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asociación comercial española de fertilizantes
1º CONGRESO SOBRE FERTILIZACIÓN
26 al 27 Septiembre 2017
Madrid - España

Patrocinadores:
FERTINAGRO BIOTECH
ICL Specialty Fertilizers
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Empresa colaboradora:
Plant Response

On the road to making nitrogen fixing plants

Stefan Burén, Sep 26th 2017

BNF Cereals

BILL & MELINDA GATES foundation

Universidad Politécnica de Madrid

POLITÉCNICA

CBGP CENTRO DE BIOTECNOLOGÍA Y GENÓMICA DE PLANTAS

EXCELENCIA SEVERO OCHOA 2017-2021

Making N_2-fixing plants

Research supported by the Bill & Melinda Gates foundation

Provide technology for farmers earning < 1 $ / day

BILL & MELINDA GATES foundation

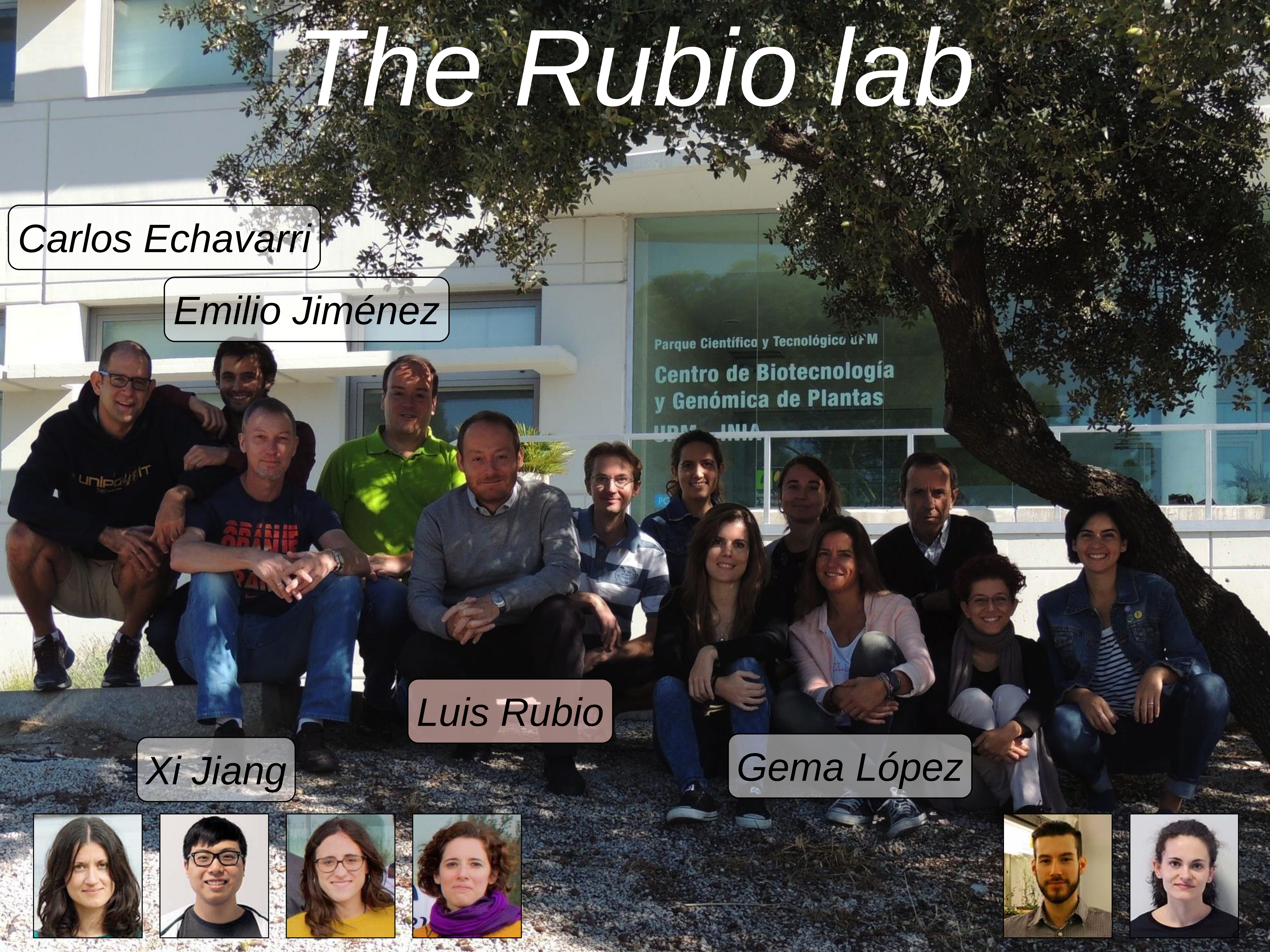
El proyecto español que ha enamorado a Bill Gates

- La Fundación Bill & Melinda Gates apoya con cinco millones de euros una investigación sobre agricultura sostenible desarrollada por la Universidad Politécnica de Madrid.

El País 2017

Luis Manuel Rubio, en el Centro de Biotecnología y Genómica de Plantas. ÁLVARO GARCÍA

The Rubio lab



Partners & collaborators

Dennis Dean

Virginia Tech

Paul Christou

Universitat de Lleida

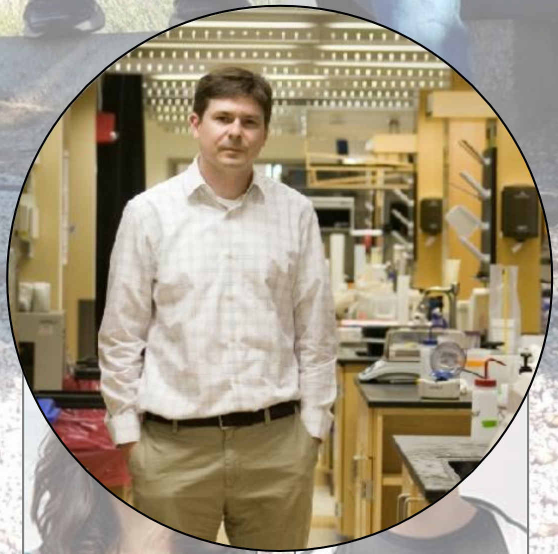
Leo Curatti

CIMA CONICET UBA

Elena Caro

POLITÉCNICA "Engineering the future" INTERNATIONAL CAMPUS OF EXCELLENCE

Chris Voigt



Massachusetts Institute of Technology

Overview talk

- Introduction nitrogen (N) chemistry
- Why we need for nitrogen
- Nitrogen fixation
  - Anthropogenic nitrogen fixation
  - Natural/biological nitrogen fixation
    - The nitrogenase protein

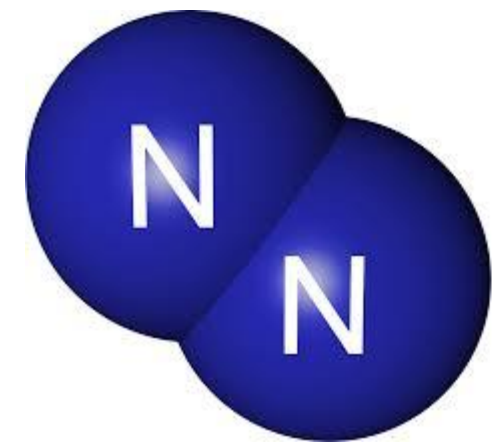
- Attempts to increase BNF using biotechnology

Our approach in the Rubio lab
    - What we accomplished and next steps

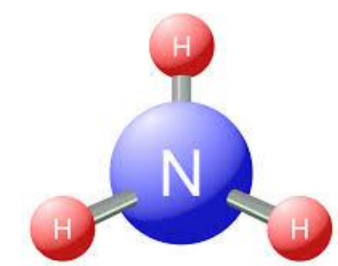
Nitrogen – plentiful but inaccessible

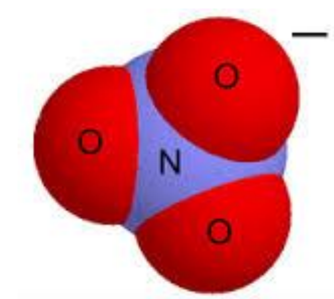
- N_2 forms 78% of earth atmosphere (**!**)
- N≡N second strongest bond (after CO)

⇨ Difficult to “break” ⇨ Inaccessible



- Most prokaryotes and all eukaryotes can only use:
  - “fixed” or “reactive” nitrogen (Nr)
  - e.g. ammonia (NH_3), nitrate (NO_3^-), nitrogen oxide (NO_x), nitrous oxide (N_2O), …



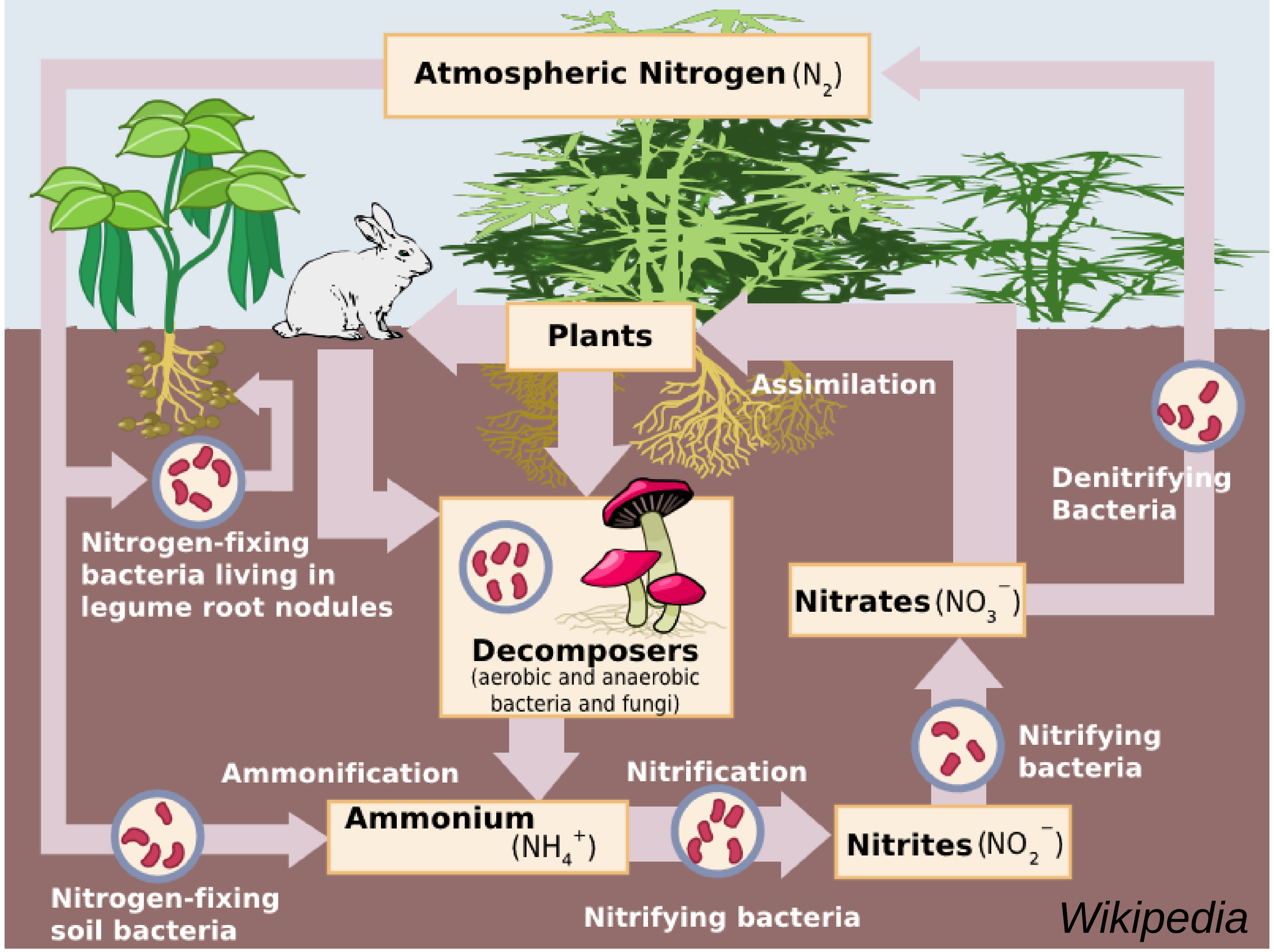


- Human body consists of 3% nitrogen:
  - Amino acids (proteins)
  - Nucleic acids (DNA and RNA)
  - Molecules for energy transfer and signalling (ATP, NADH, …)

Natural sources of nitrogen

Three natural sources of fixed nitrogen (Nr):

- Biological nitrogen fixation (BNF) >90%
  - symbiotic/non-symbiotic bacteria – **Nitrogenase protein**
- Lightning (NO_x) ~5%
- Volcanic activities (NH_3)



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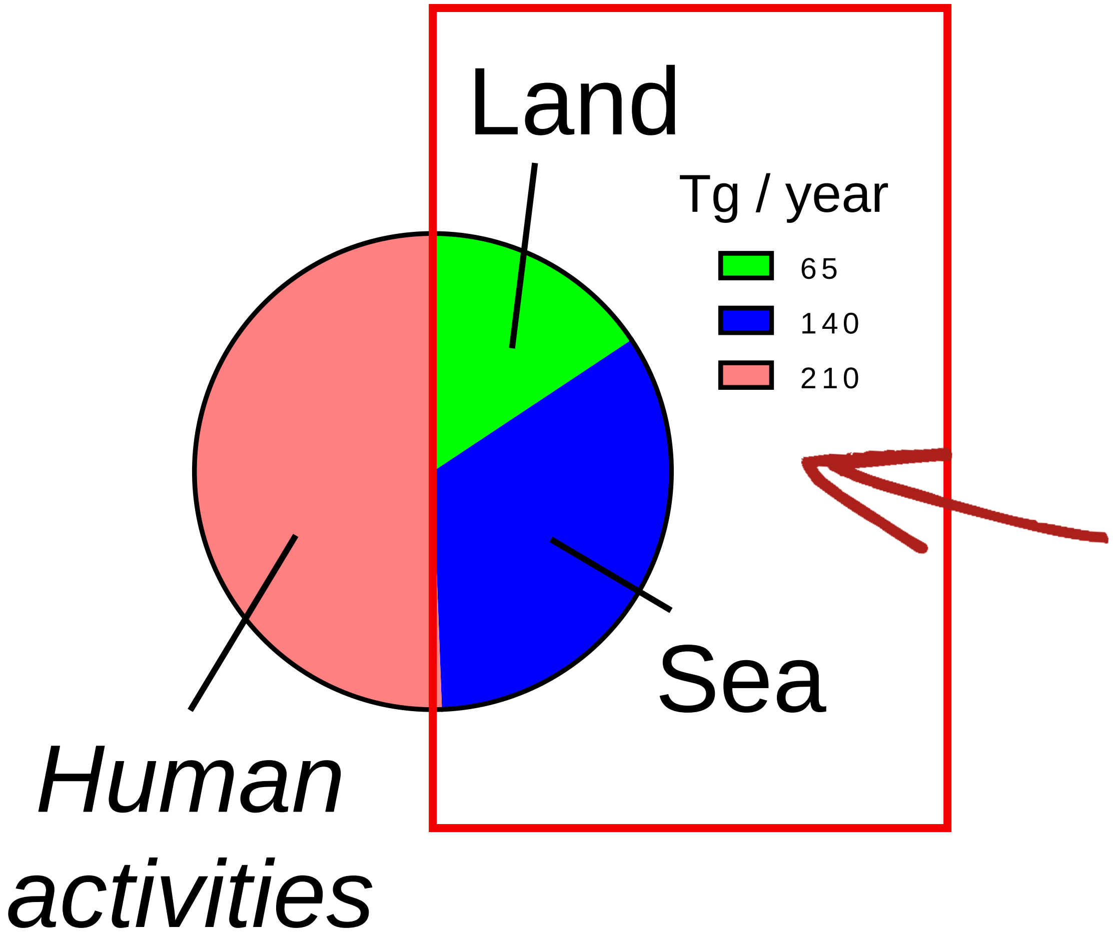
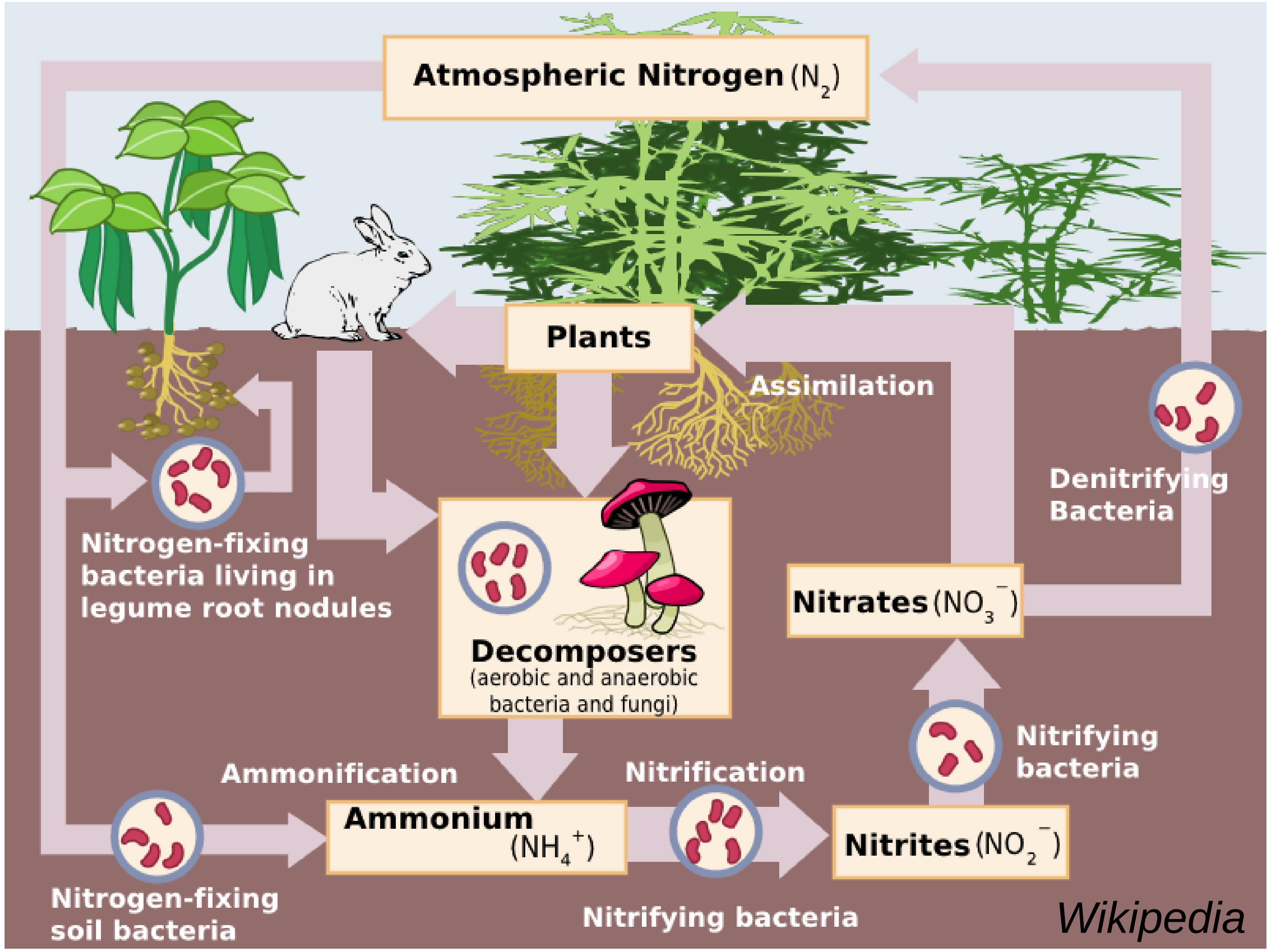




Anthropogenic reactive nitrogen

- Use of fossil fuels, biomass burning ← 15%
- BNF (due to agriculture) ← 30%
- Industrial N fixation, synthetic fertilizers ← 55%

Estimated values

⇨ 80% of N in human body from Haber-Bosch process

⇨ Negative consequences from increased Nr:

↑ Use of non-renewable resources
Water and air pollution
Greenhouse gases
Eutrophication, …

↓ Biodiversity
…

⇨ **Increase BNF using biotechnology?**

Erisman et al., WWF (2015), Howarth, Harmful Algae (2008), Fowler et al., Phil. Trans. R. Soc. B., (2013)

How can biotechnology increase BNF?

Nitrogen Fixation Research: A Key to World Food?

Investigators in a variety of disciplines are searching for new technologies for producing fixed nitrogen.

Science (1975) R. W. F. Hardy and U. D. Havelka

THE NITROGEN FIX

Few projects in plant biotechnology are harder, or promise a greater payoff, than enabling crops to make their own nitrogen fertilizer

By Erik Stokstad *Science (2016)*

genetic makeup of bacteria or the grains? Cereals that could provide their own fertilizer are beyond doubt the biggest prize of all in the gift of the new biology —far bigger in terms of lives to be saved than even the conquest of cancer or a cow that could digest sawdust.

Recent advances:

- **Biochemistry of nitrogenase**
- **Genetic tools**

Now possible?

PLANT SCIENCE

Future Prospects for Cereals That Fix Nitrogen

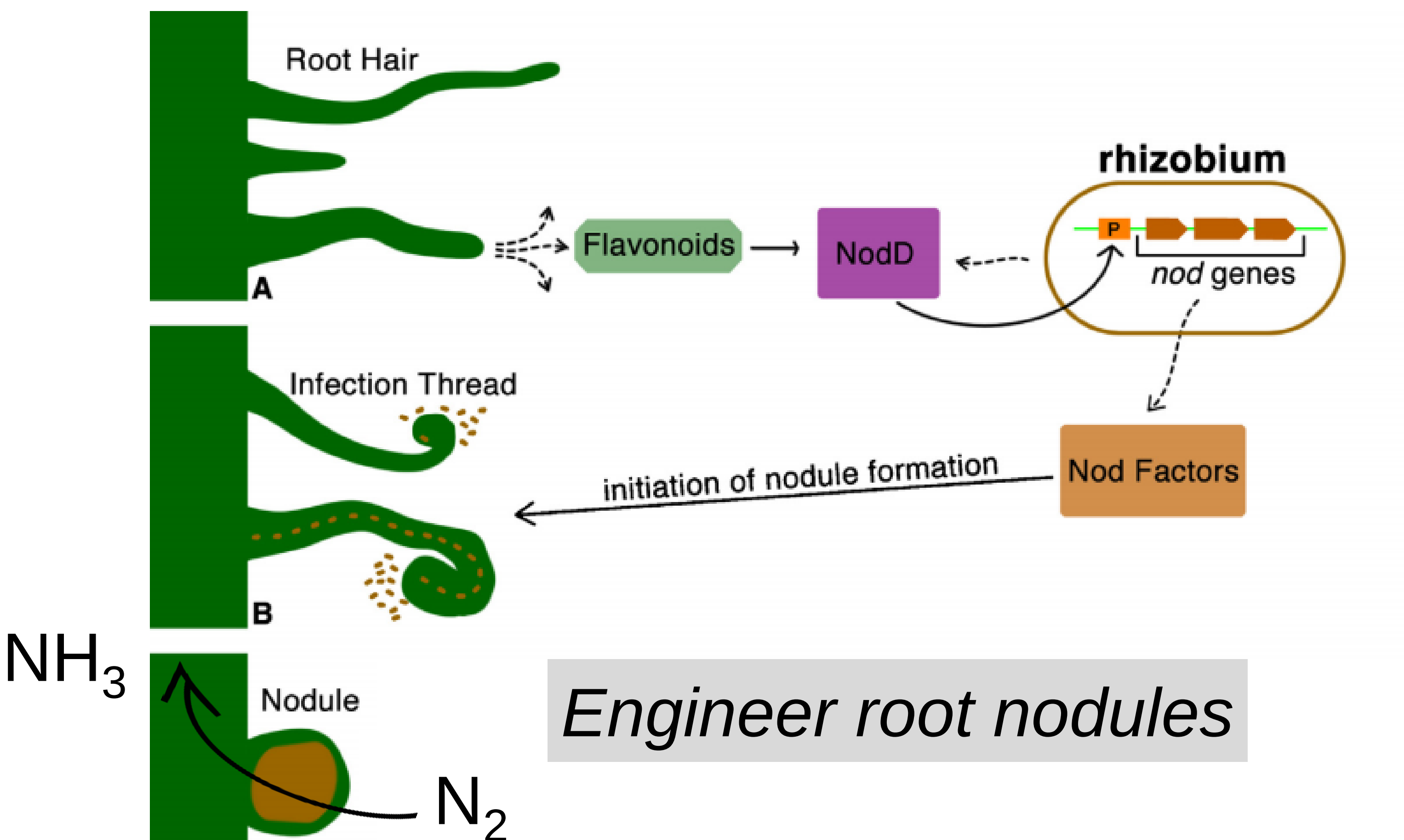
Perrin H. Beatty and Allen G. Good *Science (2011)*

Keeping the nitrogen-fixation dream alive

Emilio Jimenez Vicente[a] and Dennis R. Dean[a,1] *PNAS (2017)*

Three strategies to increase BNF using biotech

1. New symbiosis between non-legume crops and N_2-fixing bacteria:
   - **Engineer cereals** (e.g. maize) to sense/associate with N_2-fixing bacteria, and **to form nodules**
   - “Make cereals into legumes”
   - Novel symbiosis will provide fixed nitrogen



M. Laranjo et al., Microbiol. Res. (2014)

BILL & MELINDA GATES *foundation*

Prof. Giles Oldroyd
John Innes Centre
Norwich, England

Three strategies to increase BNF using biotech

1. New symbiosis between non-legume crops and N_2-fixing bacteria

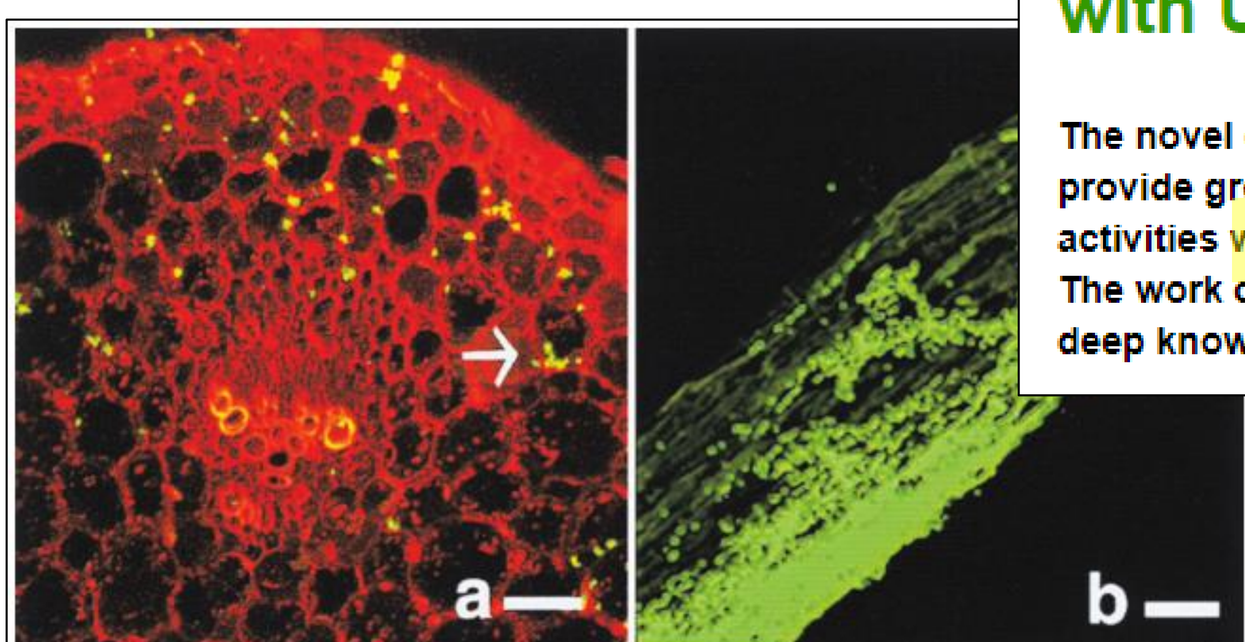
2. Enhance/improve N_2-fixing bacteria naturally associated with cereals:
   - **Engineer bacteria** (not the plant)
   - Non-symbiotic interactions (at surface or inside plant tissue)

Thursday - September 14, 2017

Bayer and Ginkgo Bioworks join forces for sustainable agriculture, forming new company with USD 100 million Series A

The novel company will focus on transformational beneficial microbes for plants to provide growers with next generation solutions to their biggest challenges / Initial activities will focus on nitrogen fixation, minimizing agriculture's environmental impact / The work combines the synthetic biology leadership of Ginkgo Bioworks with Bayer's deep knowledge and experience in agriculture and microbial products

GFP labelled *Klebsiella* in maize stem and root



Nodule-independent associations

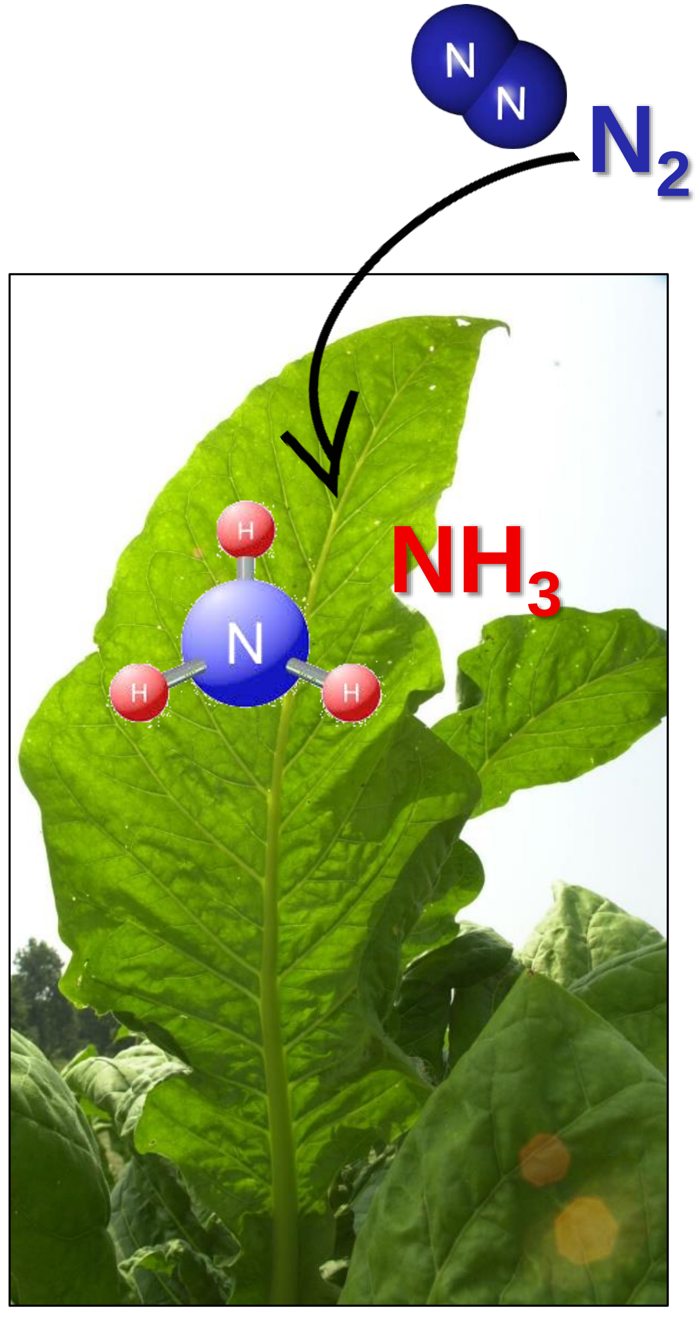
Chelius and Triplett, Appl. Environ. Microbiol. (2000)

Three strategies to increase BNF using biotech

1. New symbiosis between non-legume crops and N_2-fixing bacteria

2. Enhance/improve N_2-fixing bacteria naturally associated with cereals

3. Transfer of the prokaryotic N_2-fixing machinery into the plant cell:
    - **Nitrogenase (nif) genes to plant genome**
    - Making the plant capable of fixing N_2 from air, without involvement of symbiotic bacteria
    - Machinery available “within the plant”



The nitrogenase protein components

Nitrogenase, a complex formed by several proteins:

- two **NifD/NifK** dimers (2x NifDK)
- each with a **NifH** bound

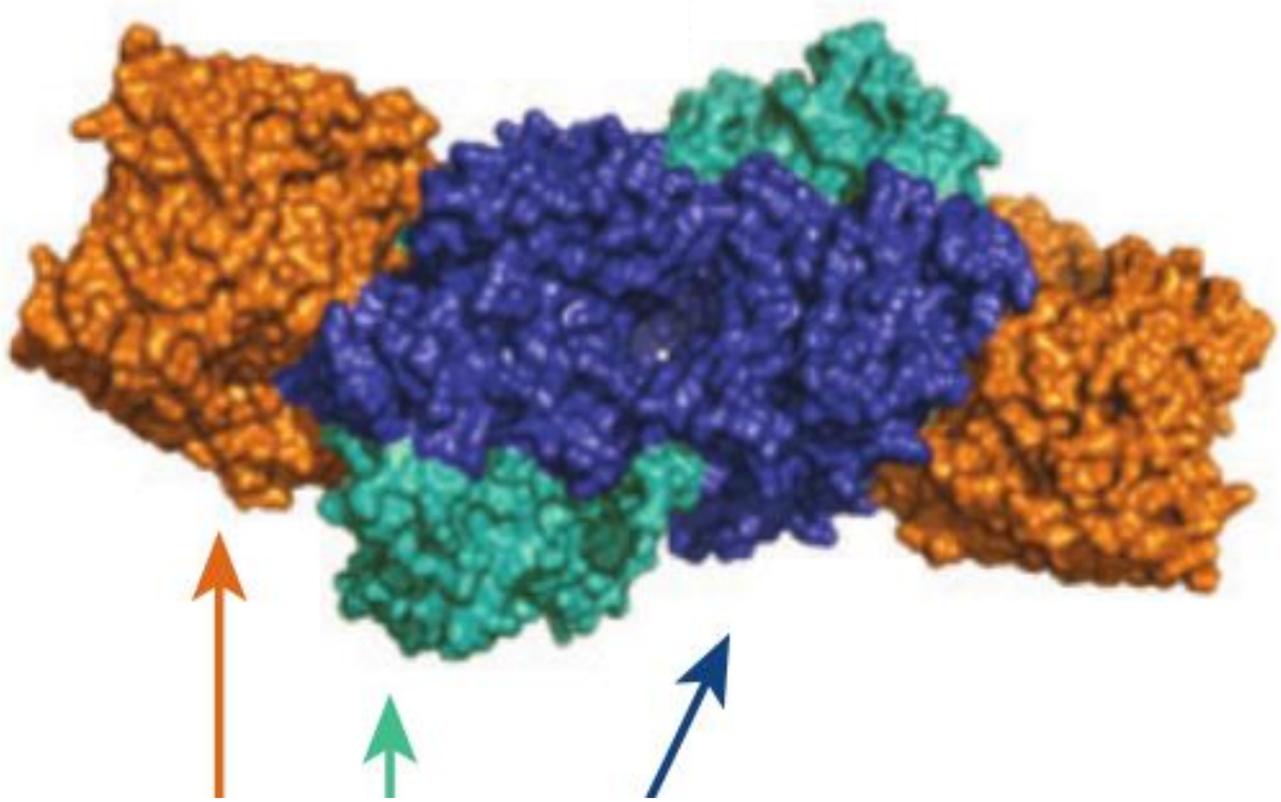
NifDK:

- Reduces N_2 to NH_3

nif genes

$$\mathbf{N_2} + 8\,H^+ + 8\,e^- + 16\,ATP \xrightarrow{\text{nitrogenase}} 2\,\mathbf{NH_3} + H_2 + 16\,ADP + 16\,P_i$$

Biological Nitrogen Fixation



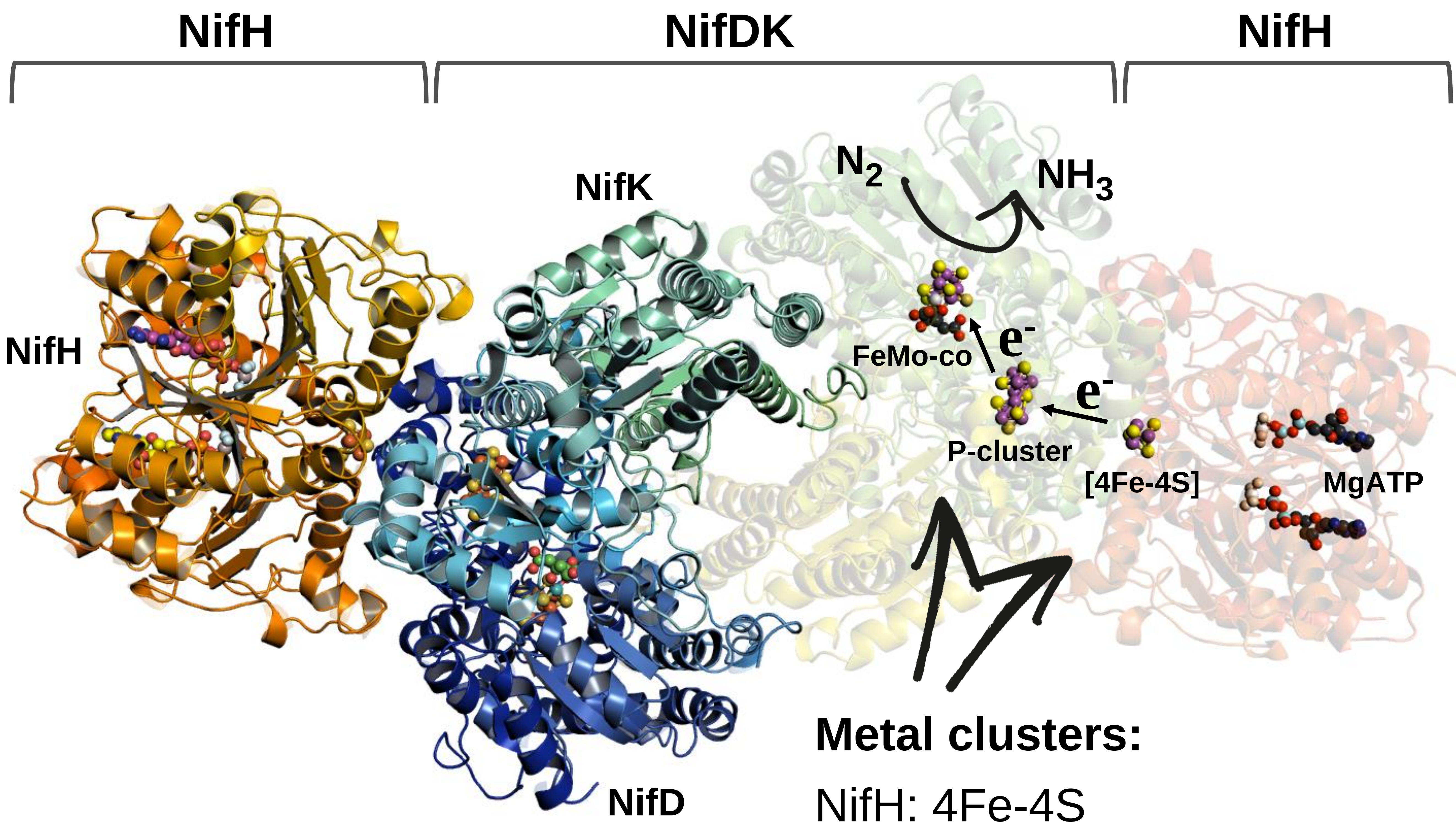
NifH NifD NifK

NifH:

- Provides $\mathbf{e^-}$ to NifDK needed for N_2 reduction
- **ATP** dependent reaction

Rubio and Ludden, Annu. Rev. Microbiol. (2008)

Mo nitrogenase and its cofactors



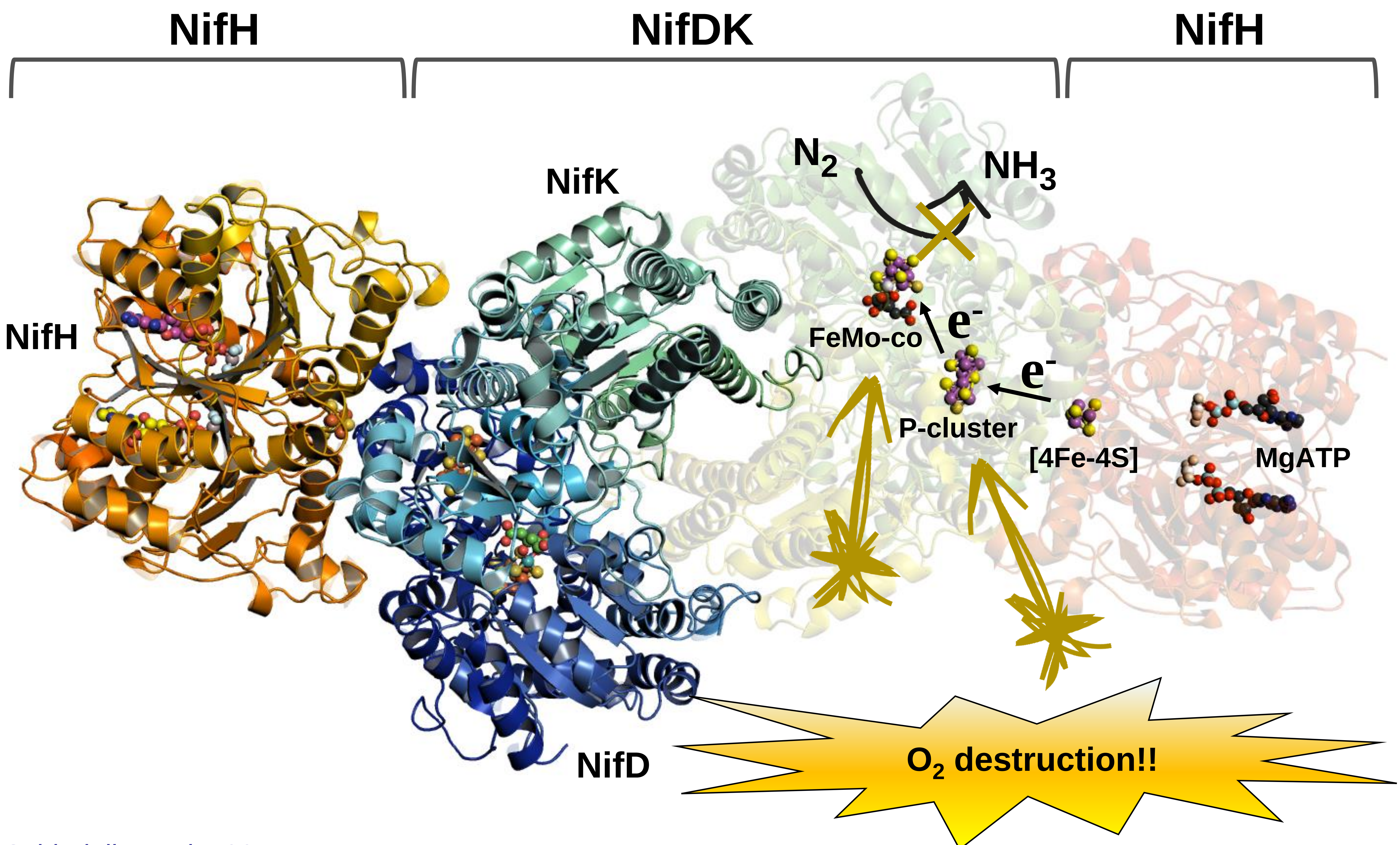
Metal clusters:

NifH: 4Fe-4S

NifDK: P-cluster and FeMo-co

Schindelin et al. 1997, Nature

Nitrogenase and oxygen



Schindelin et al. 1997, Nature

N_2-fixing plants using *nif* gene transfer

Two immediate obstacles to address:

1. Complex genetic requirements (“9-18 genes”)
   - Genetic machinery different in prokaryotes and eukaryotes

⇨ Synthetic biology

2. Oxygen damage of nitrogenase proteins
   - Photosynthesis generates O_2

⇨ 1. Express nitrogenase **when** O_2 levels are low (at night?)

⇨ 2. Express nitrogenase **where** O_2 levels are low (in roots?)

Beatty & Good, Science, 2011
Oldroyd & Dixon, Curr. Opin. Biotechnol., 2014
Curatti & Rubio, Plant Sci., 2014

Our solution to the O_2 problem

How solved in nature?

Azotobacter vinelandii (model organism):

- Free living, N_2-fixing bacteria
- **Aerobic**

- **Aerobic respiration → Energy needed for N_2-fixation**
- **Enhanced respiration → O_2 protective function**

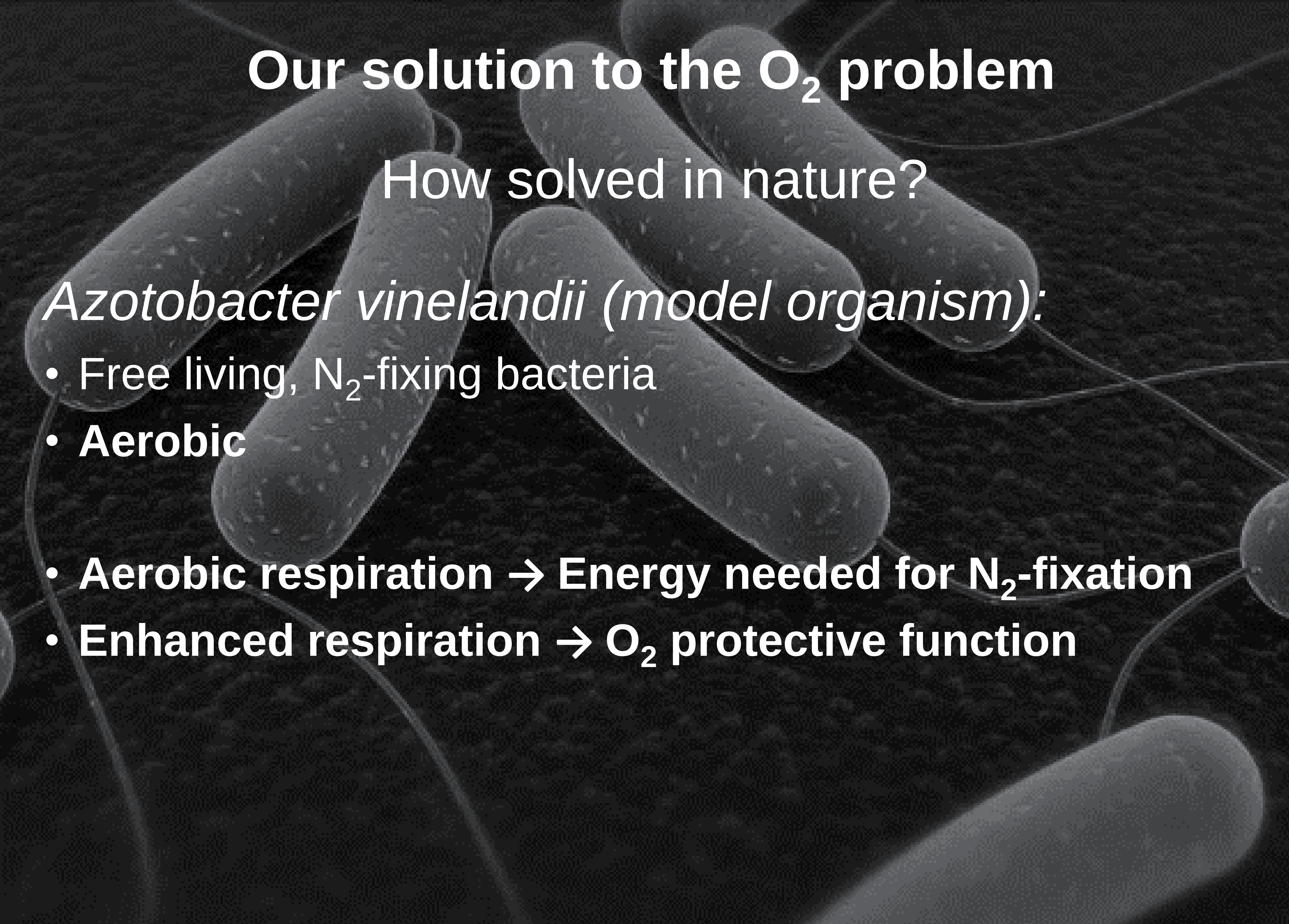


Image (modified) from AgroBio

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Mitochondria?

Image (modified) from AgroBio

Mitochondria – a “bacteria within the cell”

Why mitochondria?

- **Aerobic respiration consumes O_2 → protection**
- **Respiration → plentiful of ATP and reducing power**

Requirements for nitrogenase!

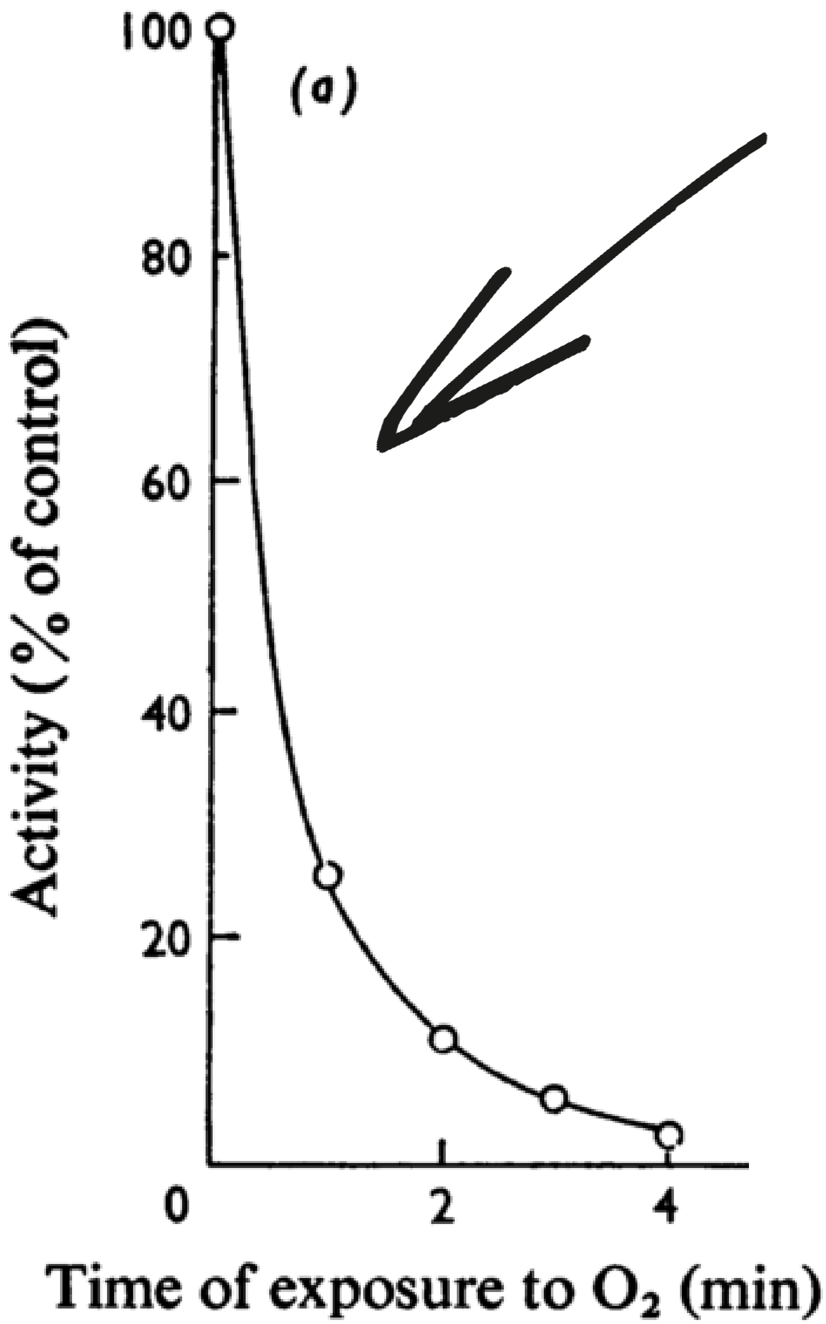
- Prokaryotic (bacterial) origin
- Bacterial Fe-S cluster biosynthesis machinery

- Also, mitochondria present in all eukaryotes

  → enables us to use yeast as model for plants

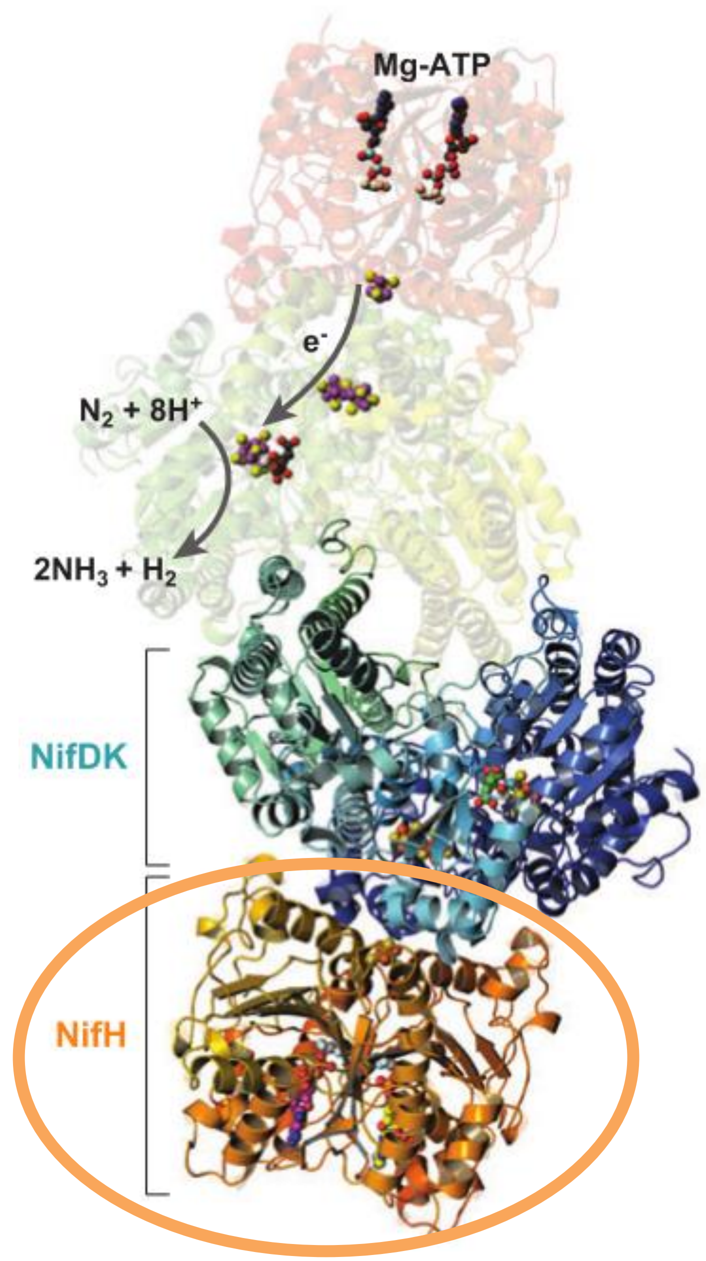
Image (modified) from Zheng Lab, Colorado

Yeast mitochondria NifH: proof of concept



NifH:

- NifH extremely sensitive to O_2
- Requires "only":
  - NifU
  - NifS
  - NifM



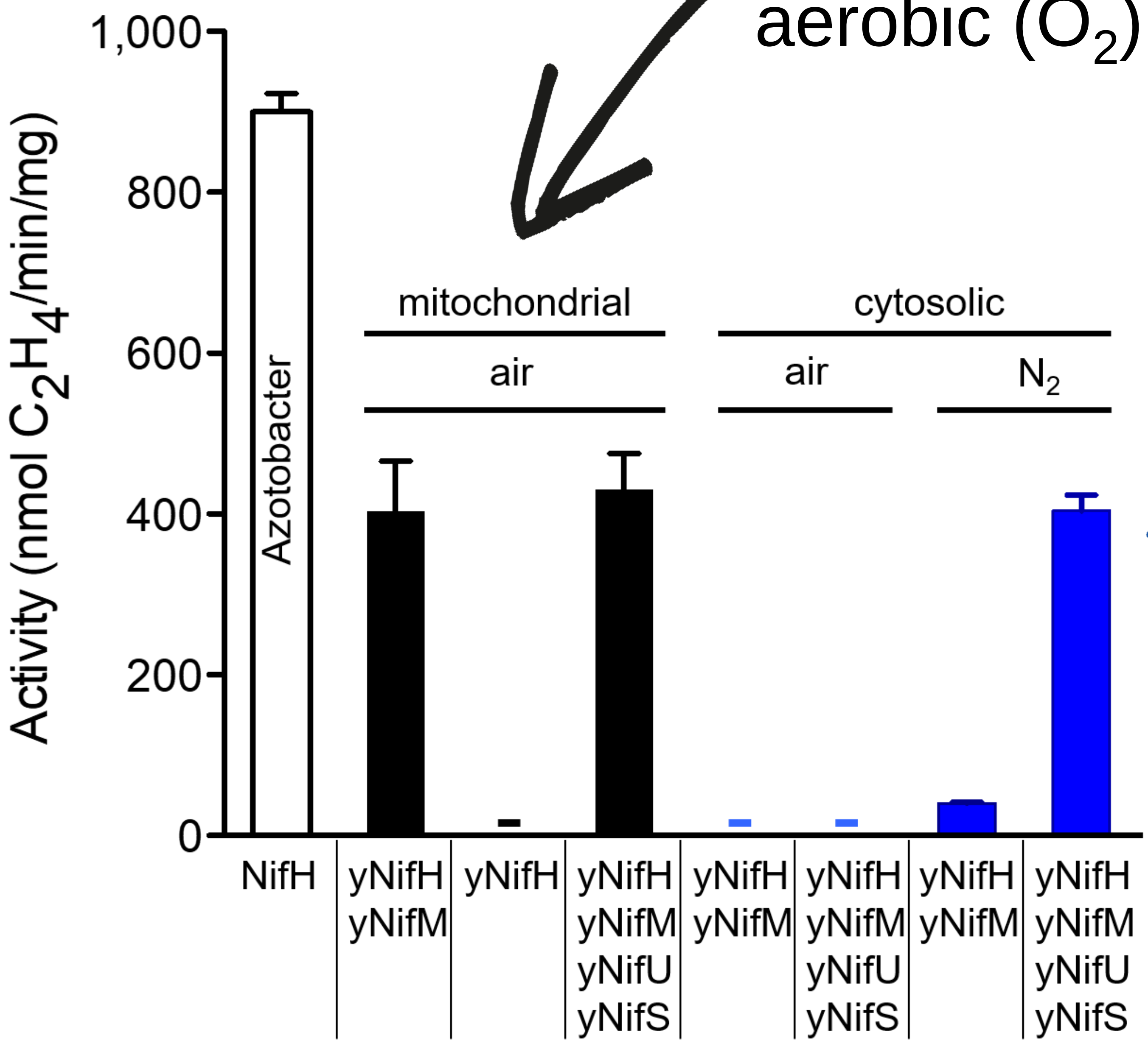
Chosen for proof of concept

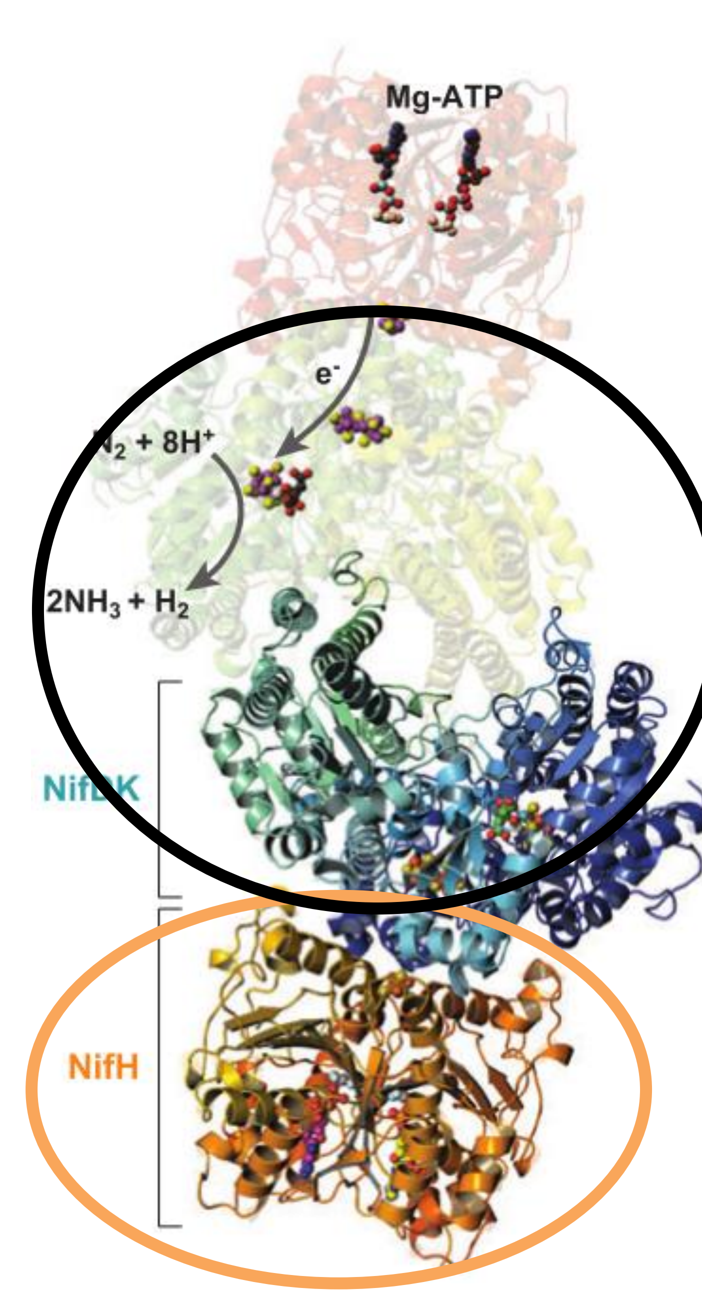
Yeast NifH protein activity

Activity assay mixture of:

Yeast NifH + *Azotobacter* NifDK

Mitochondrial NifH active in aerobic (O_2) yeast culture





Cytosolic NifH only active in anaerobic (no O_2) yeast culture

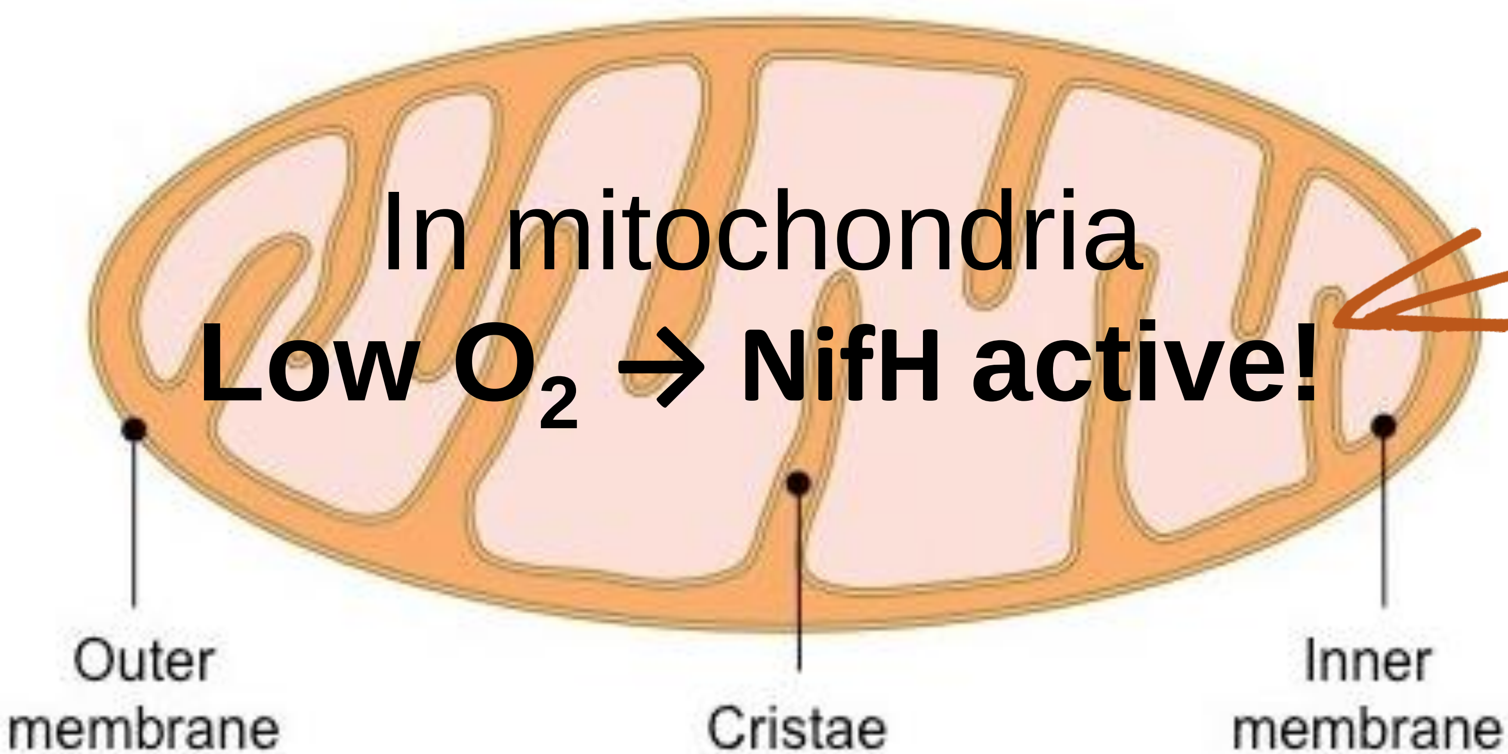
López-Torrejón et al., Nat. Commun., 2016

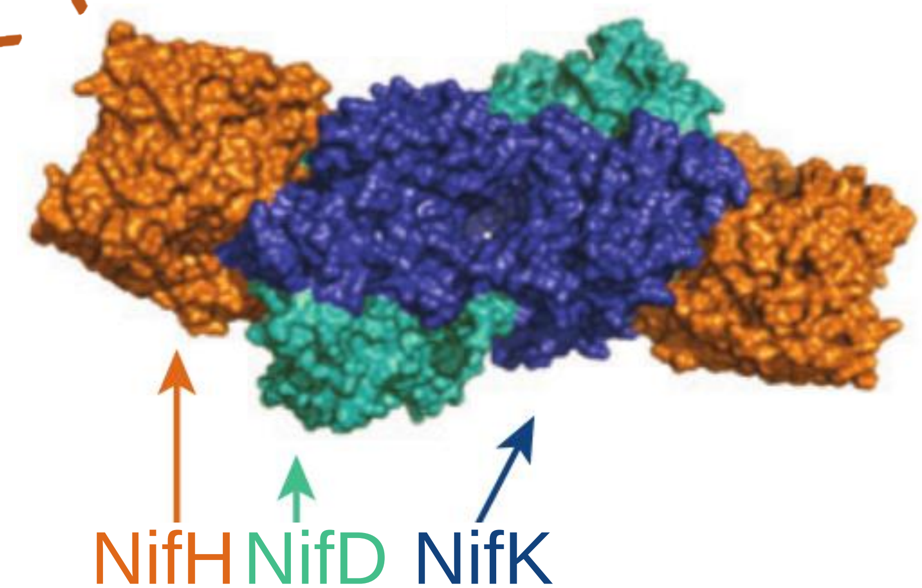
NifH: Protected in mitochondria!

Outside mitochondria: O_2 → NifH inactive

In mitochondria

Low O_2 → NifH active!





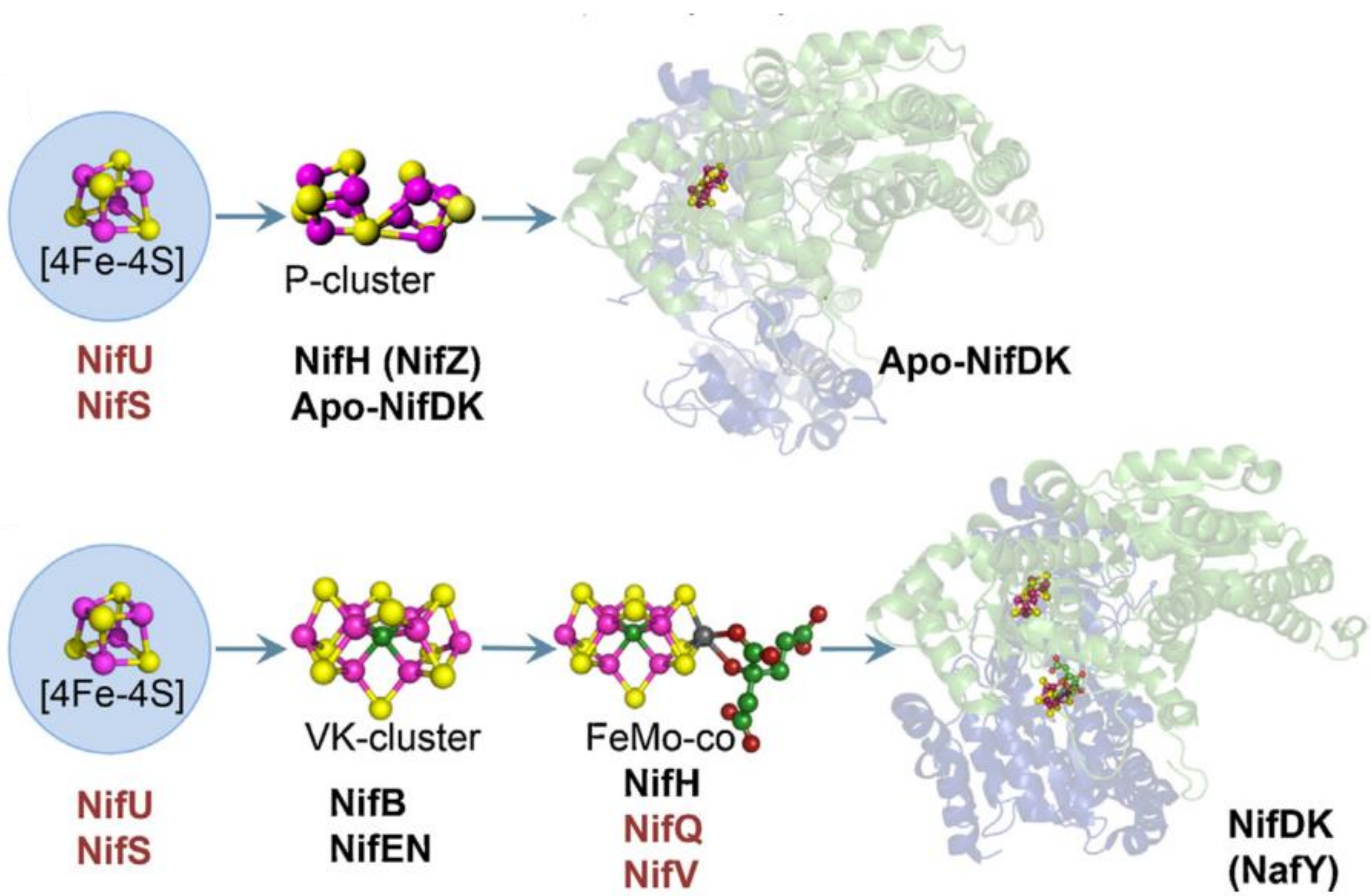
NifH active at mitochondria, what about NifDK?

López-Torrejón et al., Nat. Commun., 2016

(Minimal) genetic requirements for nitrogenase (NifDK) expression and function

NifDK synthesis more complex than NifH:

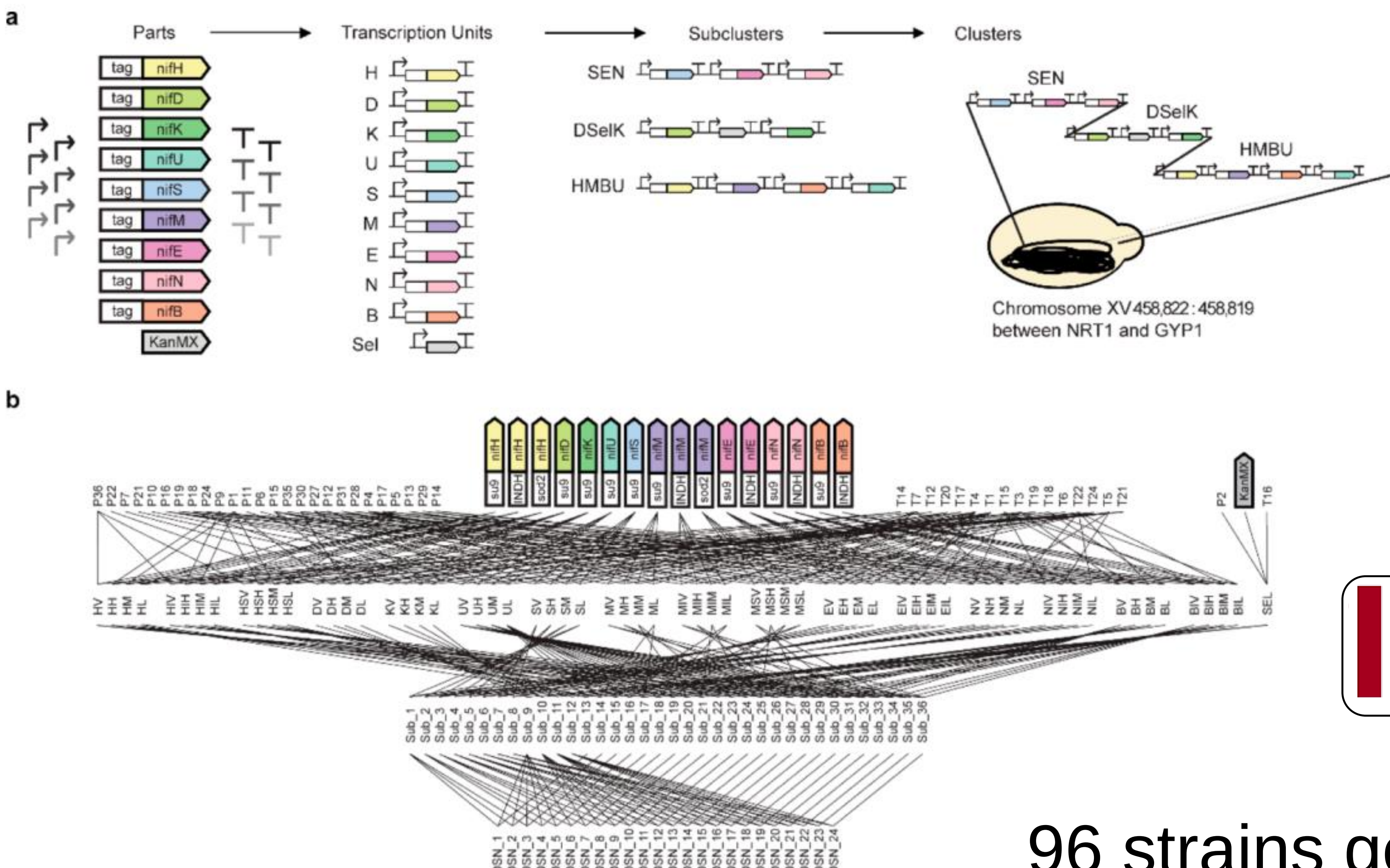
- Matured in two steps
- Each step requires its own (complex) metal cluster
- At least NifDK, NifH, NifM, NifB and NifEN proteins required, but likely several more



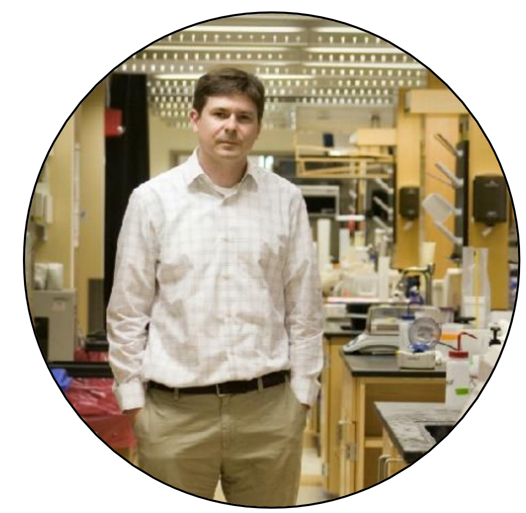
Curatti & Rubio, Plant Sci., 2014

Maturation of NifDK at mitochondria

Construction of yeasts carrying 9 *nif* genes, theoretically sufficient for active nitrogenase



Chris Voigt

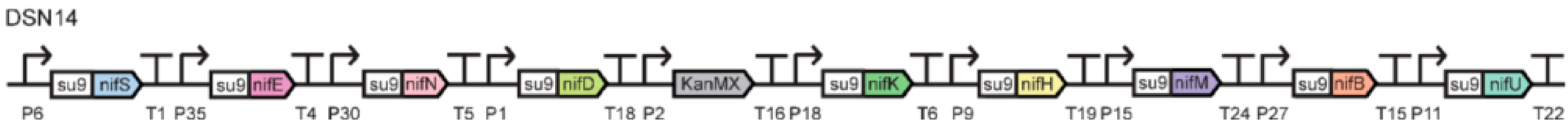


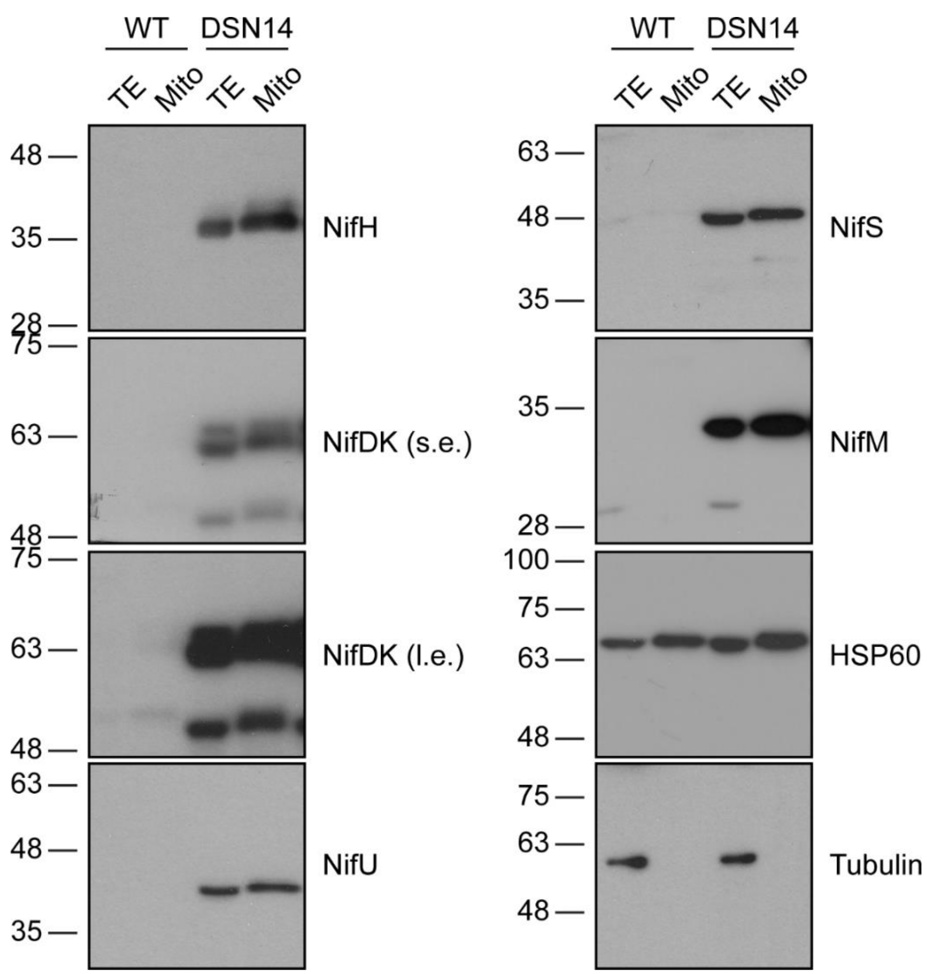
Massachusetts Institute of Technology

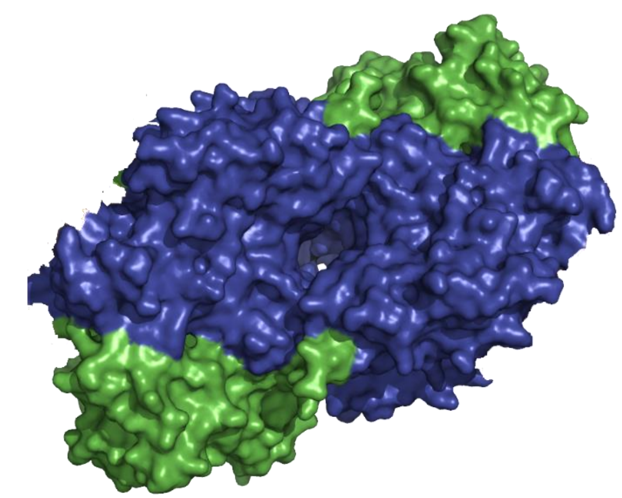
96 strains generated with varied gene expression

Burén et al., ACS Synth. Biol., 2017

NifDK complex formation at mitochondria







- All 9 proteins expressed
- NifDK tetramer formation confirmed
- *No active nitrogenase detected*

NifDK metal cluster maturation failed

More/better components needed!

Burén et al., ACS Synth. Biol., 2017

Summary, where are we?

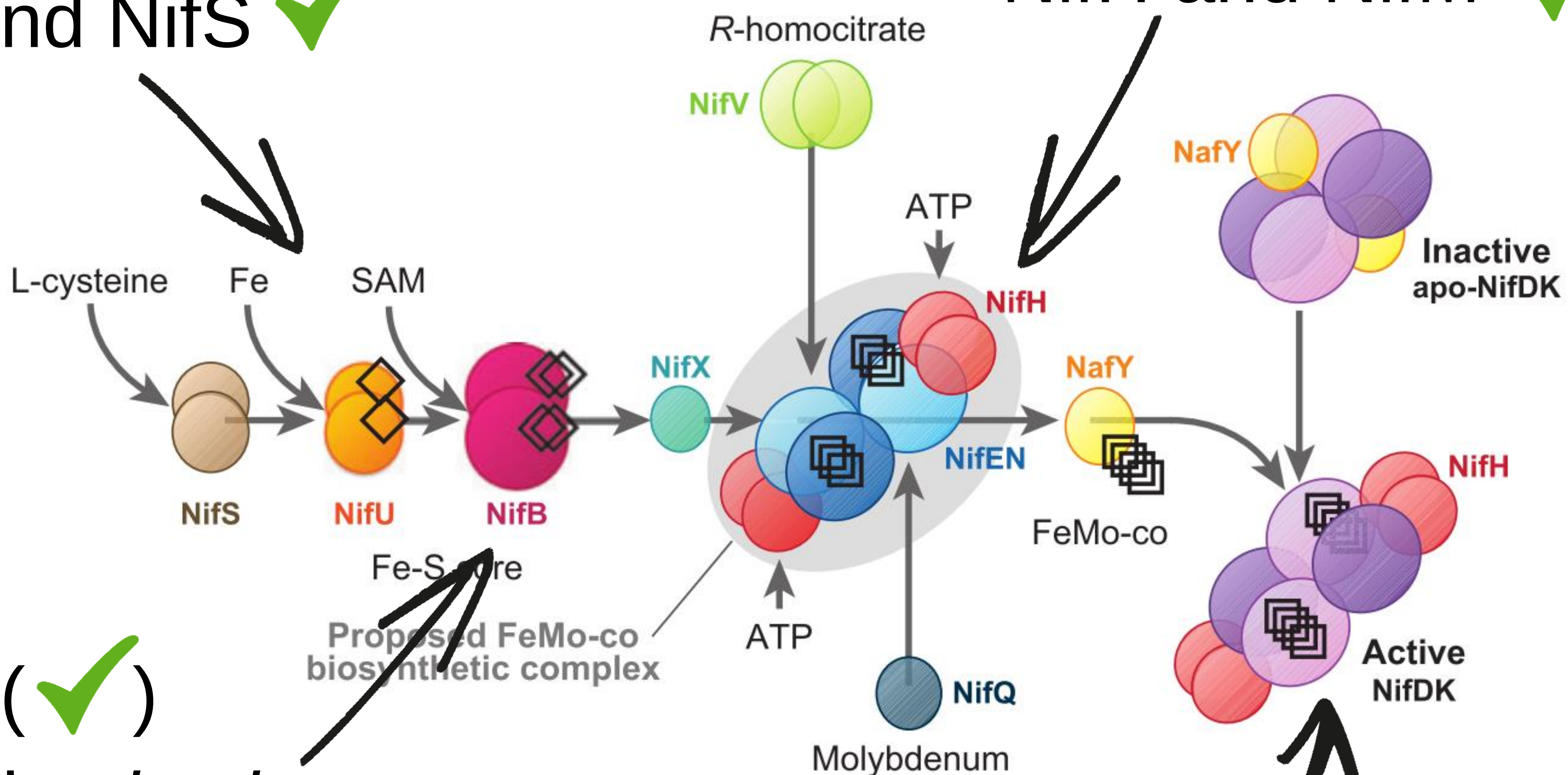
Based on yeast data:

López-Torrejón et al.,Nat. Commun., 2016

NifU and NifS ✓

López-Torrejón et al.,Nat. Commun., 2016

NifH and NifM ✓



NifB (✓)

- Active *in vitro*
- *In vivo* activity being tested

Burén et al., Front. Plant Sci., 2017

NifDK

- Tetramer formation ✓
- P-cluster and FeMo-co ✗

Burén et al., ACS Synth. Biol., 2017

What remains to do? Realistic?

O_2 problem solved by targeting to the mitochondria, shown by NifH activity in aerobically grown yeast

One major obstacle remaining:

- Nitrogenase metal cluster biosynthesis

  → More protein components needed (ongoing)

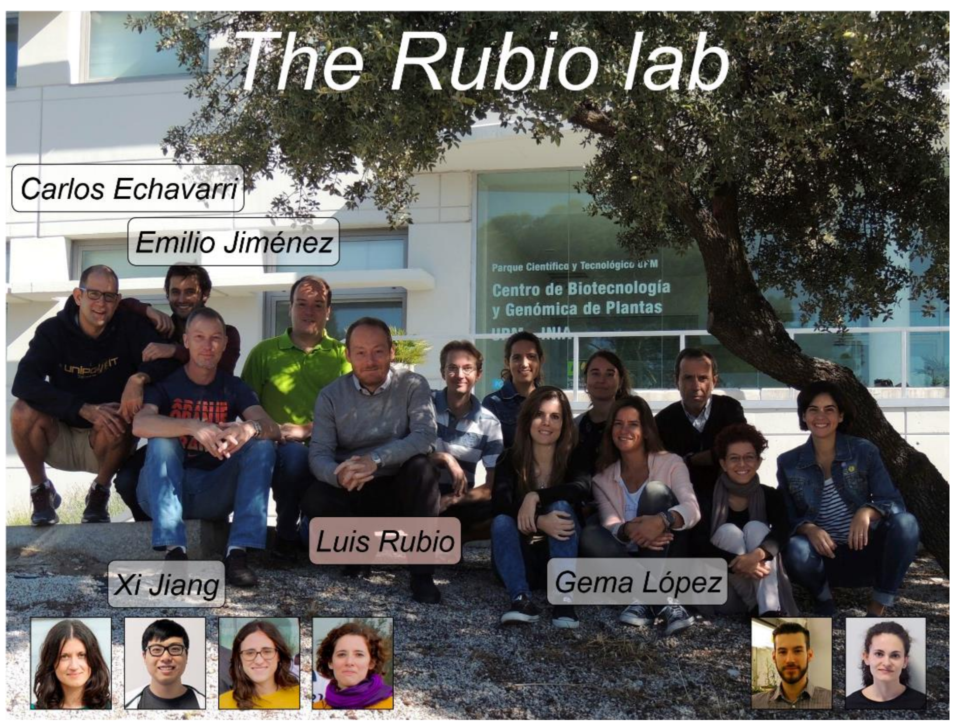
Other unknown factors:

- Levels of trace elements at mitochondria (e.g. Mo)?
- Metal transporters needed?

And (of course):

- Verify results in plants, GMO issue…

Thanks to:



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