activity without changing the DNA sequence: DNA methylation, histone modifications and chromatin accessibility.

- In the 1950s, Szilard proposed that aging is caused by a loss of genetic information due to **mutations** resulting from DNA damage ([Szilard, 1959](#)). *However…*
  - ✓ *Many types of old cells have no mutations*
  - ✓ *Many strains of mice with high mutation rates show no evidence of premature aging*
  - ✓ *Mammals can be cloned from old somatic cells to produce new individuals with normal lifespans*

- **"Information Theory of Aging"** proposes that aging is due to the loss of transcriptional networks and epigenetic information over time, driven by a conserved mechanism that evolved to co-regulate responses to cellular damage, such as a double stranded breaks (DSB).

> *"Life is based on the complex interplay between the cellular machinery and information stored in the **genome** and **epigenome**, which may be thought of as biological **hardware** and **software**. Whether aging is caused by a breakdown in the hardware, the software, or both is not yet known."*
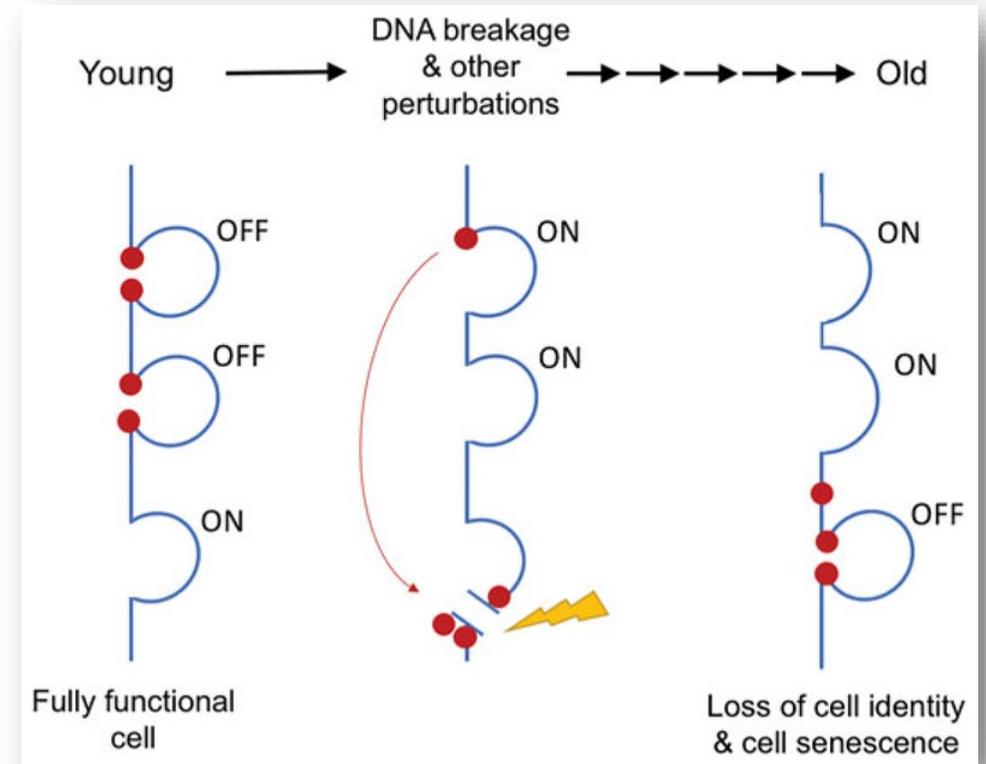
The Relocalization of Chromatin Modifiers (RCM) hypothesis.

What it says:

1. **When DNA damage occurs**, chromatin-modifying proteins (e.g., SIRT1/Sir2, HDACs) temporarily leave their normal genomic sites to help repair the break.
2. **Repeated damage with age** means these factors don't fully return, so their original targets lose proper regulation leading to epigenetic drift, mis-expression, loss of cell identity, and aging phenotypes

Key predictions/evidence:

*   Age-related gene-expression changes resemble a chronic DNA-damage response rather than new mutations per se.
*   Inducing targeted DNA breaks can accelerate epigenetic aging and aging phenotypes without increasing mutation burden, supporting the model;
*   Conversely, resetting epigenetic marks can restore function.
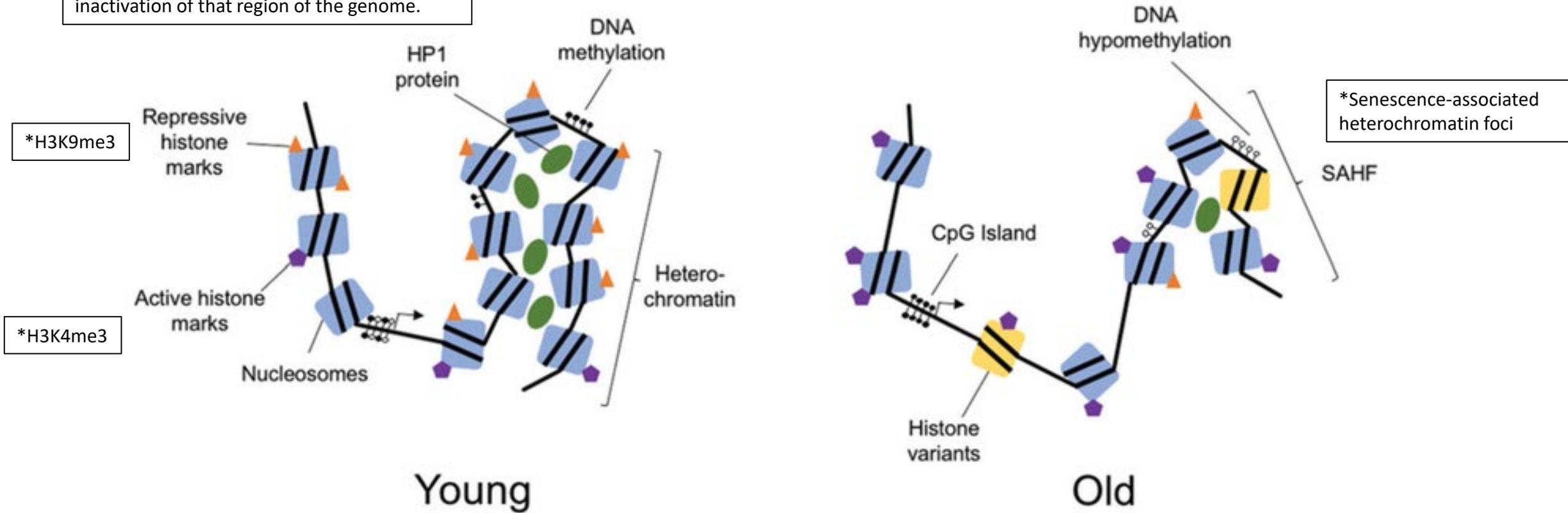

Kane & Sinclair, 2008., Crit Rev Biochem Mol Biol

The Relocalization of Chromatin Modifiers (RCM) hypothesis.

Kane & Sinclair, 2008., Crit Rev Biochem Mol Biol

*HP1 are chromatin-associated proteins (bind to H3K9me3, a mark on histone H3) to signal inactivation of that region of the genome.

*H3K9me3

*H3K4me3

*Senescence-associated heterochromatin foci

Young
- Tightly packaged heterochromatin, with repressive histone marks and HP1 protein binding.

Old
- Decrease in repressive histone marks and an increase in active histone marks.
- Global DNA hypomethylation except in CpG islands where there is hypermethylation

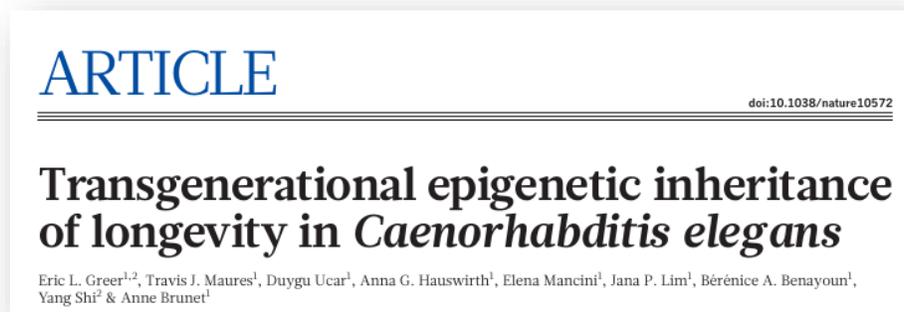# Epigenetic changes linked to aging

- Lifespan extension in worms deficient in the **H3K4 trimethylation complex** (*Greer et al., 2010, 2011*)
- Lifespan extension in flies overexpressing the **Sir2 gene** (*Jiang et al., 2013; Wood et al., 2016*)
- The curious case of the stable epigenome of long-lived **naked mole rats** (*Tan et al., 2017*).
- Many epigenetic changes follow a specific pattern, including methylation of specific CpGs of the epigenetic clock (Hannum et al., 2013; Horvath, 2013; Lu et al., 2021; Petkovich et al., 2017; Weidner et al., 2014).
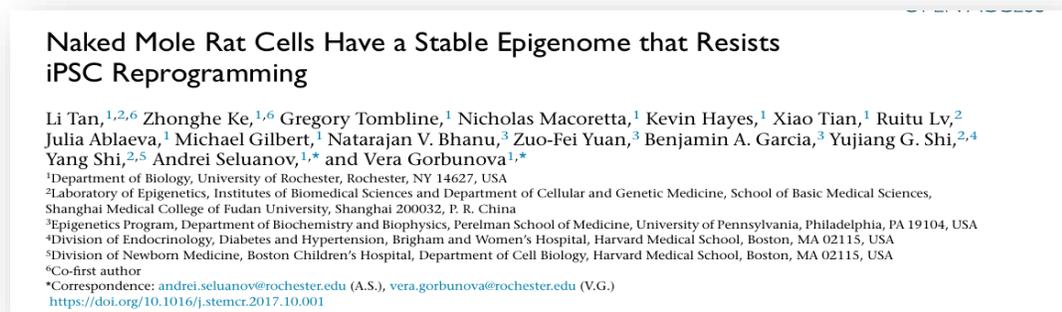

Greer et al., 2010, Nature

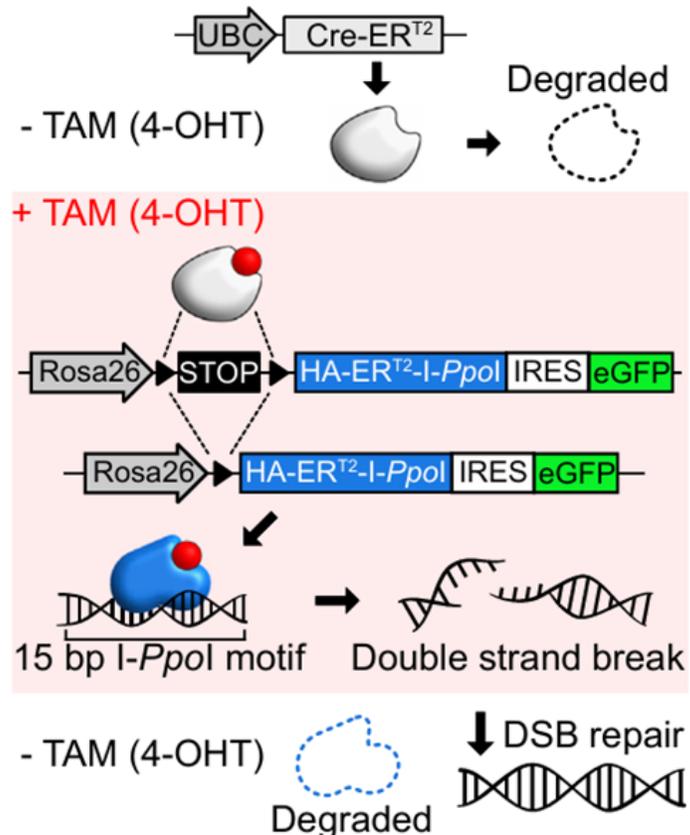

Wood et al., 2016, PNAS

Greer et al., 2011, Nature

Tan et al., 2017, Stem Cell Reports

A system to induce epigenetic aging – ICE mice
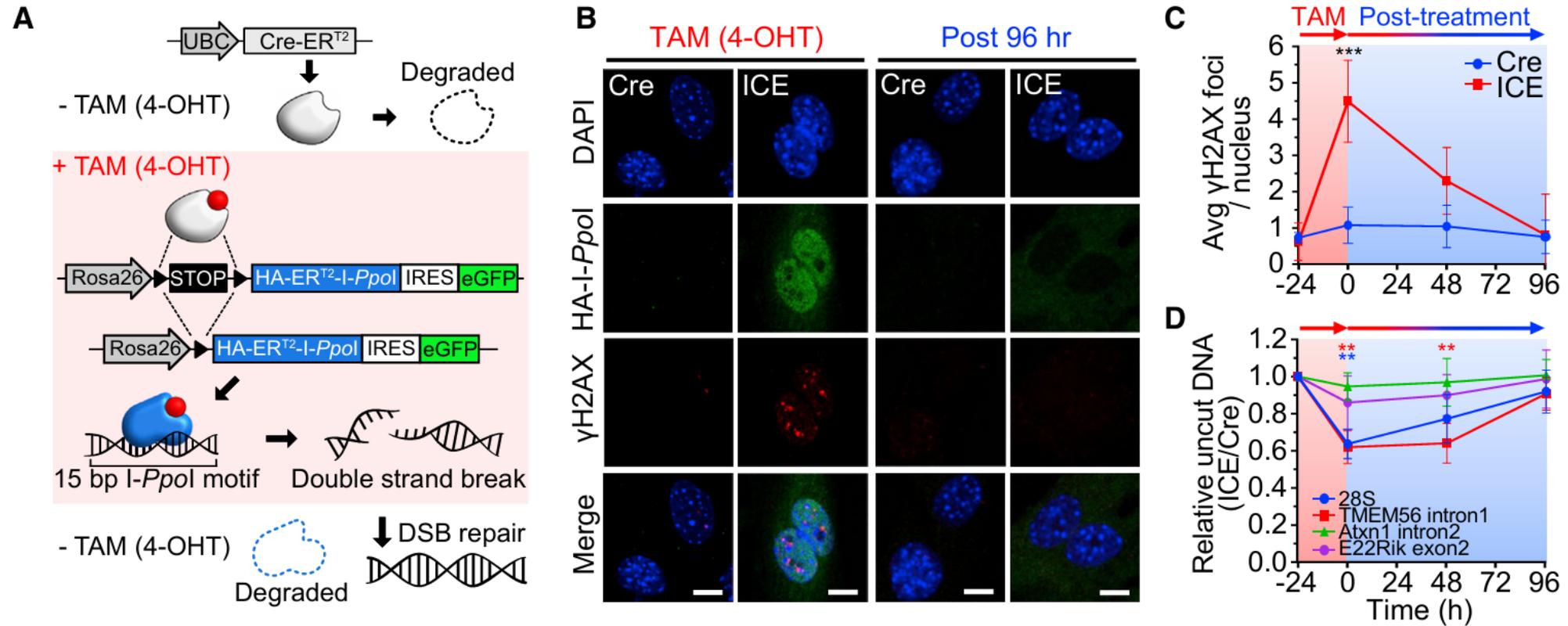
Inducible Changes to the Epigenome

1. To create DSBs in cells and mice <u>without</u> causing mutations → **I-PpoI**, an endonuclease from *Physarum polycephalum*
2. I-*Ppo*I recognizes the DNA sequence CTCTCTTAA▼GGTAGC, which occurs at 20 sites in the mouse genome, 19 of which are non-coding, and none of which occur in mitochondrial DNA.
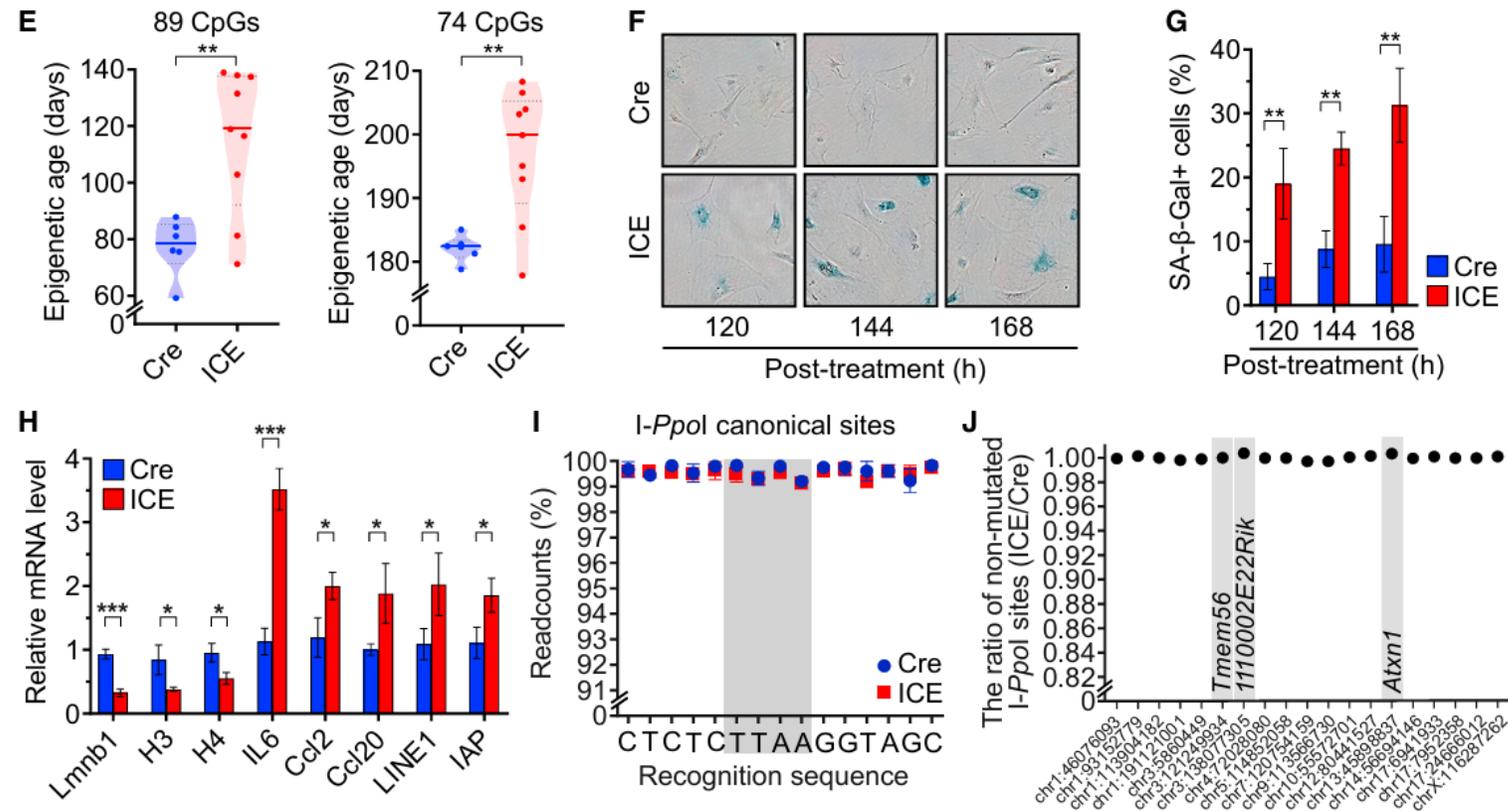

The system consists of:
- **HA-ERT2-I-PpoI:** a fusion of the I-PpoI gene to the C terminus of a tamoxifen (TAM)-regulated mutant estrogen receptor domain gene (ERT2),
- a transcriptional **loxP-STOP-loxP** cassette, and a TAM-regulated Cre recombinase gene (**Cre-ERT2**) upstream of a ubiquitin promoter for whole-body expression.
- In the presence of TAM, Cre-ERT2 excises the stop cassette, facilitating transcription of the **ERT2-HA-I-PpoI-IRES-GFP** cassette that produces nuclear localized ERT2-I-PpoI, which is degraded upon removal of TAM
- C57BL6/J transgenic mice with heterozygous ERT2-I-PpoI and Cre-ERT2 are named **inducible changes to the epigenome or ICE mice**.
- We reasoned that the four-complimentary base overhangs that I-PpoI creates would have a far lower rate of mutation than other ways of creating DSBs, such as CRISPR, chemicals, and radiation.

Fibroblasts from ICE mice have more double-stranded breaks (DSB)

- Mouse embryonic fibroblasts (MEFs) were isolated from ICE and control embryos.
- After the addition of TAM, **HA-I-PpoI** was detected in nuclei of ICE cells, but not of controls (**Figure 1B**)
- The number of serine-139-phosphorylated H2AX (**γH2AX**) foci, a marker of DSBs, reached a maximum of 4-fold above background after 24 h (**Figure 1C**).

Fibroblasts from ICE mice are epigenetically older and more senescent

- ICE cells were ~1.5-fold older than the Cre control cells (Figure 1E).
- CE cells had increased indicators of cellular senescence (Figures 1F–H).
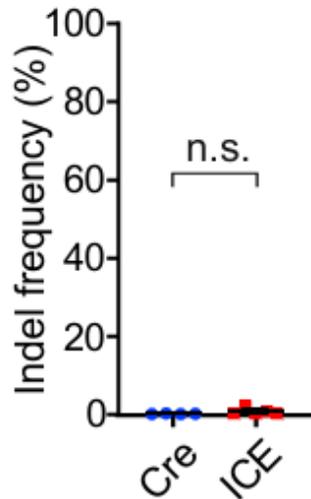- Changes are not due to mutations in the I-Ppol recognition sites (Figures 1I-J)

Thus, in MEFs, faithful DSB repair accelerates aspects of aging, including epigenetic age.

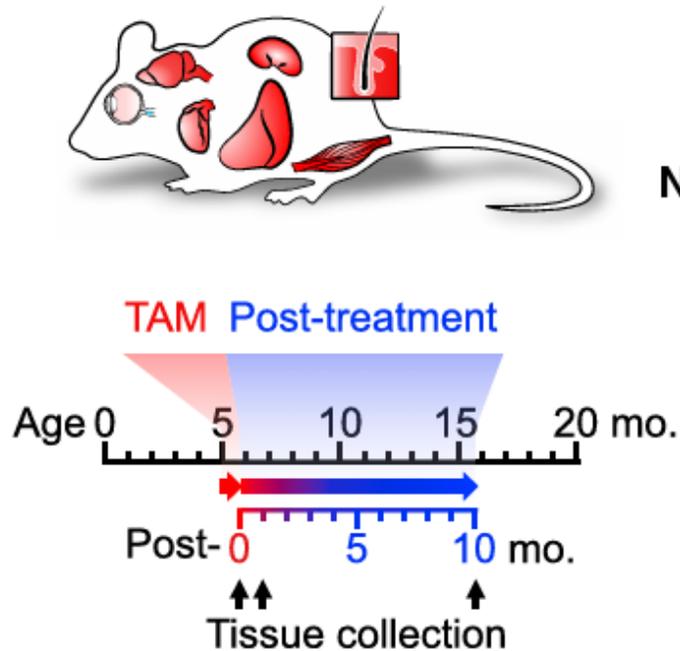# I-PpoI leads to cuts in ICE mice DNA with no further mutations/damage

K

Indel frequency

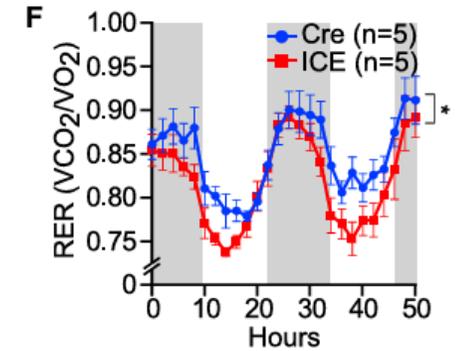| Samples	% Indel
Cre 1	0.2
Cre 2	0.26
Cre 3	0.16
Cre 4	0.14
ICE 1	2.12
ICE 2	0.72
ICE 3	0.11
ICE 4	0.13
ICE 5	0.1

L

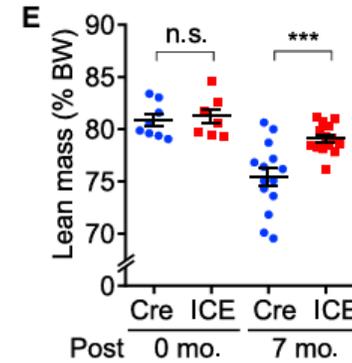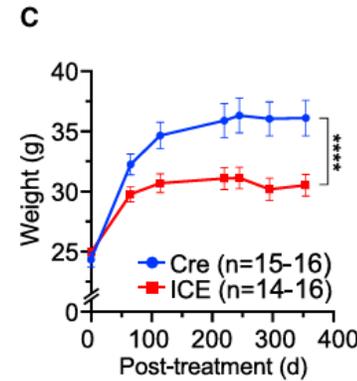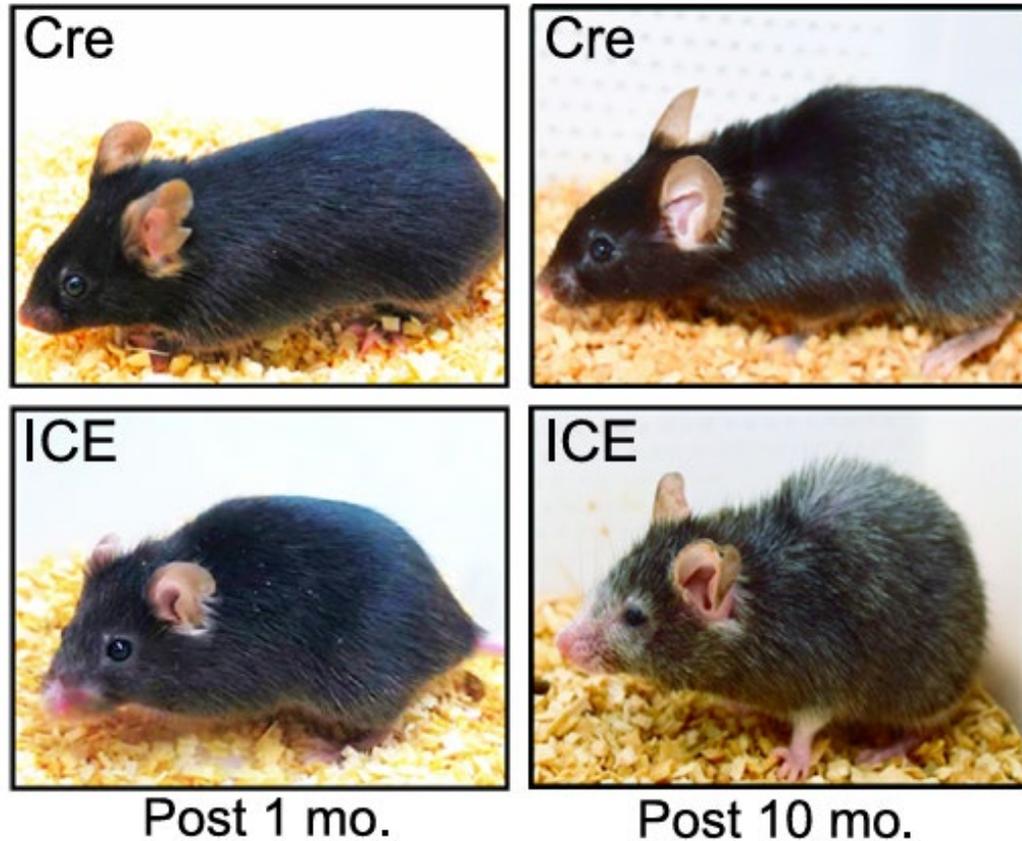**M** I-PpoI cutting   Biotin-tagged oligo   IP and analysis

N

(K) Mutation frequency of 28S rDNA in 96-h post-treated cells.
(L) Experimental design.
(M and N) Immunoprecipitation and quantification of a I-PpoI cut site (**Tmem56**) in skeletal muscle, liver, and kidney during and after TAM treatment (0-, 1-, and 10-month post-treatment). Used a biotinylated oligo with the overhang 5'-TTAA-3' to capture I-PpoI-cut DNA by IP.
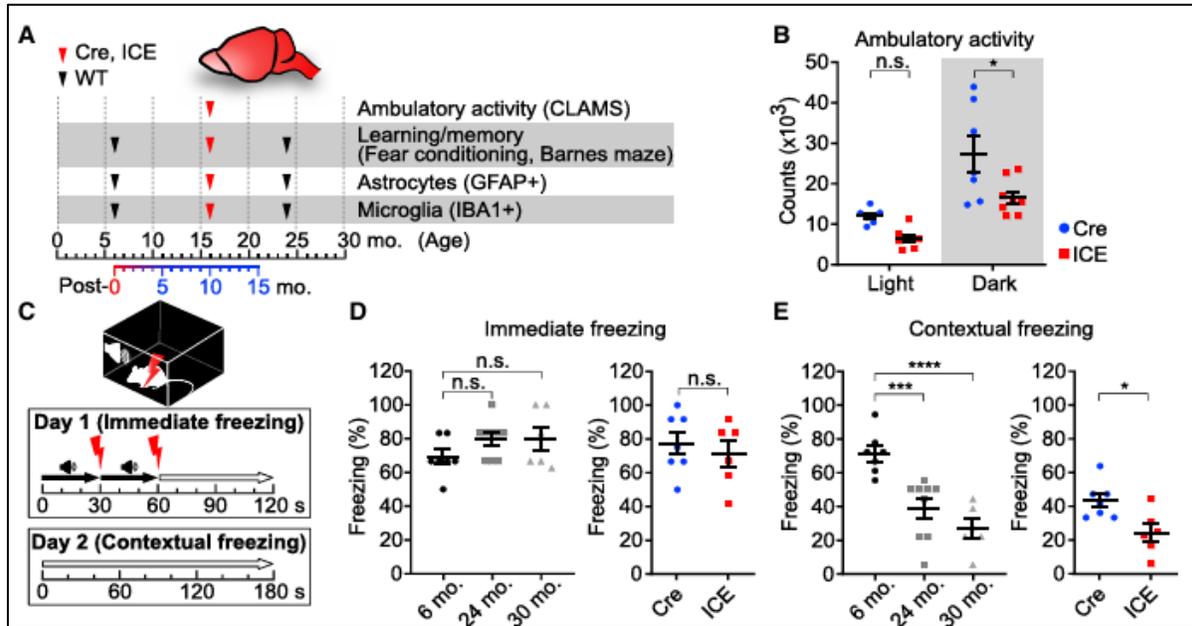
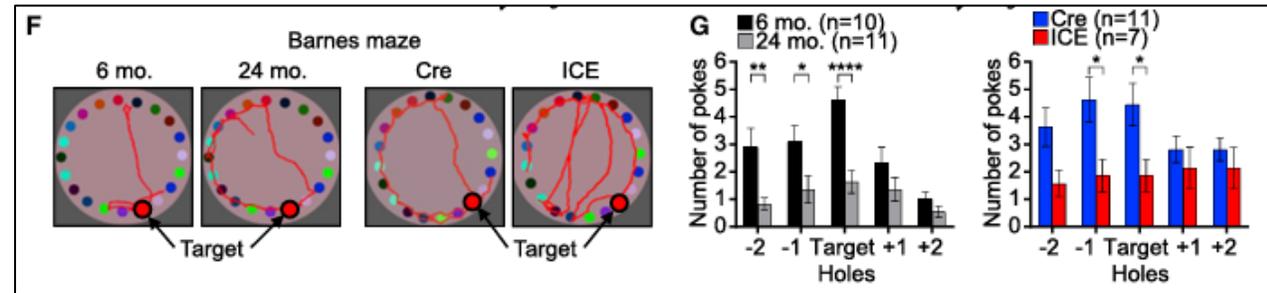# The ICE system phenocopies aging *in vivo*

At 10-month post-treatment, the ICE mice exhibited classic features of old age, including reduced body weight and fat mass, independent of food intake, a lower RER during the day, and decreased motion in the dark phase.
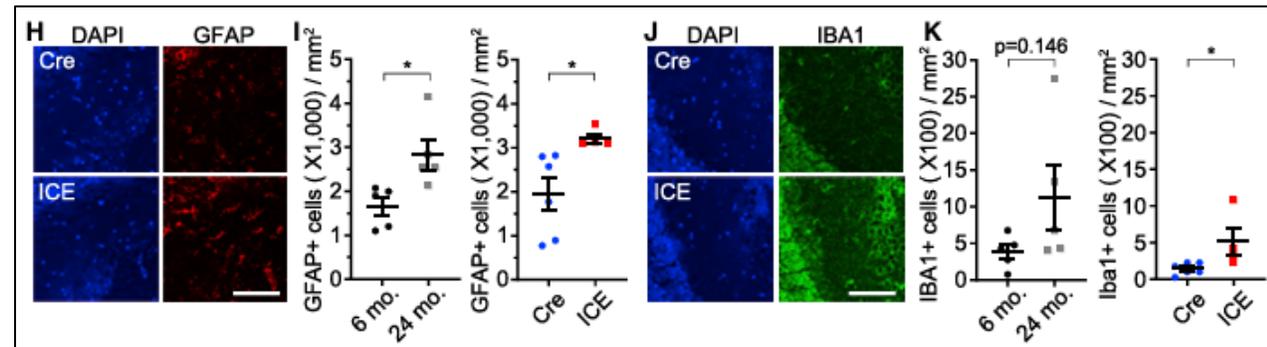
ICE mice phenocopy brain aging

- During aging, mice move less in the dark phase and have a characteristic loss of coordination. ICE mice moved ~50% less in the dark phase.

- Hippocampal function, critical for spatial and memory consolidation, declines with age and is often measured by fear conditioning, which measures short-term memory. On the second day, ~75% of the young mice and ~40% of old mice froze, indicating reduced contextual recall, with a similar difference between Cre and ICE mice

- In the Barnes maze test, a measure of long-term memory, the recall of ICE mice was about half that of Cre controls, similar to that of 24-month-old WT mice
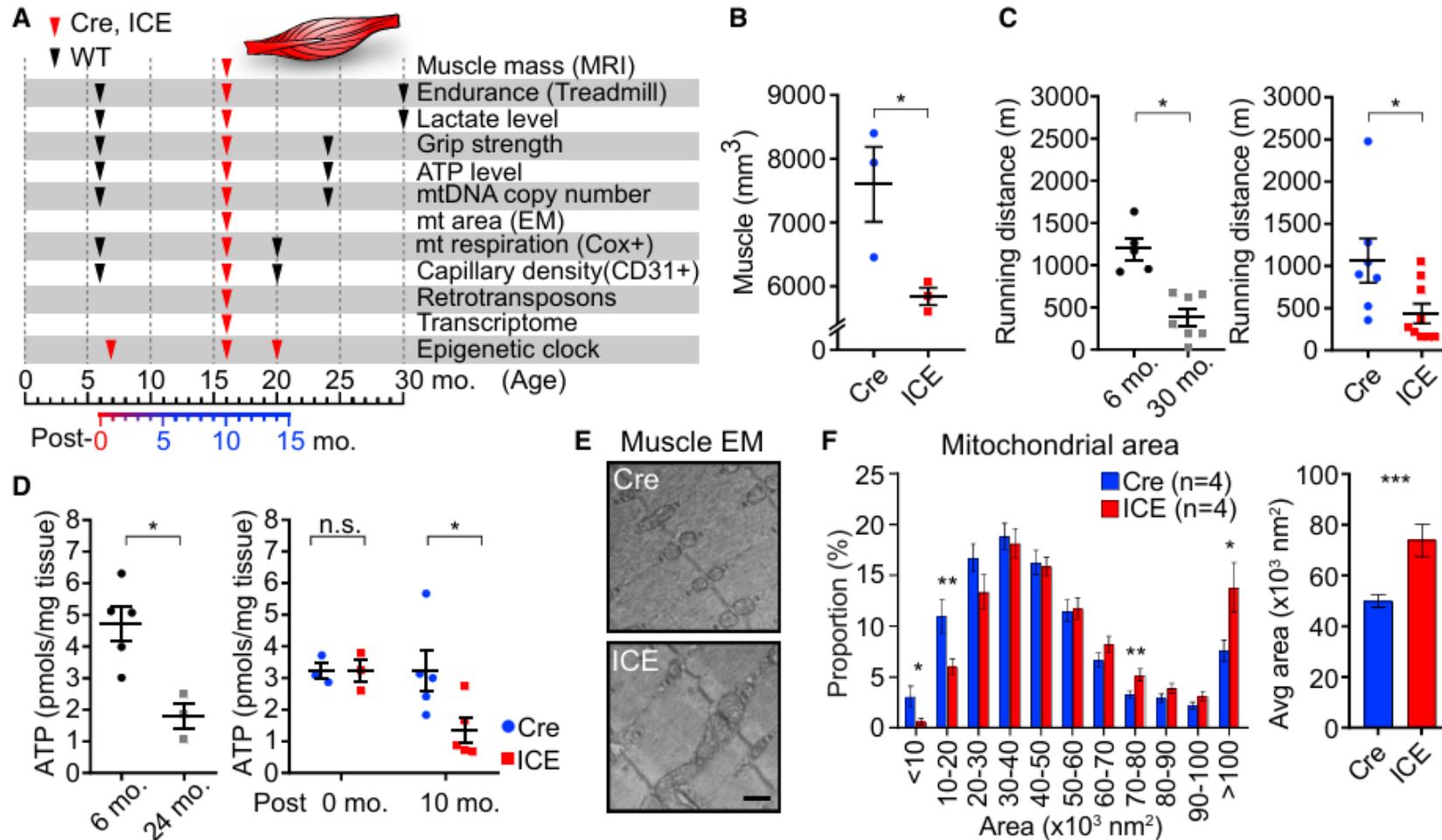
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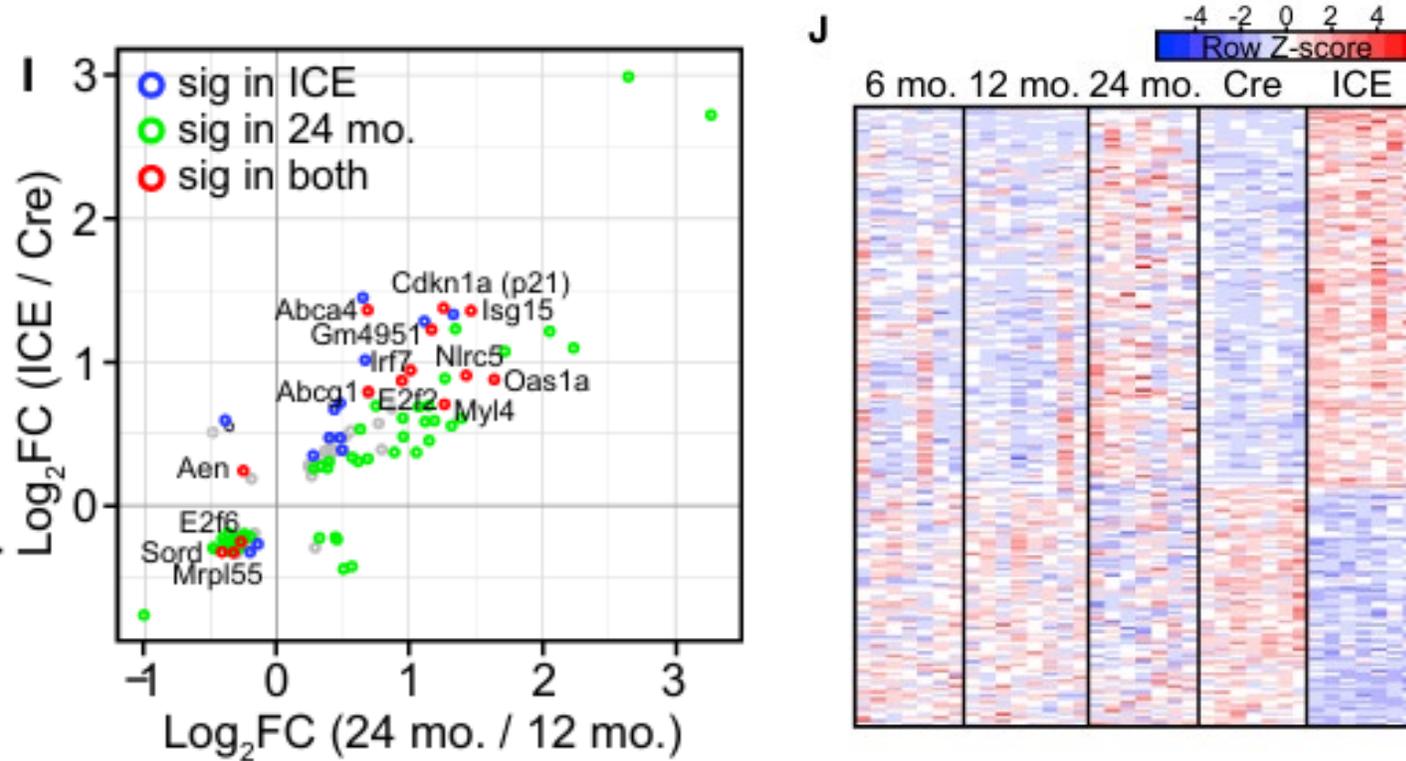
- Astrocytes and microglia, mediators of the innate immune response of the central nervous system, become hyper-activated with age.

- Similar to the hippocampi of aged mice, ICE mice had greater numbers of activated astrocytes (1.6×) and microglia (3.5×)

ICE mice phenocopy muscle aging

At 16 months of age, ICE mice had significantly **less muscle mass** (Figure 4B), **reduced endurance** (Figure 4C), **greater lactate levels** post-exercise (Figure S5A), **reduced grip strength** (Figure S5B), and other molecular hallmarks of muscle aging (Figures 4D–4F).

ICE mice phenocopy muscle aging

Gene expression and DNAme patterns of ICE mice were compared between normal young and old mice. In skeletal muscle, genes that were significantly dysregulated in ICE mice correlated with changes in old mice (Figures 4I, 4J):
- **Cdkn1a** (cyclin-dependent kinase inhibitor 1A or p21), a mediator of p53-mediated cellular senescence
- **Myl4** (myosin light chain 4), a form of myosin upregulated during aging
- **Nlrc5** (NLR family CARD domain containing 5), which inhibits NF-κB
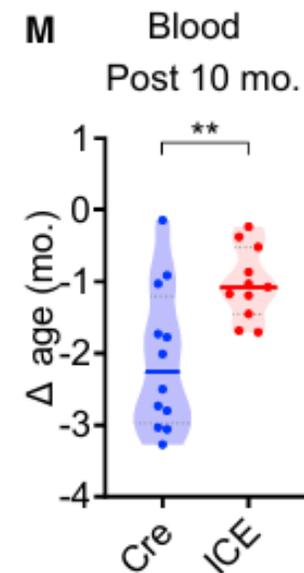- **Mrpl55** (mitochondrial ribosomal protein L55), the methylation of which is associated with longer lifespan.
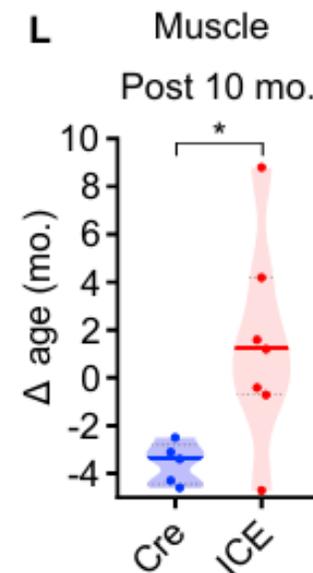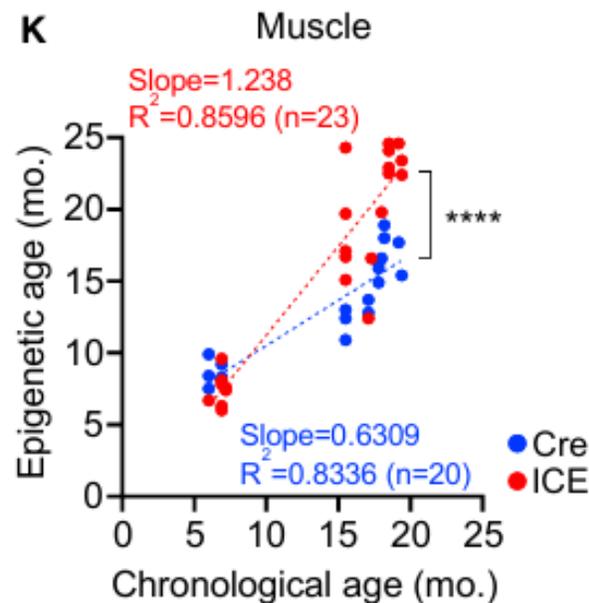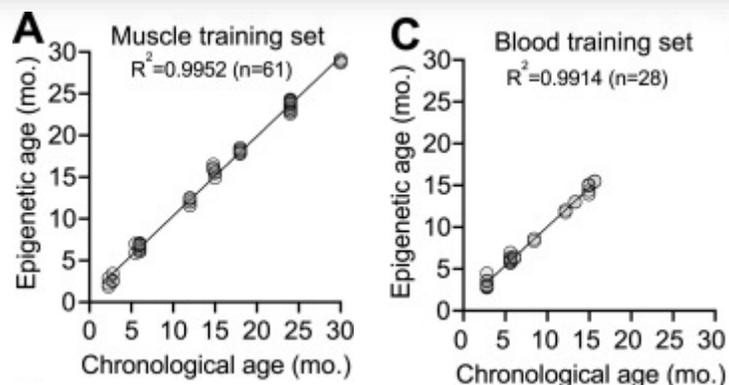
Epigenetic clocks: ICE mice undergo accelerated epigenetic aging

- **Epigenetic clocks** serve as a biomarker of biological age in mammals.
- 61 WT muscle and 28 WT blood samples from mice aged 2–30 months to define the training set.
- **RRBS (Reduced-representation bisulphite sequencing)** measures % of methylation at CpG sites to identify age-associated CpGs for blood (743) and muscle (2,048).
- Within the training dataset, **epigenetic age was correlated with chronological age** ($R2 = 0.995$ and 0.991 for muscle and blood, respectively).
- Based on the two mouse clocks, the rate of epigenetic aging was ~50% faster in the ICE mice than Cre controls ($p < 0.0001$), closely paralleling treated ICE fibroblasts.
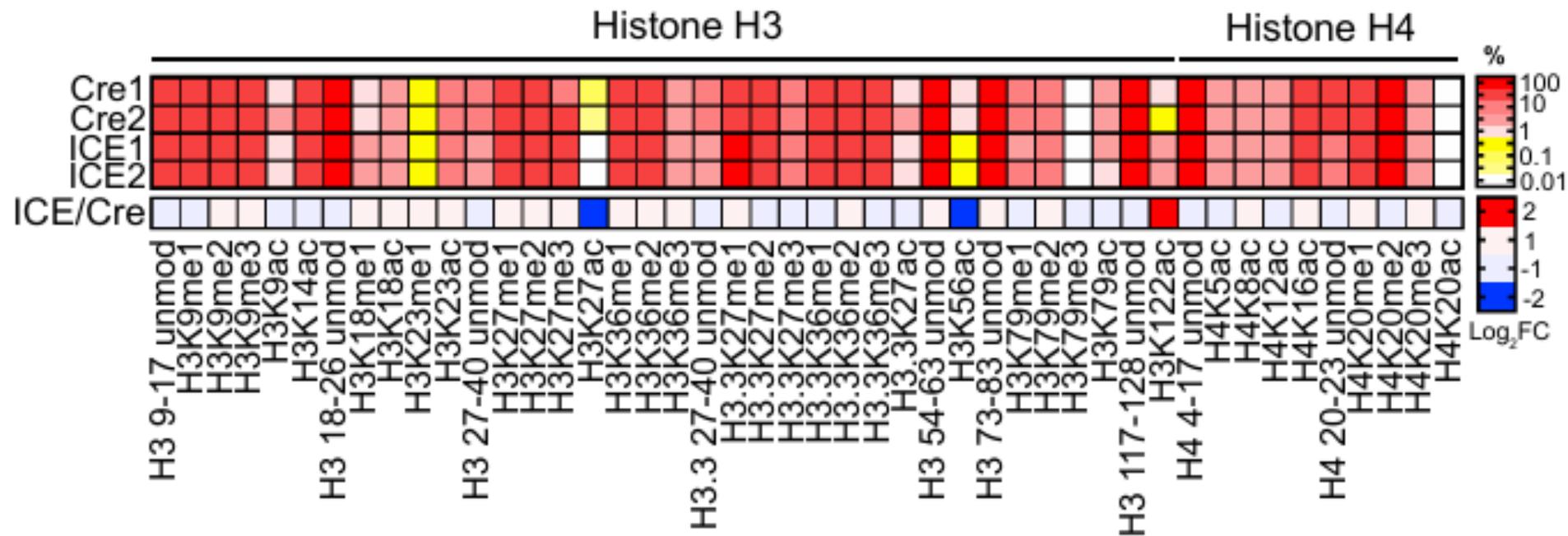

Research | Open access | Published: 03 February 2014

Aging of blood can be tracked by DNA methylation changes at just three CpG sites

Carola Ingrid Weidner, Qiong Lin, Carmen Maike Koch, Lewin Eisele, Fabian Beier, Patrick Ziegler, Dirk Olaf Bauerschlag, Karl-Heinz Jöckel, Raimund Erbel, Thomas Walter Mühleisen, Martin Zenke, Tim Henrik Brümmendorf & Wolfgang Wagner ✉

Faithful DNA repair alters the epigenetic landscape

- Aging is associated with specific changes in histone levels and post-translational modifications.
- Reduced levels of **H3K122ac** extend the lifespan of budding yeast (Sen et al., 2015)
- Levels of **H3K27ac** and **H3K56ac** decrease in human immune cells (Cheung et al., 2018; Dang et al., 2009).
- Mass spectrometric quantification of 46 different histone modifications detected **lower amounts of H3K27ac and H3K56ac and higher amounts of H3K122ac in treated ICE cells** (Figure 5A).
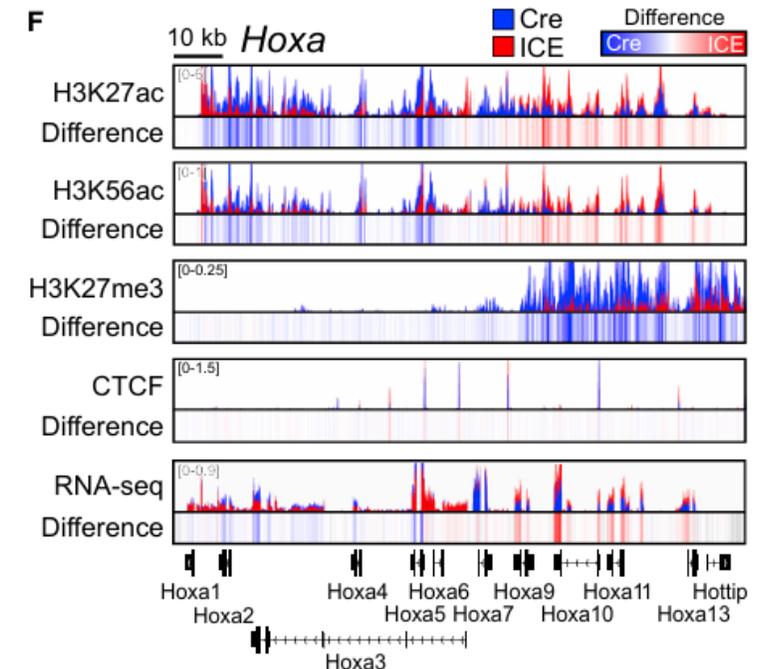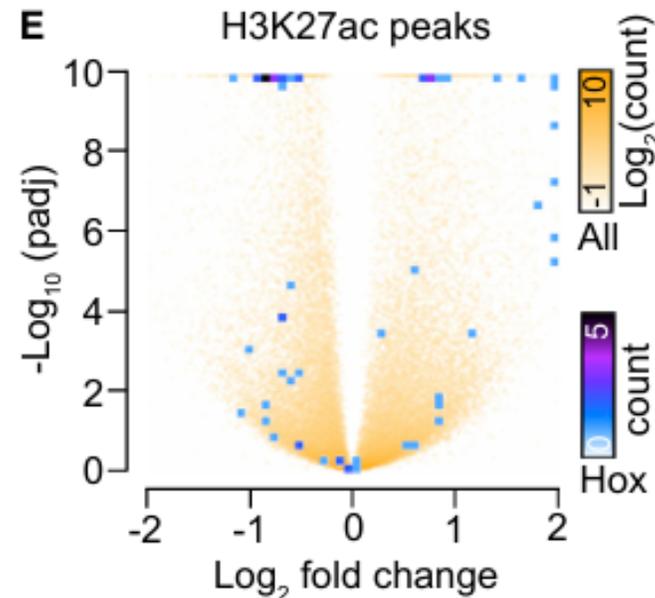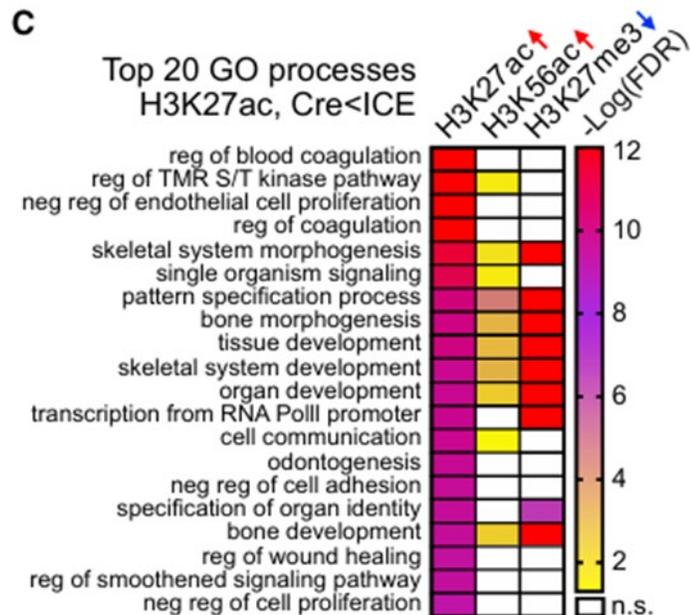

(A) Quantitative mass spectrometry of histone H3 and H4 modifications in 96-h post-treated ICE cells. %, relative abundance; me, methylation; ac, acetylation.

Erosion of the epigenetic landscape disrupts developmental genes

Remember: RCM postulates that aging is a ancient stress response that disrupts cell identity

- In the epigenetically aged ICE cells, all the *Hox* gene clusters had significant alterations in H3K27ac, H3K56ac, and H3K27me3 peaks, with coincident changes in mRNA levels
- From *Hoxa1* to *Hoxa6*, levels of H3K27ac and H3K56ac decreased, and from *Hoxa9* to *Hoxa13*, they increased, with concomitant changes in H3K27me3
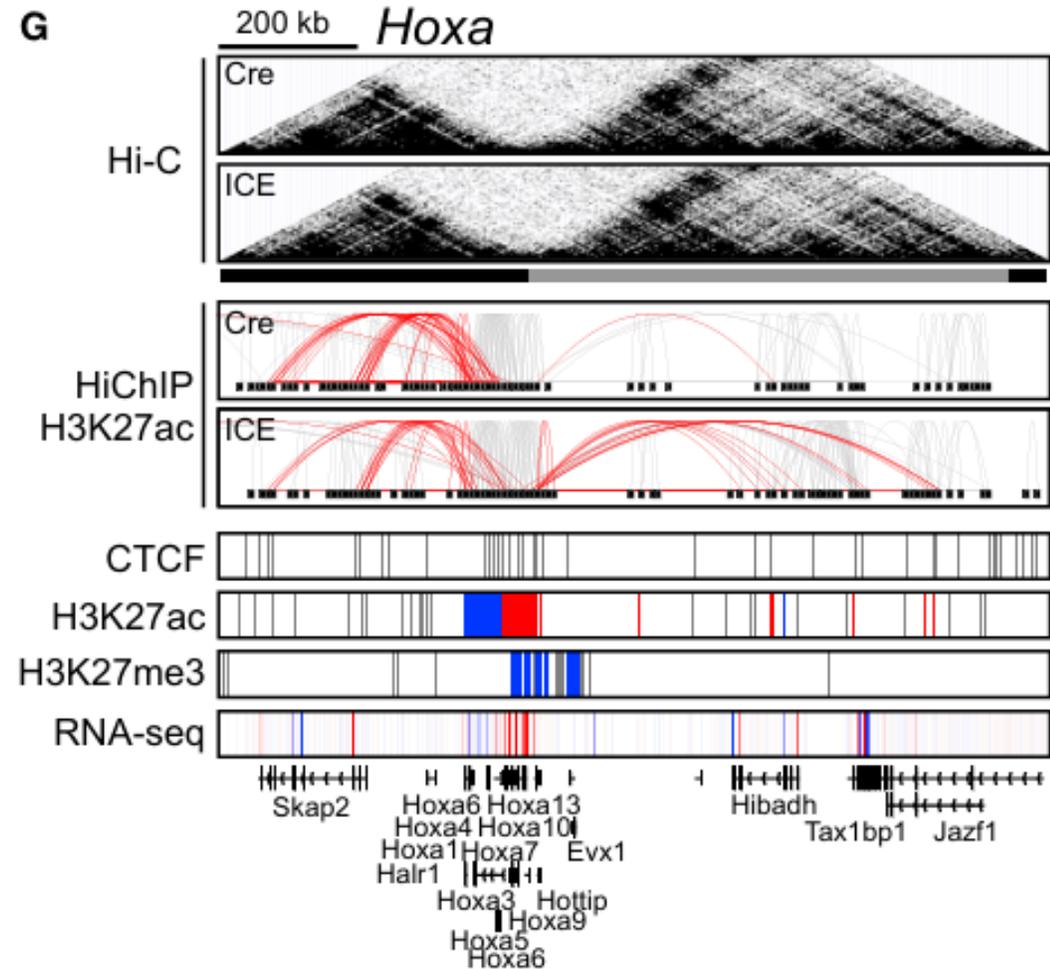

Gene ontology analysis of H3K27ac-increased, H3K56ac-increased, or H3K27me3-decreased peaks ordered by top 20 processes enriched in H3K27ac increased regions

ChIP-seq track of histone modifications and mRNA levels across the 120 kb Hoxa locus of post-treated ICE cells. Difference = ICE Cre.
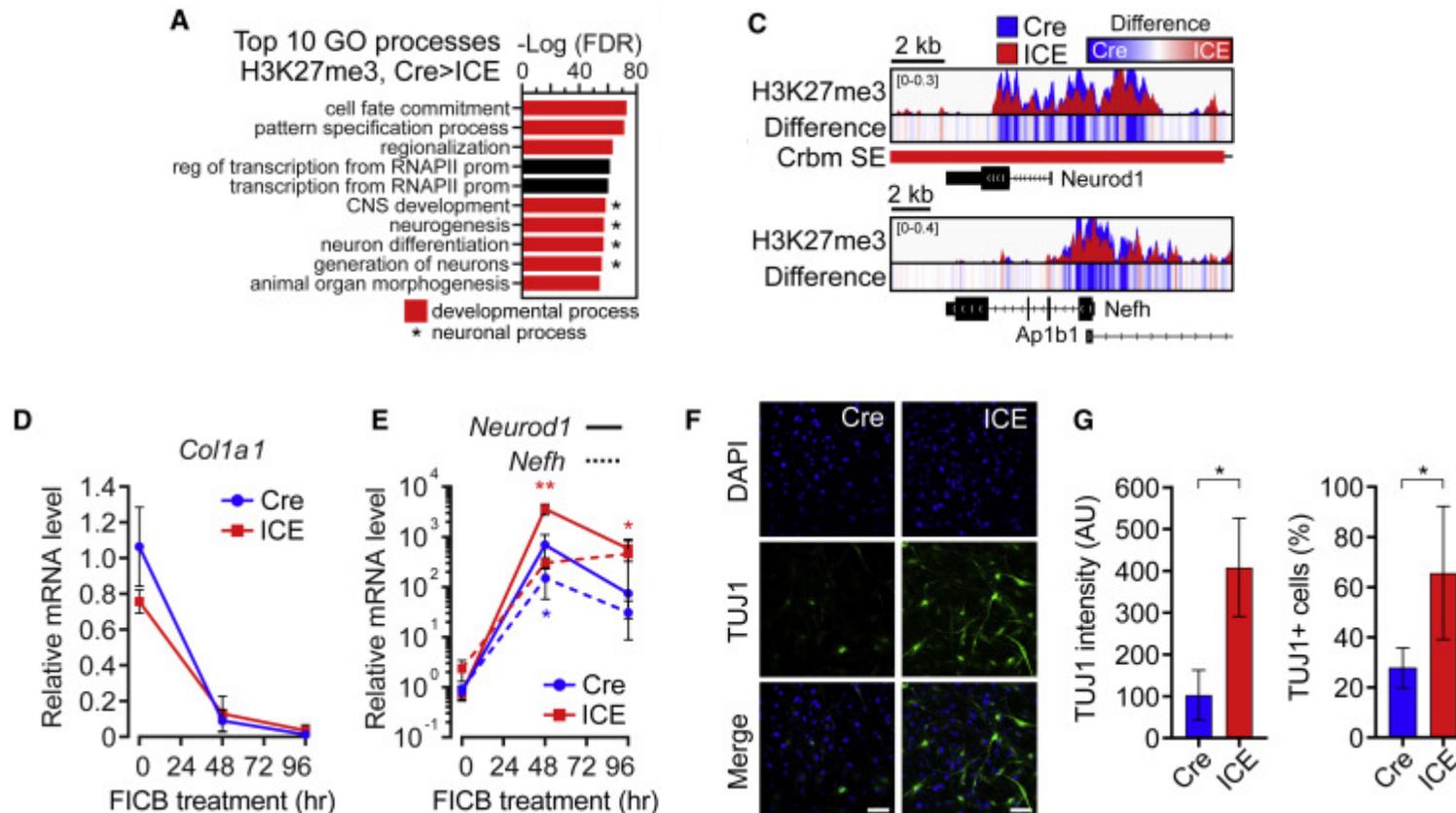
Faithful DSB repair alters spatial chromatin contacts

- High-resolution spatial chromatin contacts between promoters and enhancers were assessed by Hi-C and HiChIP to assess H3K27ac-associated chromatin contacts.

- H3K27ac-positive posterior Hoxa gene promoters (Hoxa 9–13) formed new contacts with active enhancers in an adjacent domain, with concomitant increases in mRNA levels, consistent with disordered promoter-enhancer communication.

- This is the first evidence that DNA repair alters multiple layers of epigenetic information, including spatial chromatin contacts, chromatin insulation, and Promoter-Enhancer communication.

Hi-C contact matrixes and HiChIP contact loops in Hoxa. Red, chromatin contacts between Hoxa promoters and other regions. Lower panels, regions with ChIP-seq or RNA-seq peaks. Peak regions, red (Cre < ICE), blue (Cre > ICE) or gray (unchanged).
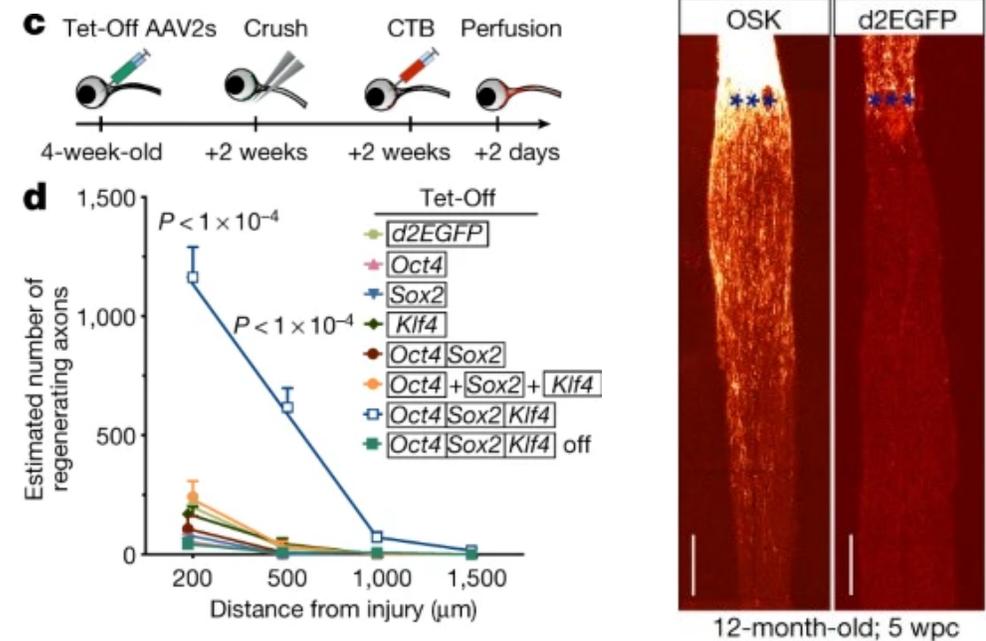
Cellular identity changes in ICE mice

- Of the genes with decreased H3K27me3, 4/10 of the top GO processes and 6/10 tissue-specific transcriptional profiles were related to neuronal processes or neuronal tissue types (Figures 6A and 6B)
- H3K27me3 signals were lower across the promoter regions of the neuronal fate genes Neurod1 and Nefh (Figure 6C)
- Reprogrammed the post-treated Cre and ICE cells into neurons by chemical means (Li et al., 2015) and found that Neurod1 and Nefh were 8- to 15-fold more easily derepressed in the ICE MEFs (Figures 6D and 6E), coincident with increases in neuron count and the neuronal cell marker TUJ1 (Figures 6F and 6G).

Epigenetic reprogramming restores a youthful epigenome in mice

- The cyclic expression of Yamanaka factors Oct4, Sox2, Klf4, and Myc (OSKM) (Takahashi and Yamanaka, 2006) alleviates the symptoms and extends the lifespan of mice.

- In a parallel study to this one, it was possible to safely reverse epigenetic age and gene expression patterns of old and damaged neurons to cure blindness in mice, a process requiring DNA demethylation (Lu et al., 2020).

- These findings revealed that cells possess a back-up copy of youthful epigenetic information that can restore cell identity
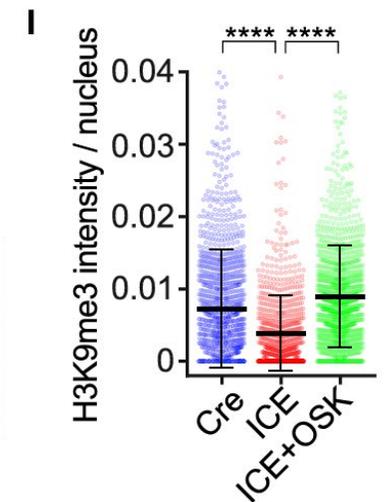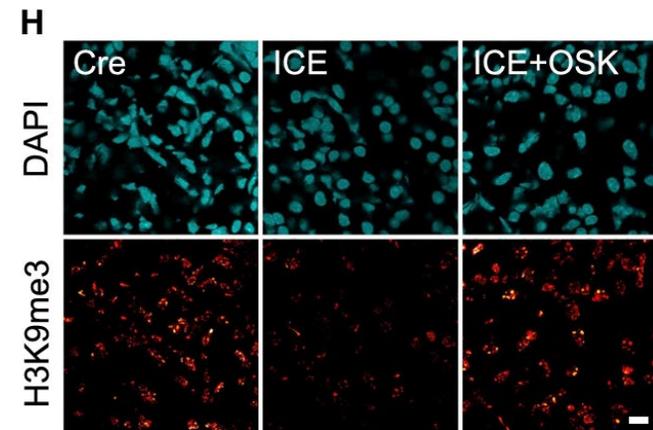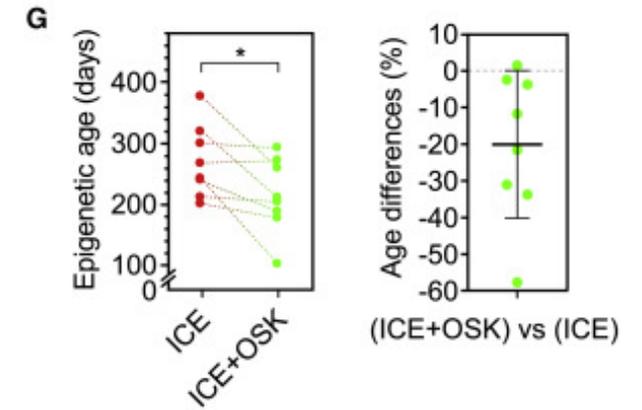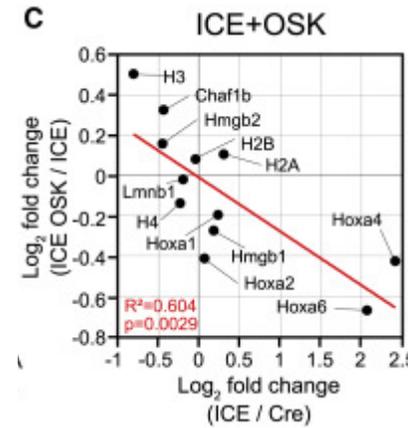
"Here we show that ectopic expression of Oct4, Sox2 and Klf4 genes (OSK) in mouse retinal ganglion cells (by injection of AAV2 carrying OSK polycistron into the vitreous body) restores youthful DNA methylation patterns and transcriptomes, promotes axon regeneration after injury, and reverses vision loss in a mouse model of glaucoma and in aged mice."

CTB-labelled axons in 12-month-old (old) mice, 5 weeks post-crush, with AAV2-mediated expression of either GFP or OSK

Lu et al., 2020, Nature

Epigenetic reprogramming restores a youthful epigenome in ICE mice

- Expression of OSK in post-treated ICE cells reversed age-associated mRNA changes, including those for Hmgb, Chaf1, Hoxa, and canonical histone genes (Figures 7A–7C)

- Based on four different mouse clocks, the epigenetic age of ICE cells was reversed by up to 57% (Figure 7G)

- During normal aging, levels of H3K9me3 in kidneys decrease and H3K36me2 in muscle increase. After 5 weeks of inducing adeno-associated virus (AAV)-delivered OSK in the whole body of ICE mice, the levels of these aging markers in kidney and muscle were rejuvenated to a point where they resembled negative controls (Figure 7H, 7I)

Epigenetic reprogramming restores a youthful epigenome in ICE mice

- **Old mice:** opaque lenses and lose retinal ganglion cells (RGCs) in the innermost retinal layer
- **ICE mice:** lens opacity was greater, and the number of RGC axons in the myelinated region were fewer than controls.

- Ectopic expression of OSK in RGCs returned mRNA levels to a more youthful pattern ($p < 0.0001$).

- 7/10 of the top upregulated processes in the RGCs of 12-month-old mice were involved in development, the majority of which (86% of nervous system developmental genes) were restored by OSK (Figure 7Q).