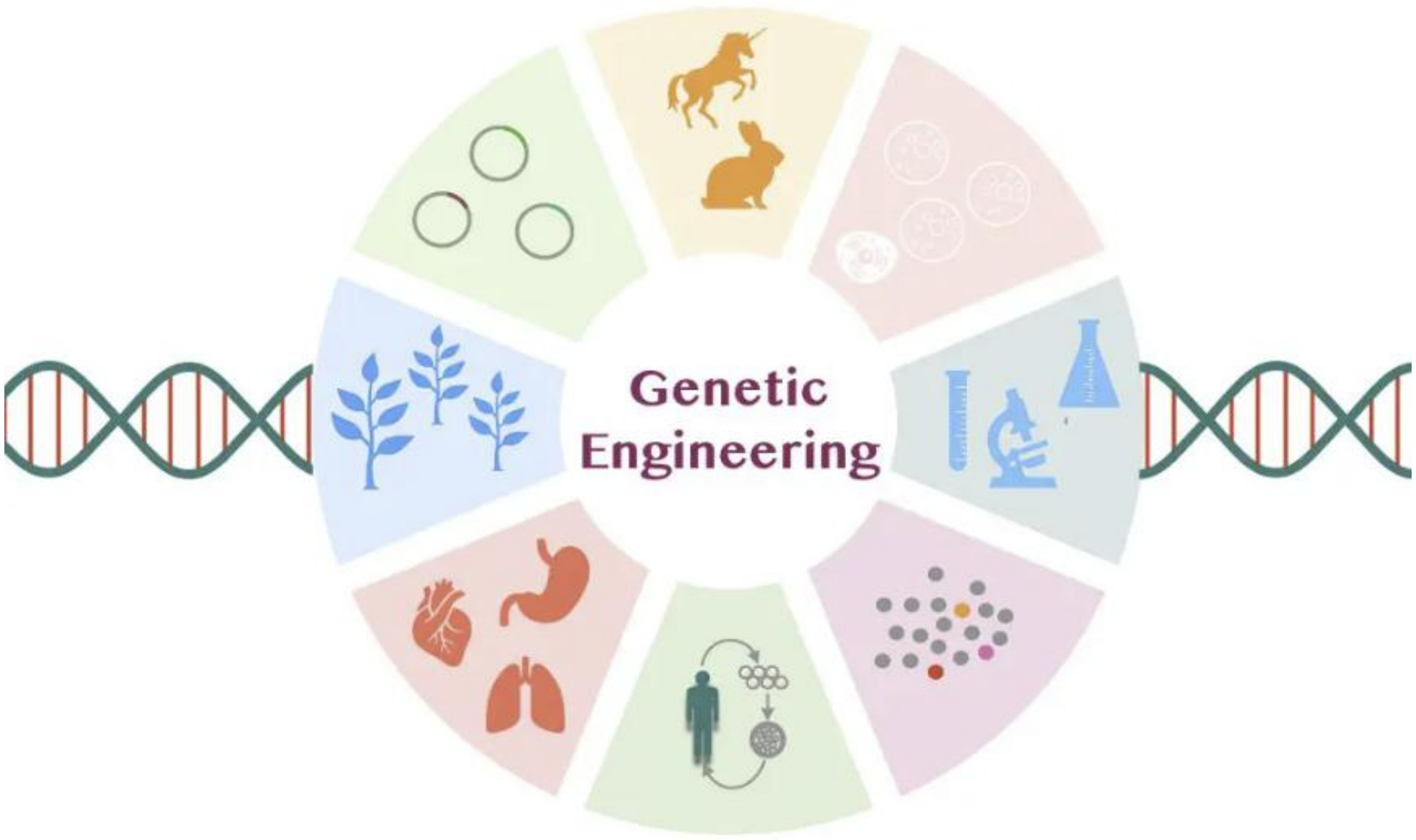
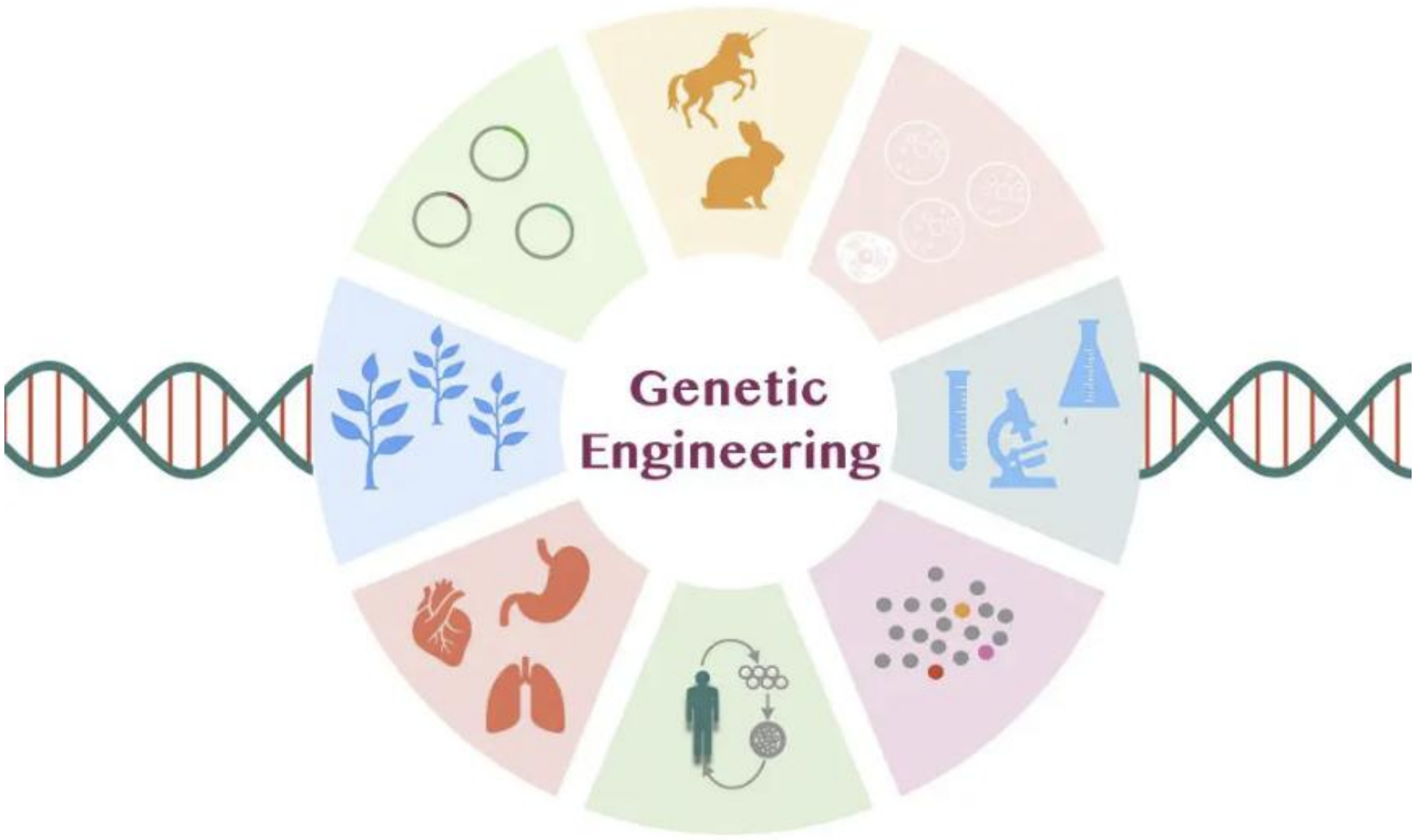
From Idea to Impact: Tackling a New Genetic Engineering Project

What Can We Accomplish Using GE Tools?



Step 1: The Idea



Why is Extinction Important?








Consider the IUCN Red List of 42,108 species across the world


Many are in danger of eradication altogether.

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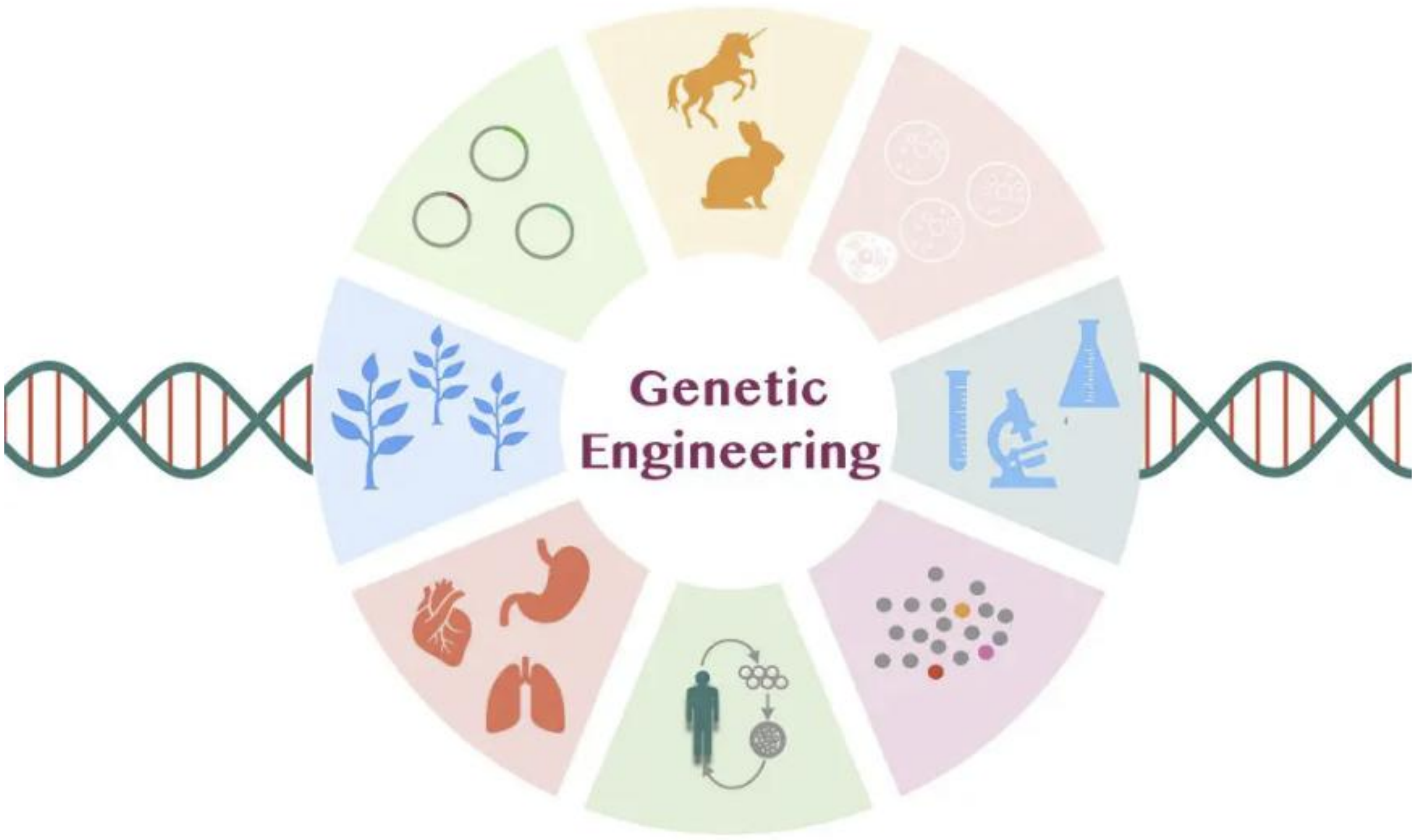
Step 2: The Need



Why does Extinction Matter?

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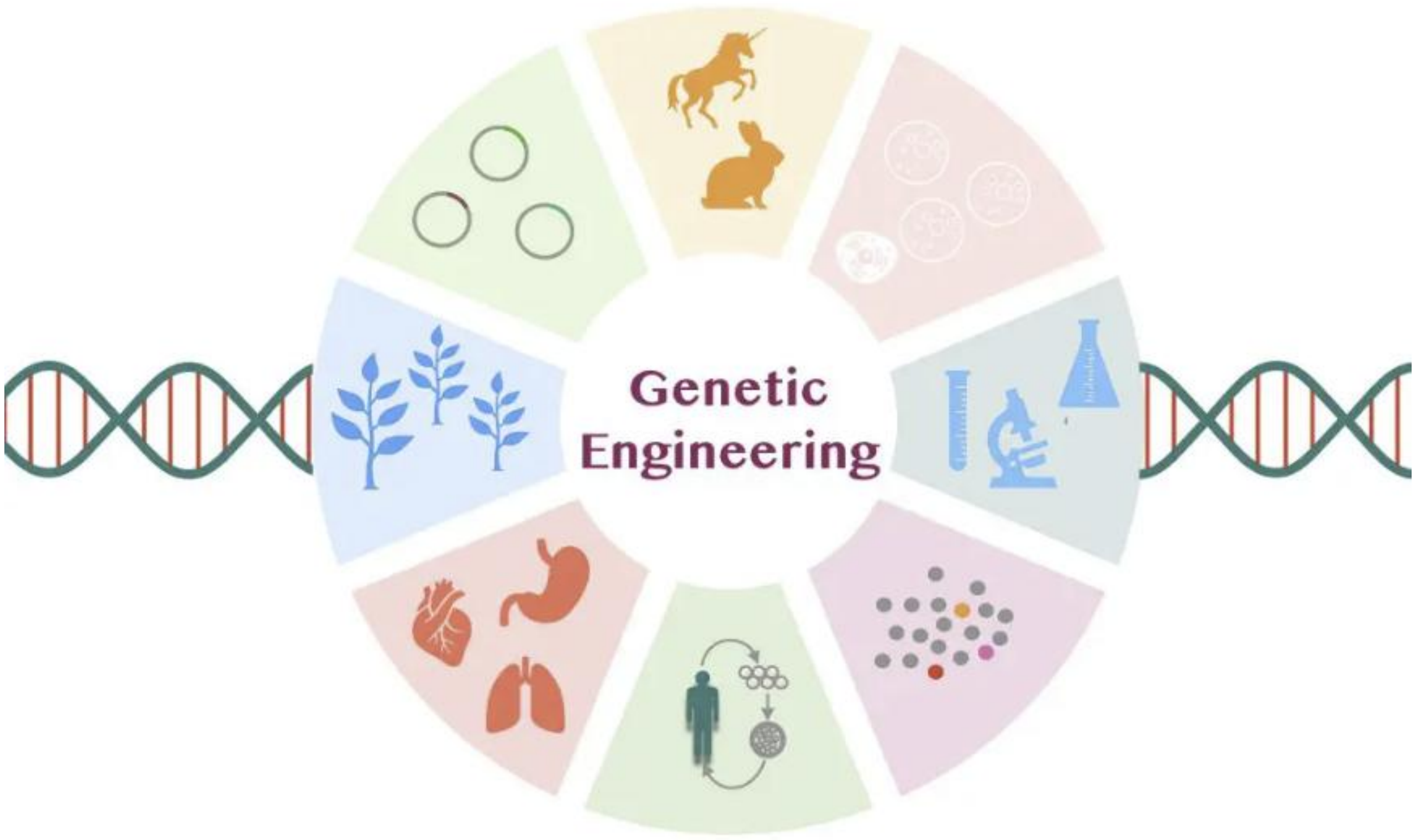
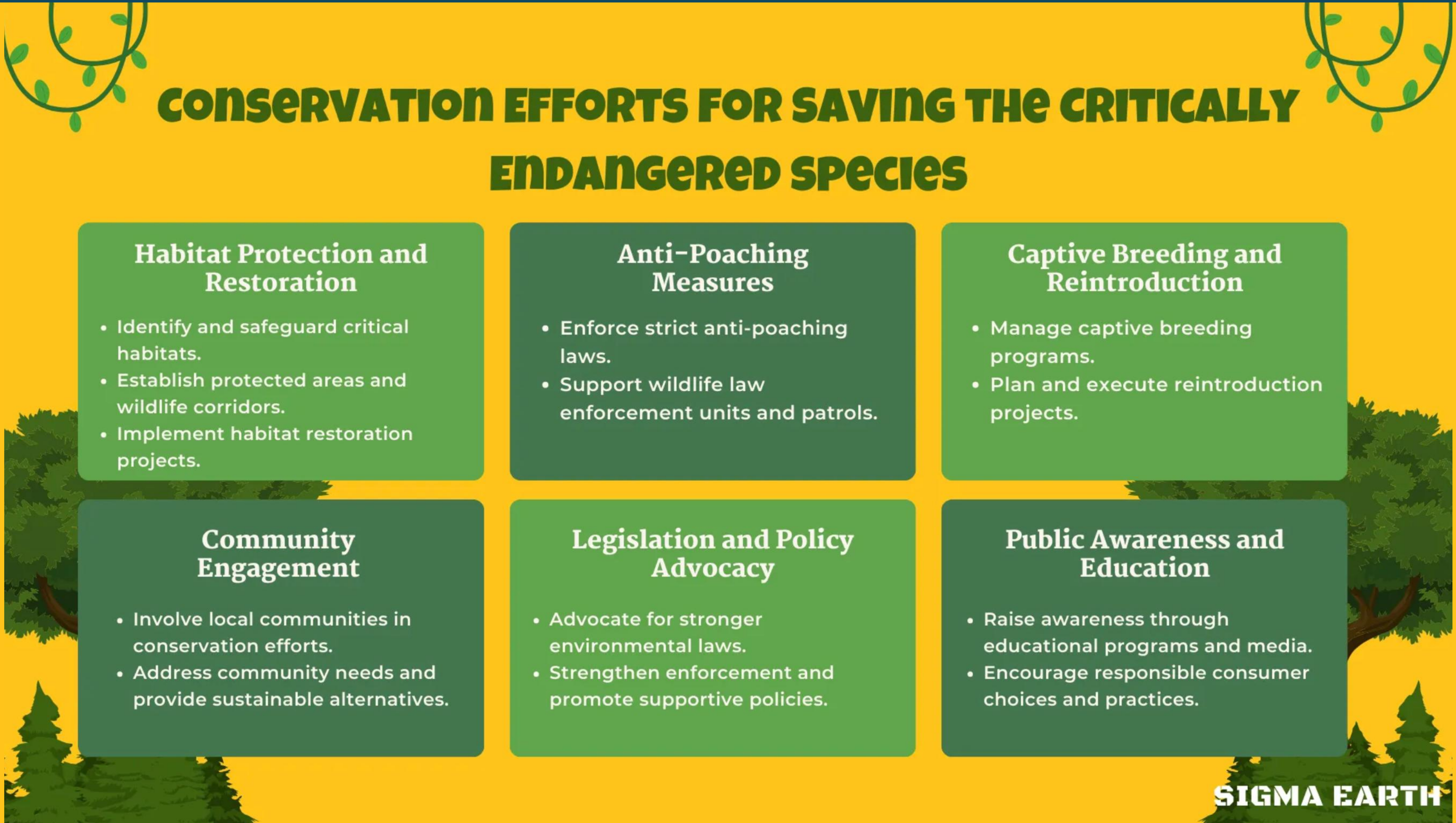
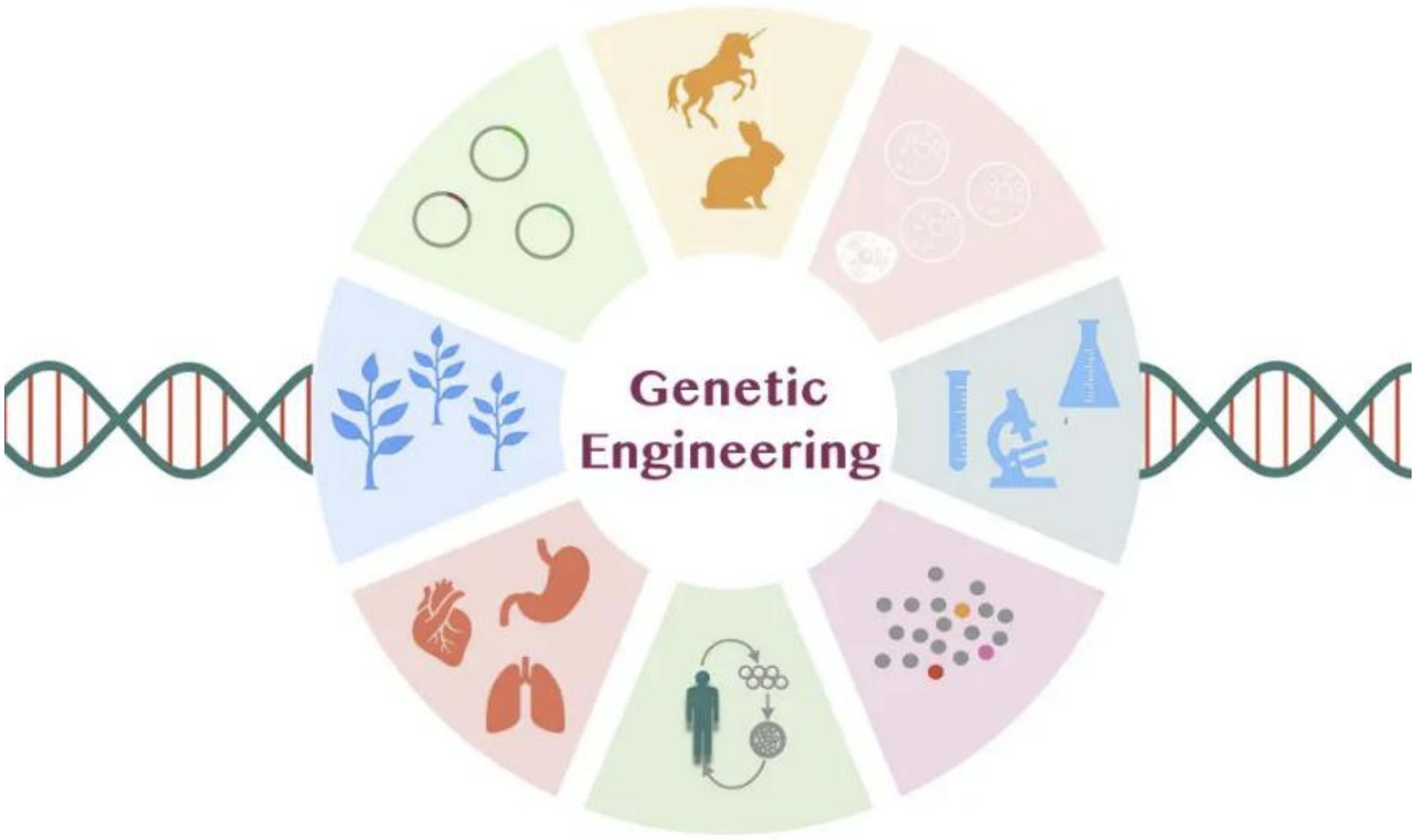
Step 3: What Are the Current Solutions?



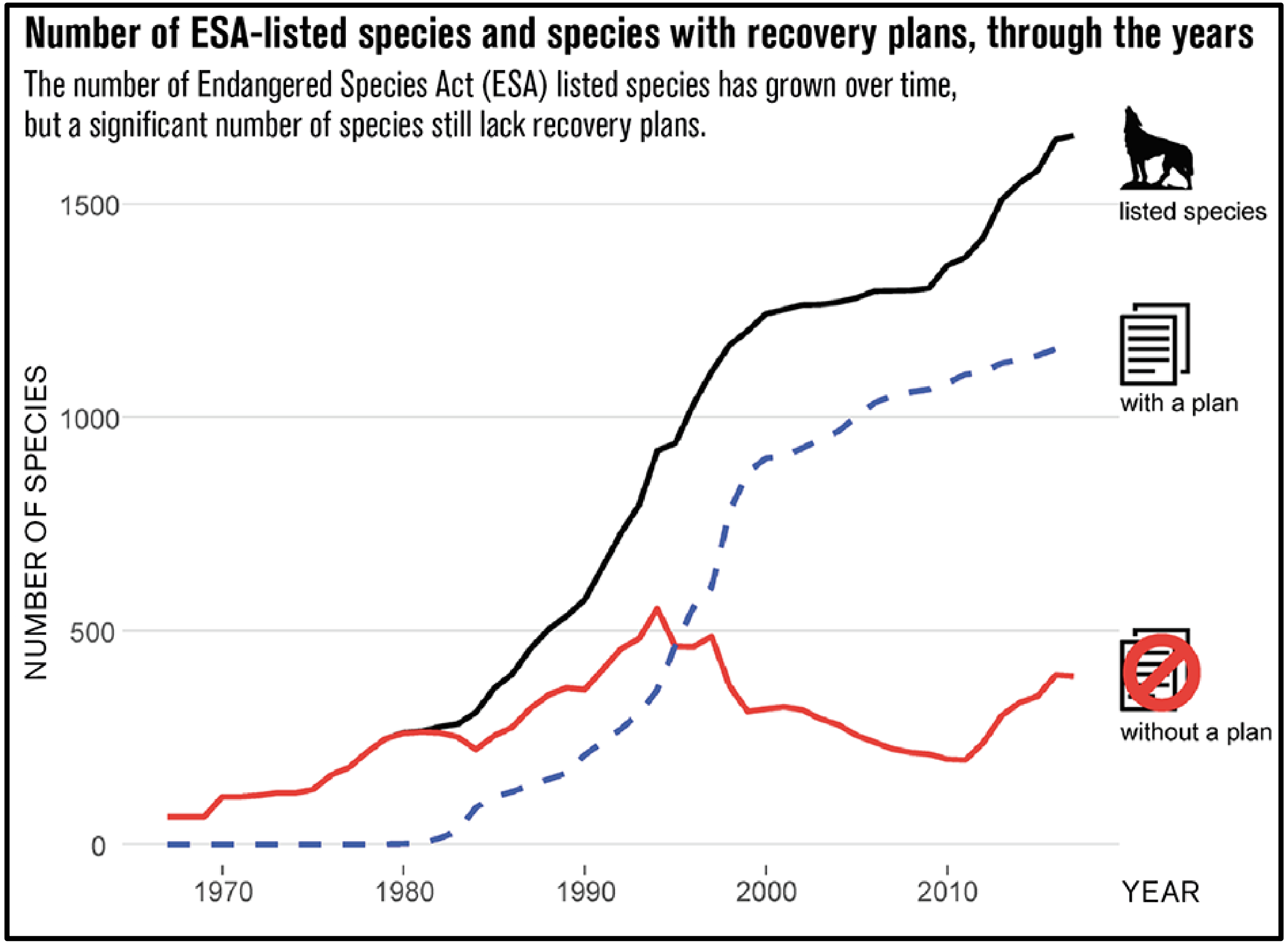
Biodiversity Conservation in Action



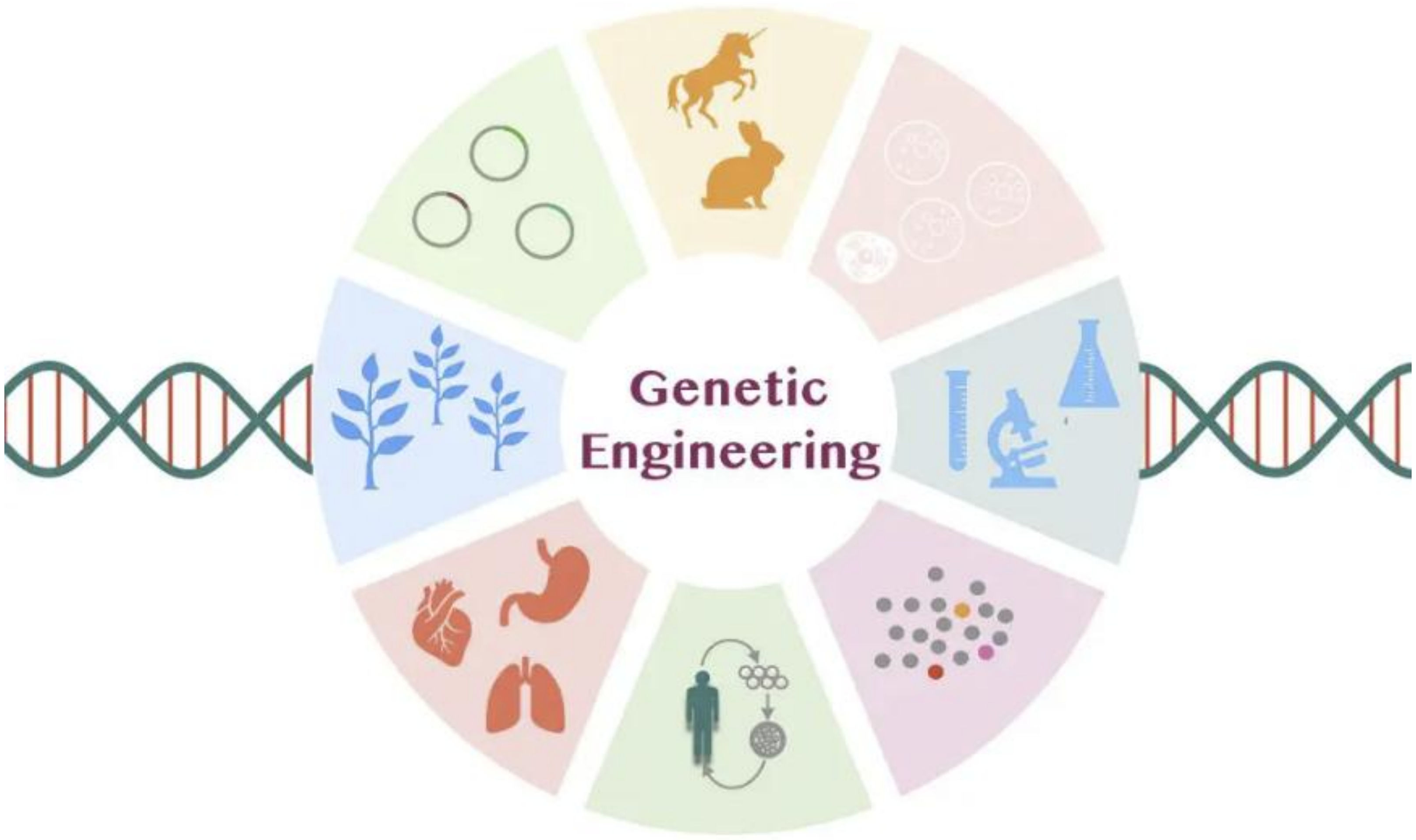
Step 4: Gaps in the Existing Solutions: Why New Research Is Necessary



Why It's Not Enough?

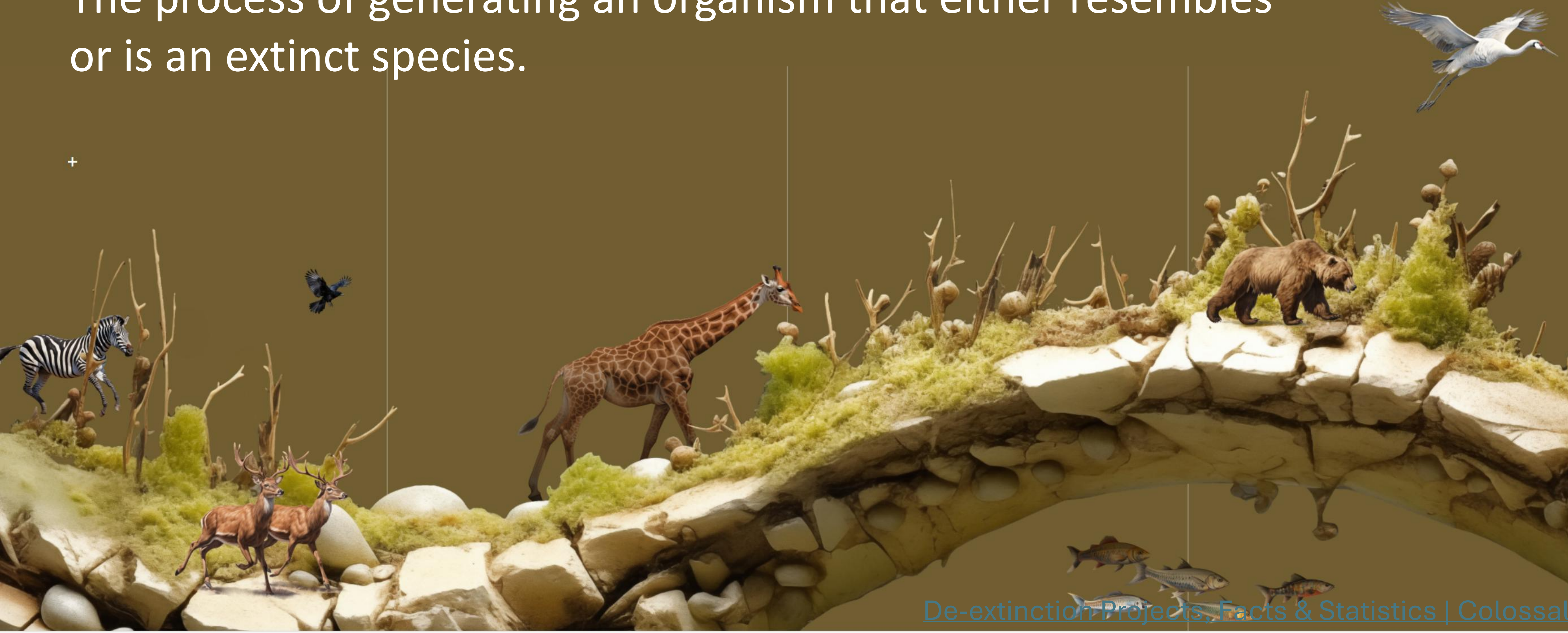


Step 5: Your New Research Idea



What is De-Extinction?

The process of generating an organism that either resembles or is an extinct species.



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Colossal Biosciences

As the world's first and only de-extinction company, Colossal is closer to restoring the past, preserving the present and safeguarding the future than anyone before. To truly illustrate our success, we must not only redefine de-extinction, but establish standards for the science behind it, as well.

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Undoing Extinction: Promise or Problem?

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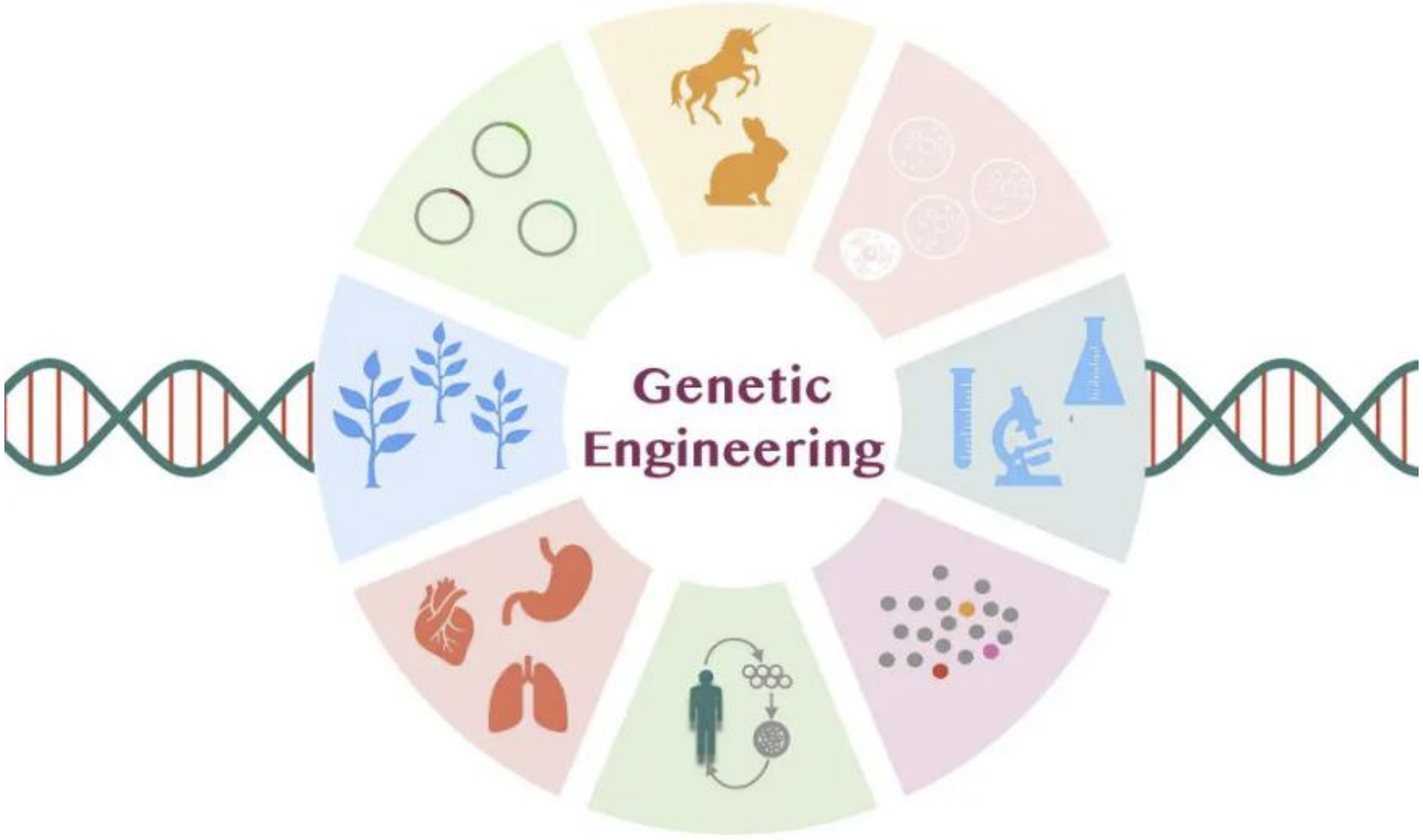
Step 6: Project Design



Key Considerations in Choosing a De-Extinction Candidate

Ecological Function
Did the extinct species play a key role in its ecosystem?

Scientific Value
Does the project advance tools and methods that can be applied to current conservation or biomedical challenges?

Ethical and Conservation Justification
Was the extinction caused by humans? Will the project align with conservation goals or raise ethical concerns (e.g., animal welfare, ecological disruption)?

Practical and Public Support
Will the project attract funding or public engagement?

Genetic Feasibility
Is there high-quality ancient DNA available? Is there a living species close enough to serve as a genetic or reproductive match?

Reproductive Compatibility
Can a living relative serve as a surrogate for reproduction, or are alternative reproductive methods possible?

Why the Dire Wolf?

Genetically Distinct and Symbolic
The dire wolf was a genetically distant species with a unique evolutionary lineage. Its revival showcases the ability to reconstruct truly extinct species.

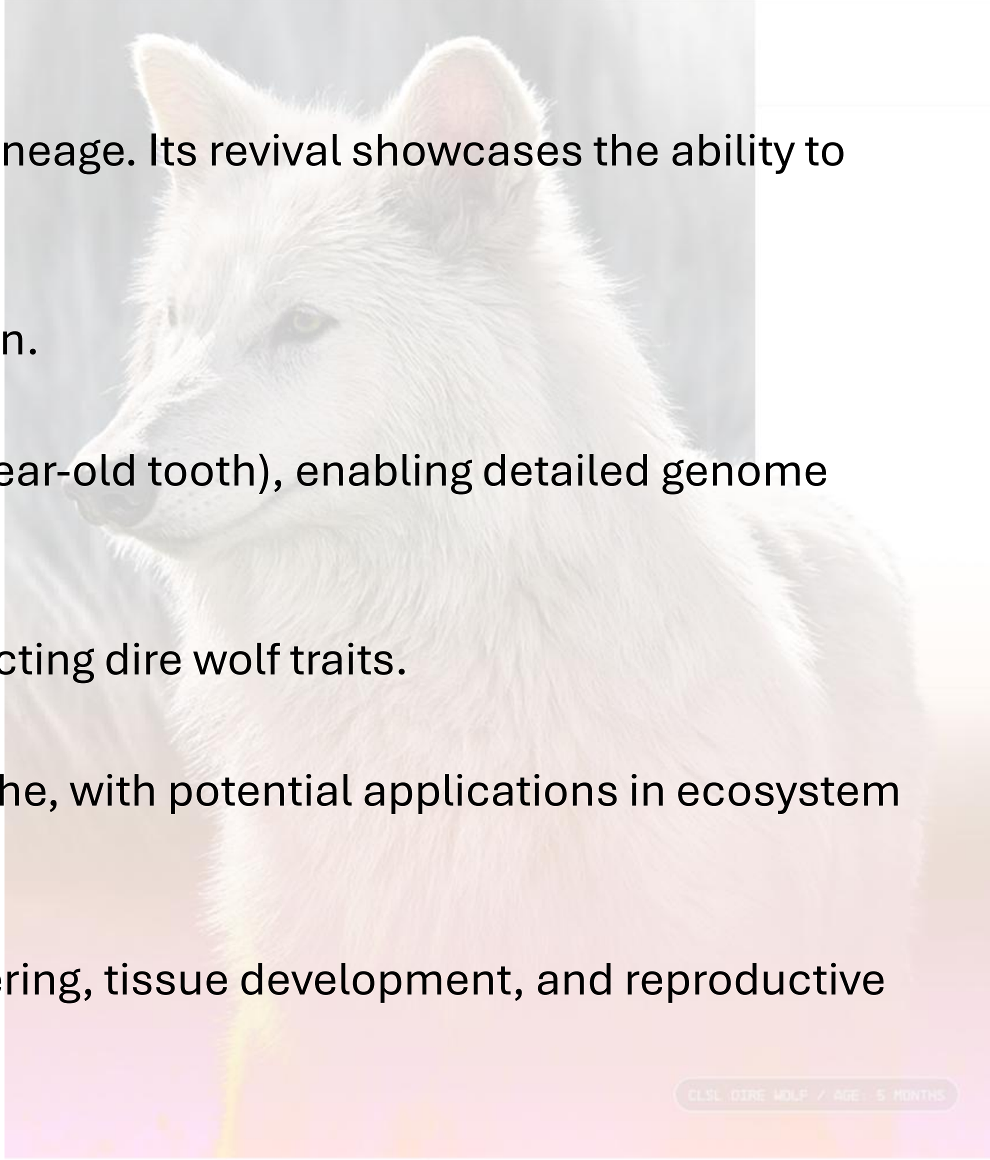
Strong Public and Cultural Resonance
Popularized by fiction and folklore, the dire wolf captures public imagination.

High-Quality Ancient DNA Available
Colossal obtained exceptionally preserved fossil samples (e.g., a 13,000-year-old tooth), enabling detailed genome reconstruction—far higher quality than many other extinct species.

Viable Living Relative for Reproduction
The gray wolf provides a practical surrogate and genetic base for reconstructing dire wolf traits.

Ecological Relevance and Potential Utility
As a large carnivore, the dire wolf could represent a functional predator niche, with potential applications in ecosystem modeling and restoration (though not yet intended for release).

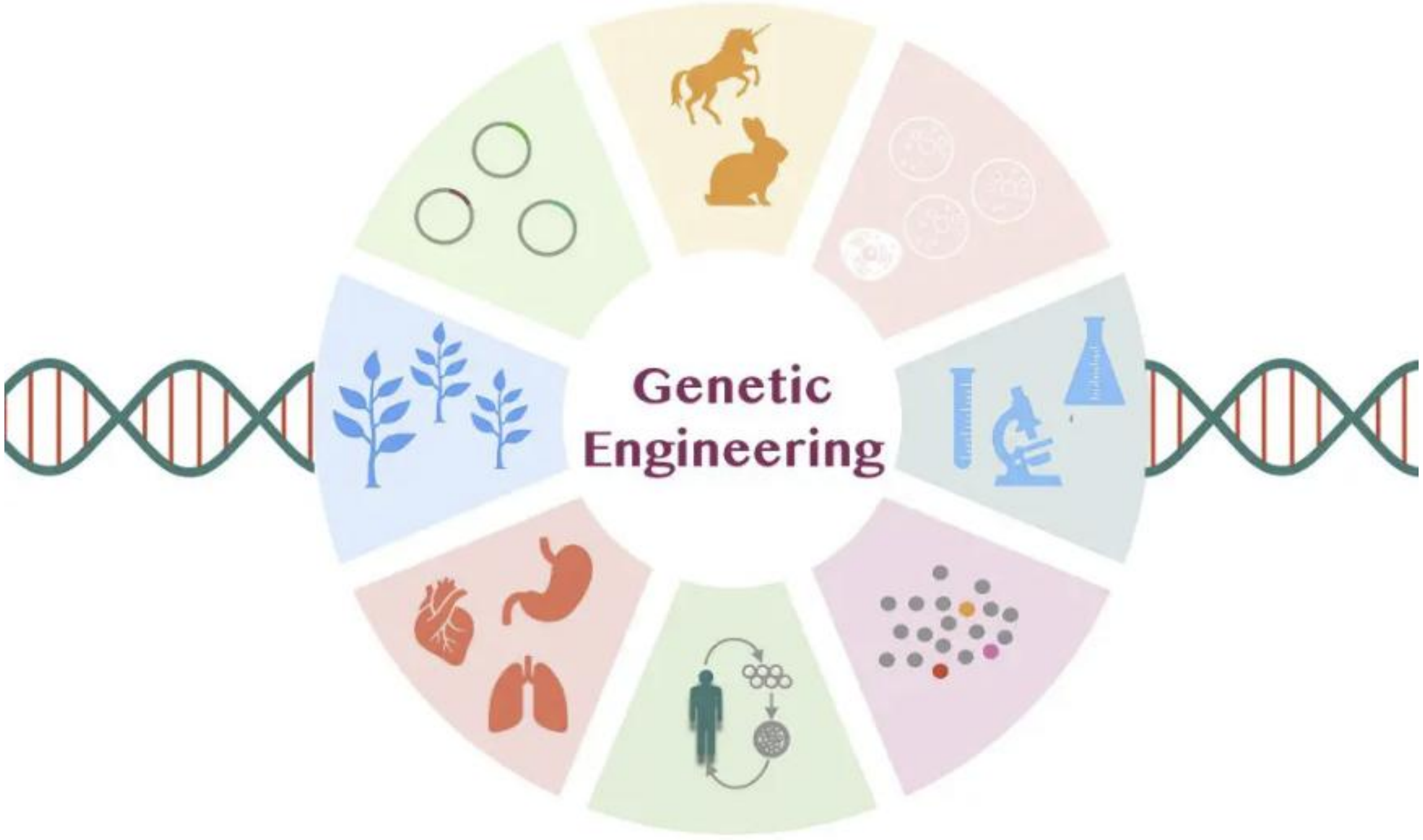
Platform for Technical Innovation
The dire wolf project serves as a proving ground for multi-gene trait engineering, tissue development, and reproductive biotechnology—all transferable to conservation efforts.



Why Not the Mammoth or Dodo?

Species	Challenges and Limitations
Woolly Mammoth	• Extremely ambitious — requires advanced gestation in endangered Asian elephants. • Long gestation and reproductive cycles make experimentation slow. • Large size, cold-climate adaptations, and habitat limitations add complexity.
Dodo	• Poor-quality or incomplete DNA due to humid fossil environments. • Limited close relatives; pigeons exist but have large physiological differences. • Ecological role unclear; may not justify functional revival effort.
Dire Wolf	• High-quality ancient DNA available. • Genetically distinct yet has a viable modern surrogate (domestic dog). • Technically feasible, ecologically meaningful, and culturally resonant.

Step 6: Project Design



Step 6: Project Design

De Novo Synthesis Is Theoretically Possible...

Why Not Build a Dire Wolf from Scratch?

Why editing was the better solution?

DNA Fragmentation in Fossils
Ancient dire wolf DNA is too degraded to reconstruct a full genome with high confidence.

Gaps in the Genome
Many regions are missing or unreadable in ancient DNA, making de novo synthesis incomplete or error-prone.

Cost and Complexity
Synthesizing a mammalian genome is extremely expensive and technically difficult.

Functional Uncertainty
Even if the full DNA was built, it's unclear whether a synthetic dire wolf genome would produce a viable, functional organism without testing and iteration.

Biological Feasibility
Using a modern wolf genome as a template allowed scientists to target and insert key phenotype-driving genes, producing a living organism with dire wolf-like traits.

Challenges of Working with Ancient DNA

DNA Damage Over Time
Ancient DNA is fragmented and chemically altered, making sequencing difficult.

Deamination Errors
Cytosine degrades into uracil, often misread as thymine—introducing false mutations.

Reference Bias in Mapping

- Ancient DNA is aligned to a modern genome (e.g., dog or gray wolf).
- Divergent fragments may fail to align, while only similar sequences map.
- This artificially inflates similarity and can distort genetic comparisons.

Impact on Evolutionary Inference
Biased mapping can lead to **incorrect conclusions** about species relationships and population history.

Problematic for Distantly Related Species
Especially severe when the reference genome is millions of years apart—as with the dire wolf.

DNA Sequencing

1. Two fossil specimens of dire wolves:

DireSP: A ~13,000-year-old tooth fossil from Ohio.

DireGB: A ~72,000-year-old skull fossil from Idaho.

2. Depth of Coverage

DireSP: 3.4× coverage — relatively low, but usable.

DireGB: 12.8× coverage — very good for ancient DNA.

3. How Many DNA Reads Did They Get?

2.8 billion usable reads from DireSP

33.3 billion usable reads from DireGB

This is a huge improvement over previous attempts (which had only 0.2× and 0.3× coverage).

4. Mapping the Reads to a Reference Genome

Because ancient DNA is fragmented, they aligned these reads to the genome of a modern gray wolf.

Problem: Dire wolves are genetically distant from gray wolves, so not all reads will map well.

Only **6%** of the genome had good depth (≥5×) in DireSP.

But **82%** of DireGB's genome was covered at ≥5× — excellent.

DNA Sequencing

5. Rebuilding a Better Genome

Since gray wolves and dire wolves are different, they rebuilt a dire wolf-specific reference genome. They ran 6 rounds of refinement to gradually move away from the gray wolf reference. They found:

- Over **14.5 million differences**, including:
  - ~13.6 million single nucleotide changes (SNPs)
  - ~388,000 insertions
  - ~515,000 deletions

They created a “pseudo-haploid” consensus genome, picking the most frequent variant at each position.

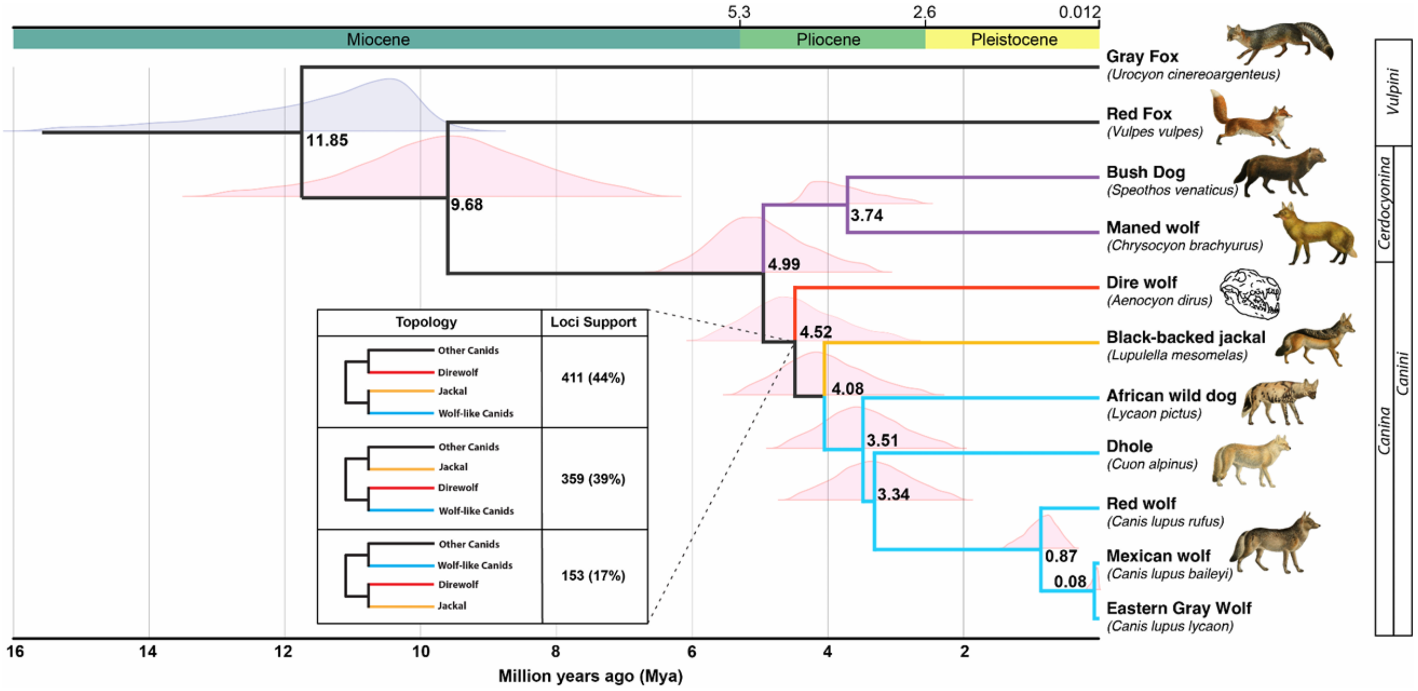
6. Mitochondrial Genome

Separately, they also reconstructed the mitochondrial genome at **very high depth: 158.4×**. This helps trace evolutionary history and maternal lineage with high accuracy.

DNA Sequencing

Final Assembly

- By combining the nuclear and mitochondrial genomes, they produced a near-complete dire wolf genome assembly.
- About **16.8% of the genome** is still masked as unknown (“N content”), which is normal for ancient DNA.
- **71% of all protein-coding genes** were retained compared to the modern gray wolf genome.
- Missing genes were mostly in low-coverage or damaged regions, not due to real absence.
- Mitochondrial and nuclear DNA was used to build evolutionary trees of the dire wolf.



Where Do Dire Wolves Fit in the Evolutionary Tree?

- Nuclear data placed dire wolves in a **separate, early-diverging branch** — before jackals and modern wolves split.
- But: Only **44% of DNA regions** supported this placement; **39%** suggested a closer relationship to other wolves.
- Mapping ancient DNA to the gray wolf genome **introduced bias** — made dire wolves appear more wolf-like than they really are.

If the reference genome can affect where a species appears on the tree, how might that impact our scientific conclusions?

- **Reference bias** can lead to **wrong assumptions** about species' relationships.
- If you start with the wrong assumptions about where the dire wolf fits on the evolutionary tree, you might:
- Compare it to the wrong species.
- Misidentify which genes were **truly unique** vs. shared.
- Introduce **errors into any gene-editing plan** aimed at recreating its traits.

Step 6: Project Design

Now that we have the sequenced DNA and know where the dire wolf fits on the evolutionary tree, what would you do next?

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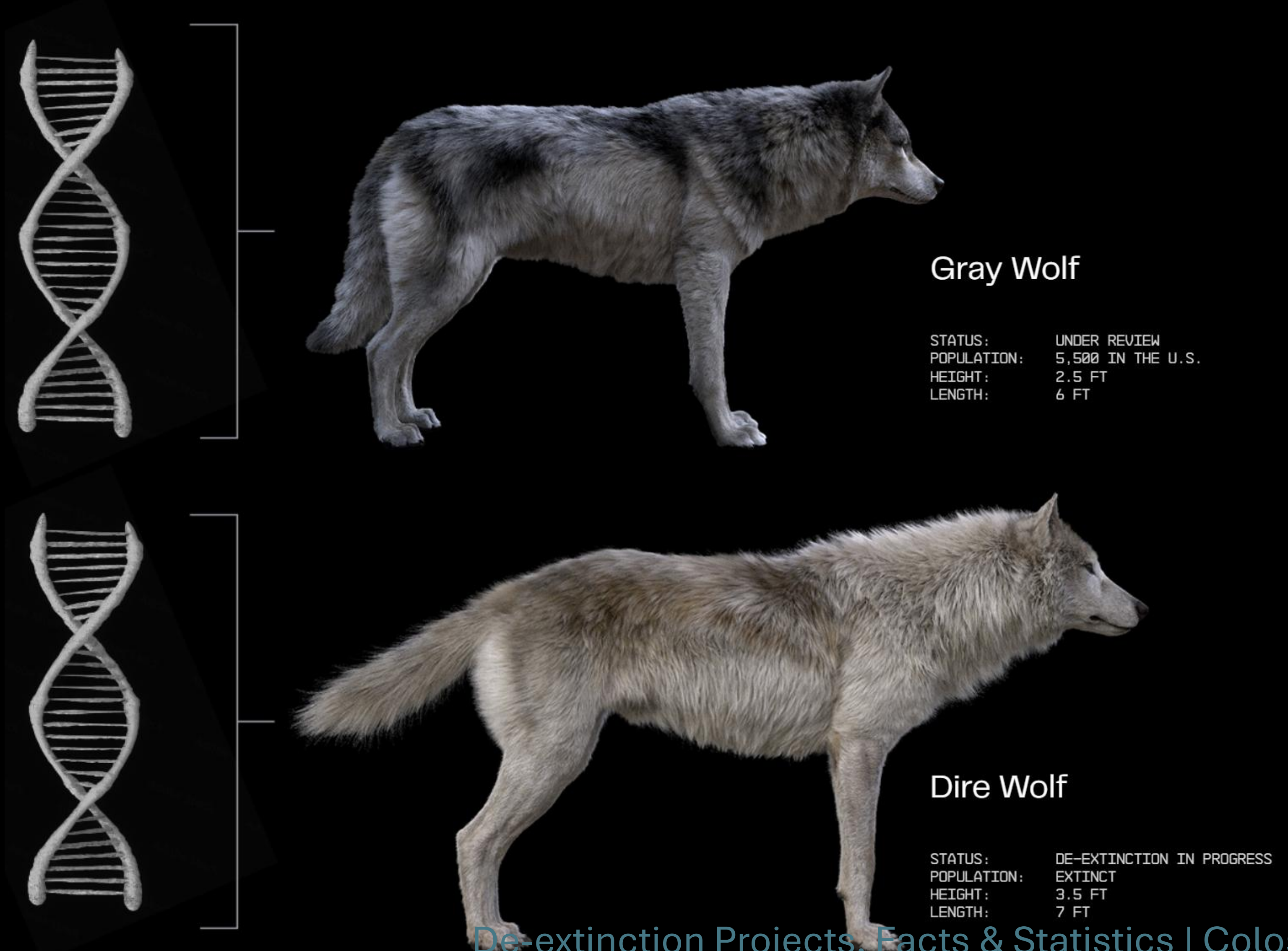
Step 6: Project Design

What makes the dire wolf genetically unique compared to other canids, especially the gray wolf?

Is it enough to just look at all the differential genes?



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What Makes the Dire Wolf Unique?

Gene Selection in Dire Wolves

80 genes under positive selection.

Functional Relevance

Mutations found in protein domains.

Why It Matters?

- Highlights genes that shaped dire wolf traits.
- Provides targets for gene-editing.
- These genes may be critical to recreate dire wolf-like physiology or behavior.

Would you prioritize editing these 80 genes in a de-extinction effort?

Why Not Edit All 80 Positively Selected Genes?

1. Not All Positively Selected Genes Are Functionally Important

- Reflect neutral evolution or mild adaptation.
- Be redundant or have minimal functional impact.
- Affect traits irrelevant to dire wolf identity (e.g., minor metabolism tweaks).

2. Lack of Functional Annotation

Many genes have unknown or poorly characterized functions. Without knowing what the gene does, researchers can’t predict:

- What trait it affects.
- Whether editing it will help or harm.

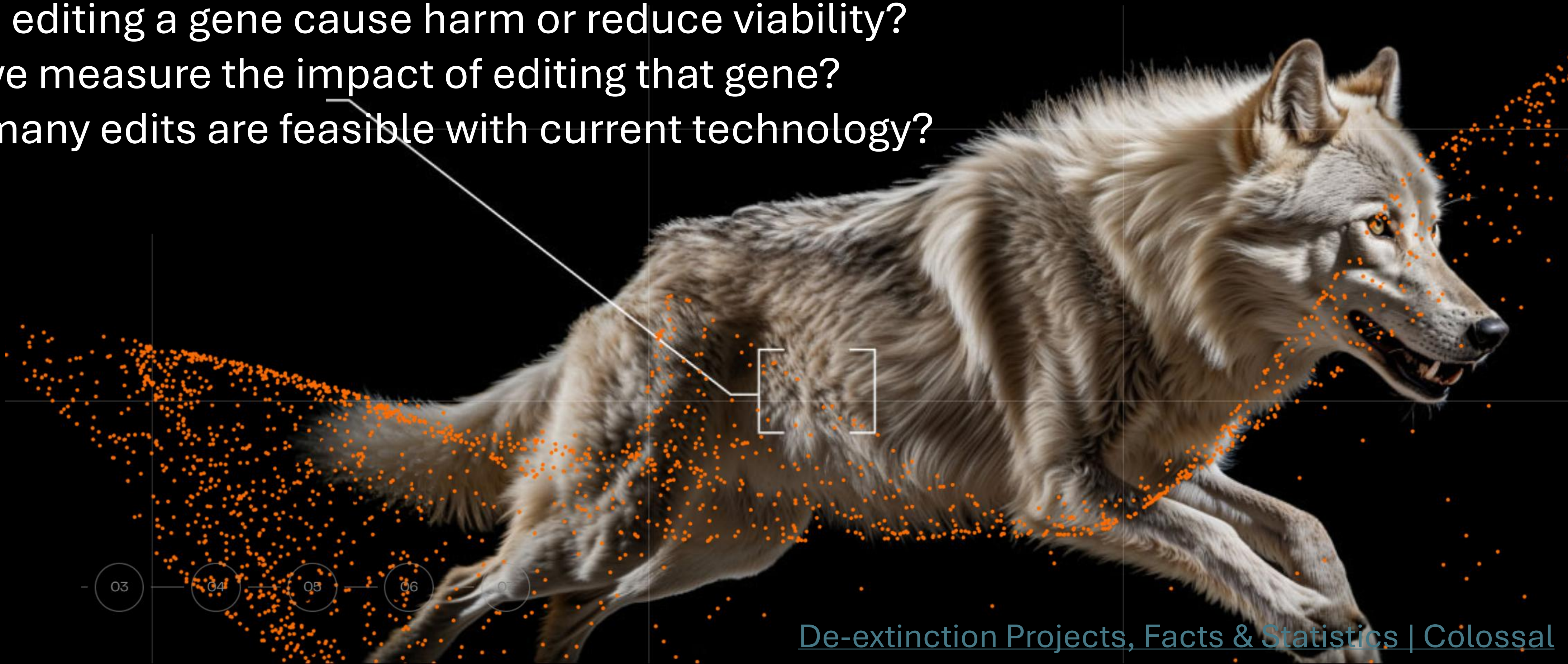
3. Focus on Feasibility and Safety

Editing dozens of genes:

- Increases the risk of off-target effects.
- Makes it harder to troubleshoot outcomes.
- Could reduce viability or fertility in edited animals.

How Do You Choose Which Genes to Edit?

- Which genes are most likely to affect visible or functional traits?
- Do we know what each gene actually does?
- Could editing a gene cause harm or reduce viability?
- Can we measure the impact of editing that gene?
- How many edits are feasible with current technology?



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How Did Scientists Choose Just 6 Genes to Edit?

1. Function Matters

- Focused on genes with known links to skull shape, limbs, senses, or behavior.
- Excluded genes with unclear or irrelevant functions.

2. Unique and Likely Functional Changes

- Picked mutations that change the protein (not silent).
- Chosen variants were present in both dire wolves and absent in modern wolves.

3. Tissue Relevance

- Genes had to be active in tissues like bone, brain, or muscle.

4. Feasibility and Safety

- Edits had to be technically possible.
- Avoided genes that could harm embryo development or fertility.

What Gene Editing Method Would You Choose?

Scientists selected **20 gene edits** in the gray wolf DNA in **14 genes**, to express multiple dire wolf–like traits, including larger size, wider head, strong jaws, more muscular limbs, unique vocalizations, and pale coat coloration.

- To engineer specific traits into a modern wolf genome, we need to edit precise locations in the DNA.
- What gene-editing tool would you use?
- CRISPR-Cas9 was chosen because of its high efficiency, ease of use, and ability to make multiple edits at once.

How Would You Create a Dire Wolf Embryo?

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How Would You Create a Dire Wolf Embryo?

Method	Description	Pros	Cons
SCNT (Somatic Cell Nuclear Transfer)	Transfer nucleus of edited cell into enucleated egg	Proven, full genome transfer, viable in mammals	Technically complex, low efficiency
iPSC + Embryo Formation	Reprogram edited cells into induced pluripotent stem cells and form embryo	Potential for more flexibility	Still experimental in many mammals
Direct Embryo Editing	Inject CRISPR into early-stage embryos	Can be simpler than SCNT	Limited to a few edits, high mosaicism risk
Germline Editing in Lineage	Edit embryos of a closely related species over generations	May achieve long-term traits without cloning	Requires breeding over many generations

Why was SCNT Selected?

Nucleus is extracted from a somatic cell (any non-reproductive cell).
This nucleus is inserted into an egg cell that had its own nucleus removed.
The resulting egg is stimulated to begin dividing into an embryo.

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Why was SCNT Selected?

1. Guarantees All Edits Are Present

SCNT starts with a **single, verified, fully edited donor cell** (like a fibroblast), ensuring the resulting embryo carries **all intended modifications** in every cell.

2. Avoids Mosaicism

Direct embryo editing often results in **mosaic embryos**—where some cells have the edit and others don't. SCNT avoids this by reprogramming a **fully edited adult somatic cell** into an embryo, producing a **genetically uniform organism**.

3. High Gene-Editing Complexity

Projects like Colossal's involve editing **dozens of genes simultaneously**. SCNT allows to **screen edited cells in vitro**, confirming all edits and ruling out off-target effects **before** making an embryo.

4. Species Viability and Surrogacy

SCNT allows scientists to **implant the cloned embryo into a surrogate** (e.g., an Alaskan gray wolf) and track development from day one.

What Somatic Cell Should You Use for SCNT?

To clone a dire wolf, researchers needed a donor cell that:

- ❖ Is easy to culture and expand in vitro
- ❖ Can be efficiently edited
- ❖ Supports reprogramming into a viable embryo

Common Somatic Cell Options:

- Fibroblasts (from skin)
- Blood-derived cells (e.g., EPCs)
- Urine-derived epithelial cells
- Adipose-derived stem cells

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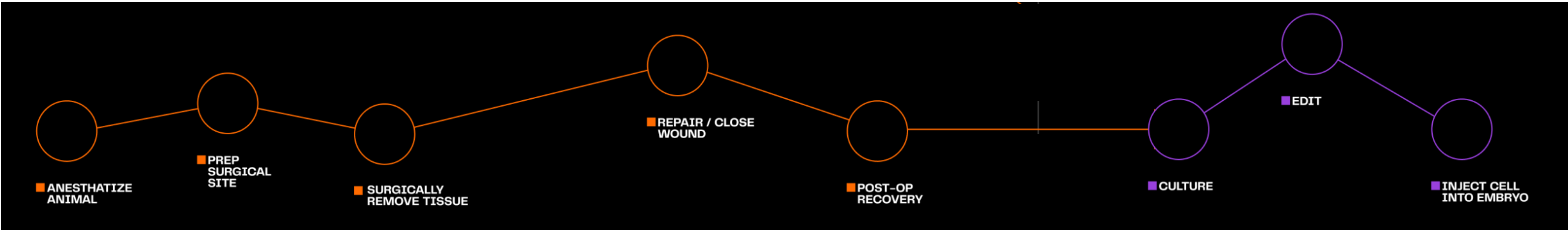

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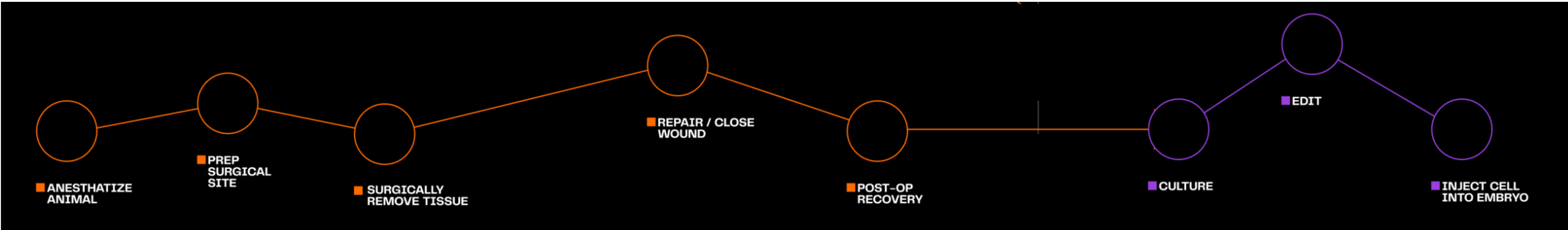

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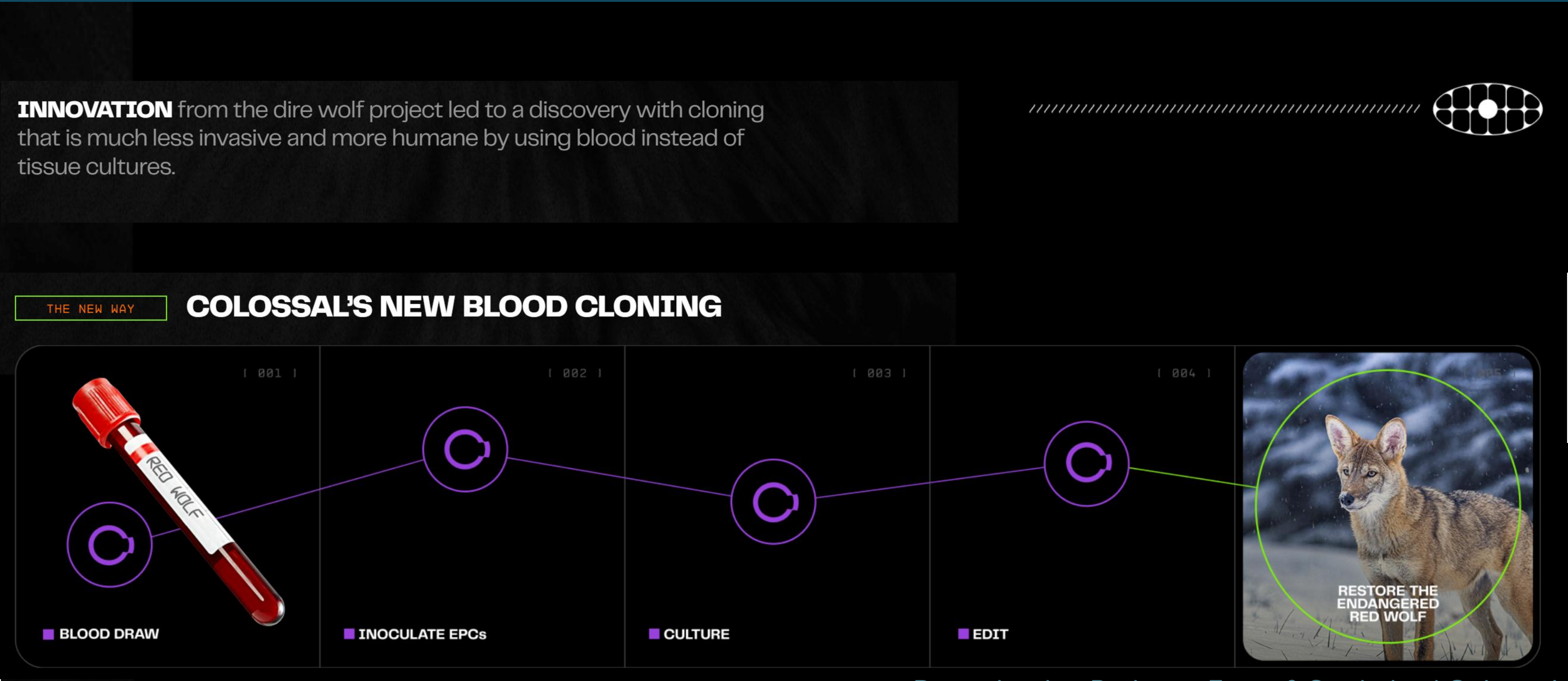

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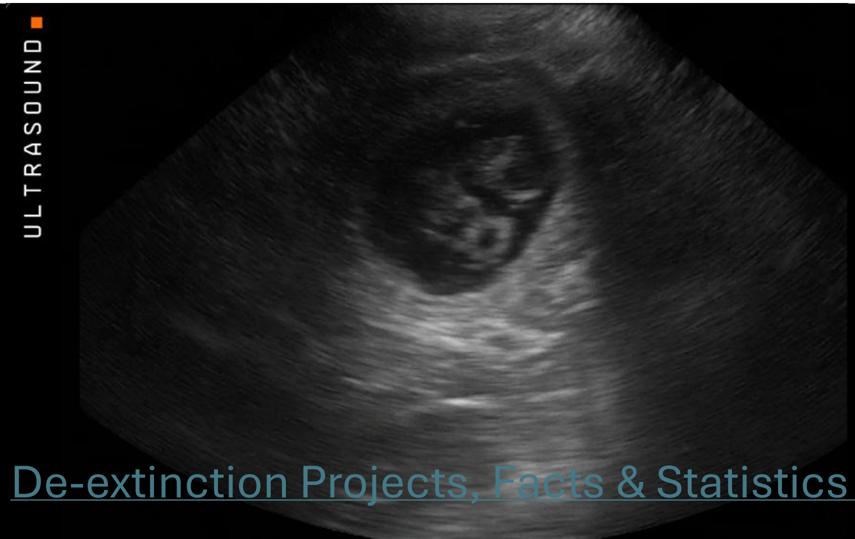
Implanting the Embryo into a Surrogate Mother

- After SCNT, the edited embryo is ready for implantation.
- Embryo is transferred into a surrogate animal (e.g., Alaskan gray wolf).
- Proper timing of implantation is crucial for pregnancy success.
- Development is monitored throughout gestation.



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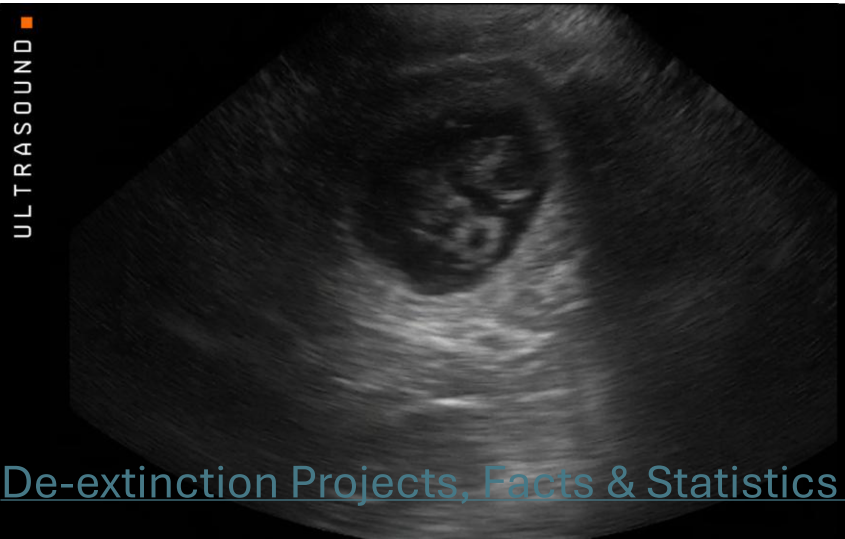
Implanting the Embryo into a Surrogate Mother

Challenges of Cross-Species Surrogacy:

- Uterine environment may differ (hormones, signaling molecules)
- Timing of implantation and gestation must align
- Potential immune rejection
- Risk of developmental mismatch between embryo and surrogate



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The Dire Wolf Pups — Romulus, Remus, and Khaleesi

- From 45 engineered ova, four viable embryos were implanted into surrogates.
- Three pups born:
❖ Romulus and Remus (males) — born October 1, 2024
❖ Khaleesi (female) — born January 30, 2025
- All three pups survived infancy, marking a successful outcome

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Pupdate!
A Howl Lost
to Time,
RESTORED
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Can We Call It a Species Yet?

Is one or even three animals enough?
What would have happened if only males were born?

TIMELINE OVERVIEW

NEONATAL 0-2 WEEKS	TRANSITIONAL 2-4 WEEKS	SOCIALIZATION 3-12 WEEKS	RANKING 3-6 MONTHS	ADOLESCENCE 6-18 MONTHS	ADULT +18 MONTHS
+ Born blind, deaf and with little sense of smell.	+ Eyes open and are blue at 11-15 days.	+ Ears begin to raise; hearing improves significantly.	+ Period of rapid growth.	+ Can now travel with pack.	+ Developmental growth complete.
+ Nursing pups feed four or five times a day.	+ Begin to stand, walk, growl and chew.	+ Gradual process of weaning begins.	+ Pups begin to join hunting trips.	+ End of skeletal growth.	+ Intelligent, with strong problem-solving skills.

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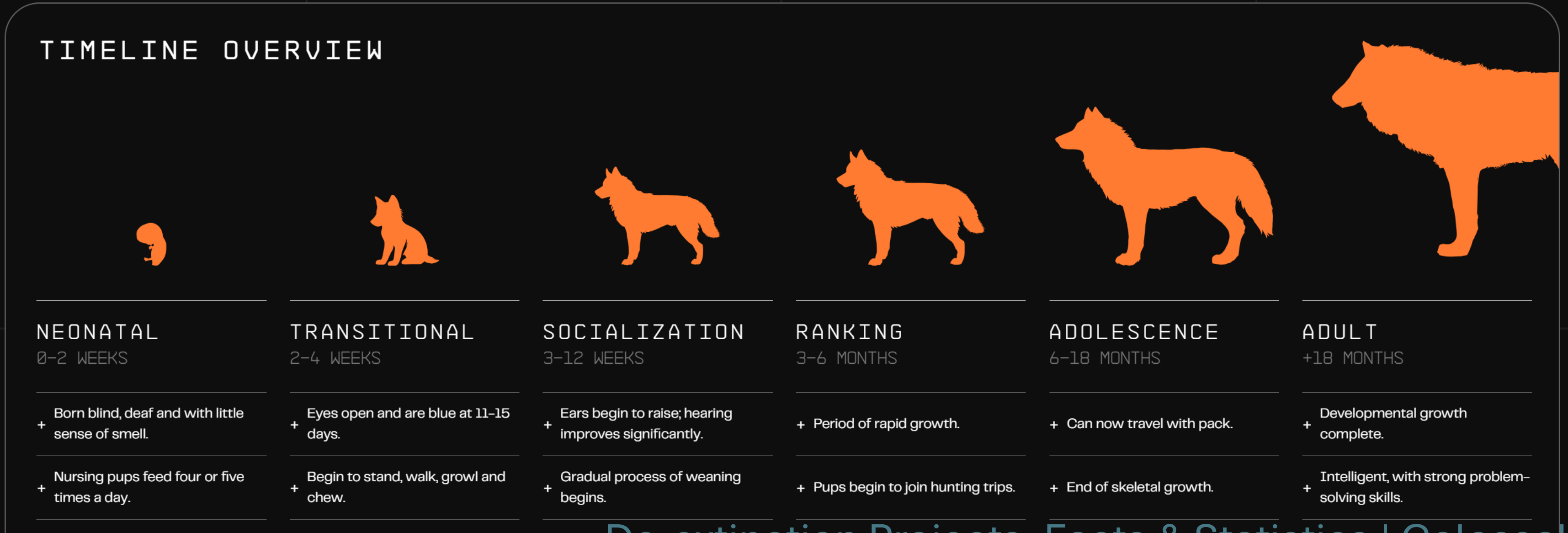
Can They Reproduce? The True Test of De-Extinction

- Fertility: Will these hybrids be fertile at all?
- Compatibility: Are their mating behaviors and cycles in sync with each other or with modern wolves?
- Parental behavior: If surrogates raise the first generation, will those pups learn species-typical behaviors?
- Genetic bottleneck: Starting with just three animals risks inbreeding — so more edited animals or hybrid crossings may be necessary to boost genetic diversity.



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