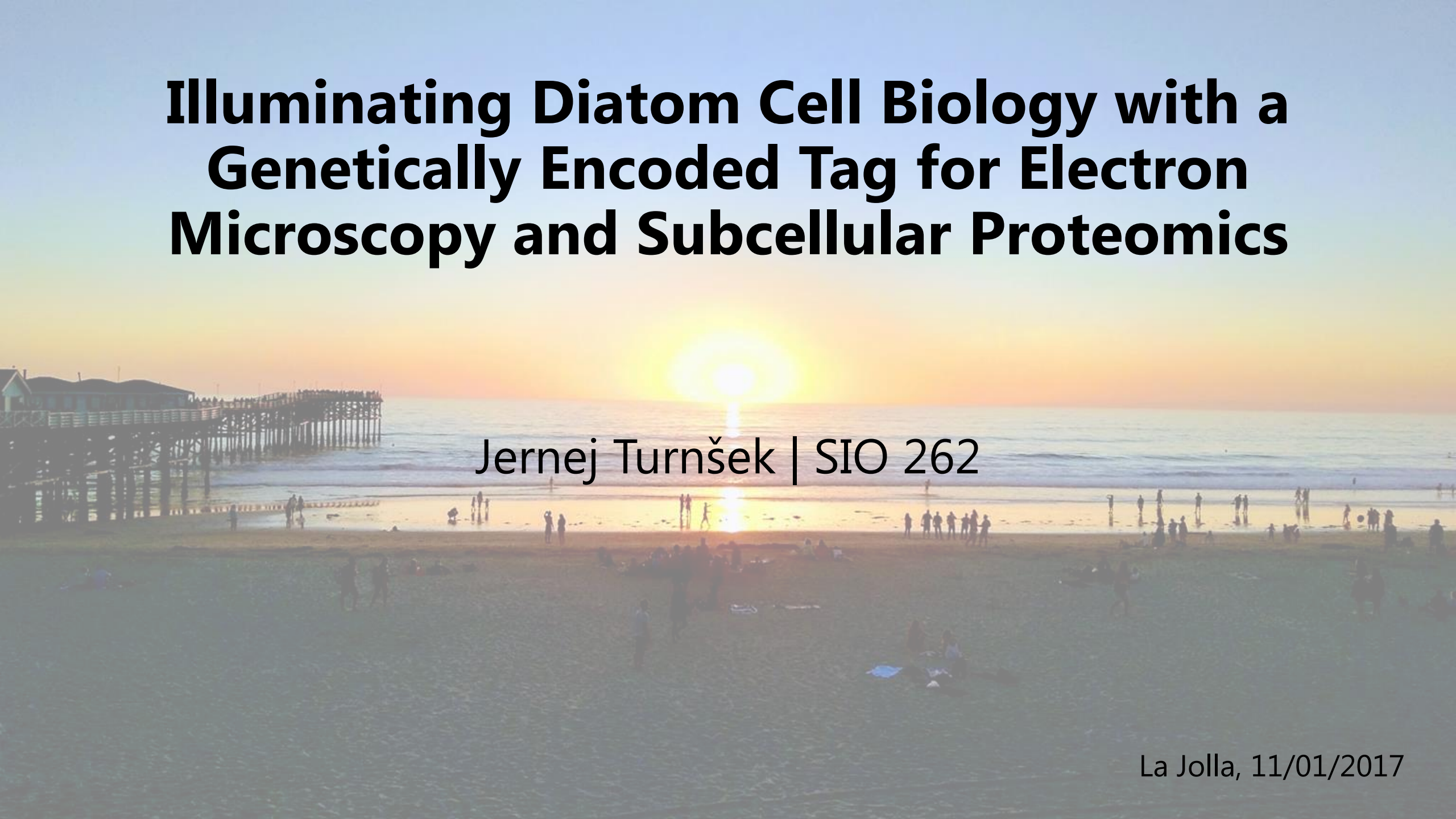
Illuminating Diatom Cell Biology with a Genetically Encoded Tag for Electron Microscopy and Subcellular Proteomics



Jernej Turnšek | SIO 262

La Jolla, 11/01/2017

- **Part 1: Biology**

Inorganic iron in oceans is scarce, but marine microeukaryotes have evolved high affinity acquisition systems to access it.

- **Part 2: Technology**

APEX2 is a genetically encoded tag for electron microscopy and subcellular proteomics.

- **Part 3: Technology to illuminate biology**

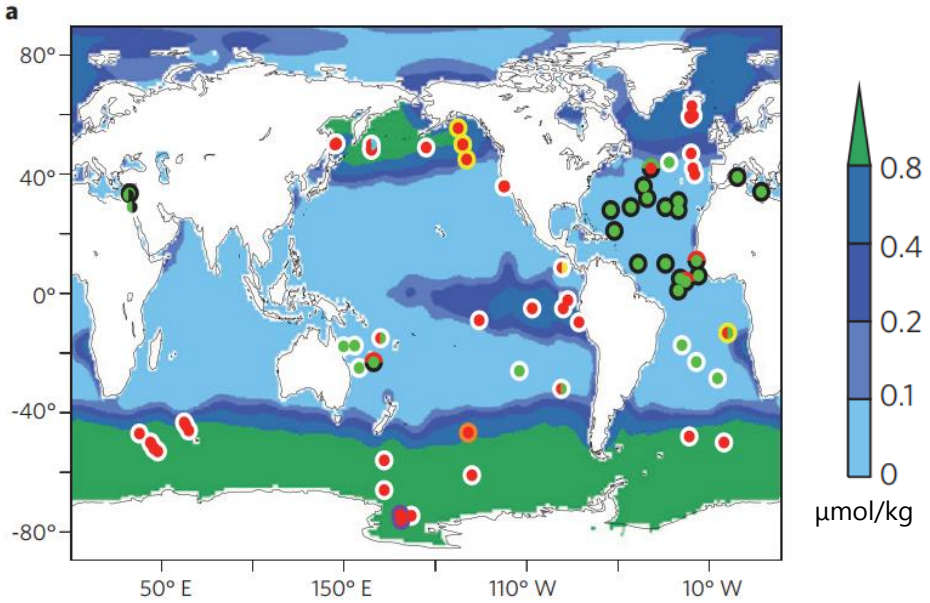
APEX2-based approach to identify candidate proteins involved in inorganic iron acquisition in the model diatom species *Phaeodactylum tricornutum*.

- **Future directions**

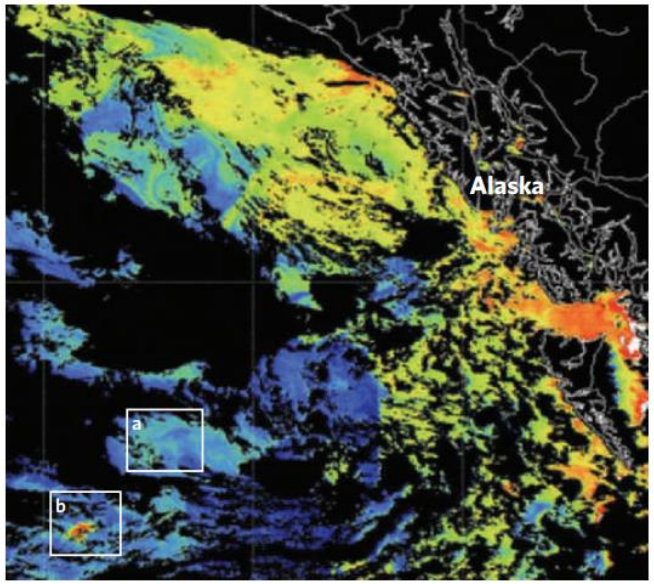
- **Vision** for diatom biology and other marine systems.

Part 1: Biology

Iron controls ocean productivity



(●) = iron controls growth; contours = [NO_3^-]



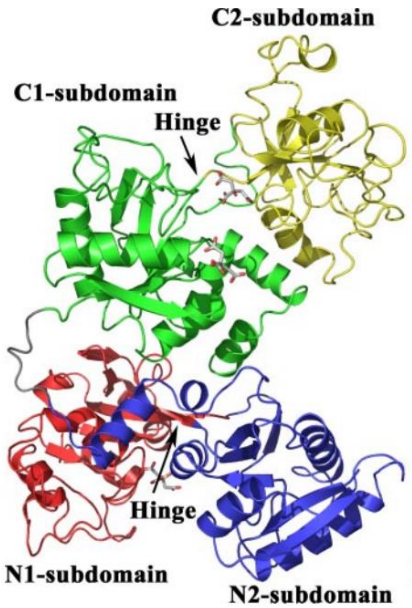
warm colors = high phytoplankton concentration

- ~30% of the ocean's surface area consists of high-macronutrient, iron-limited systems.
- ~99% of iron is complexed to organic ligands, ~1% comes as dissolved inorganic iron Fe^{3+} (Fe').
- [Fe'] is estimated to be in the 10^{-15} M range.

Accessing this scarce ion?

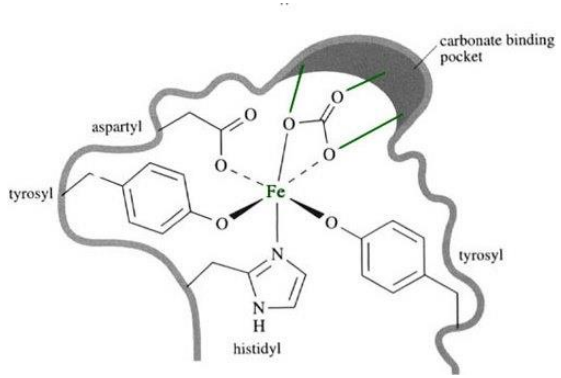
1. Moore *et al.* Processes and patterns of oceanic nutrient limitation. *Nature Geoscience* 6: 701–710 (2013).
2. Geider, R.J. Complex lessons of iron uptake. *Nature* 400: 815–816 (1999).
3. Tagliabue *et al.* The integral role of iron in ocean biogeochemistry. *Nature* 543: 51–59 (2017).
4. Armbrust, E.V. The life of diatoms in the world's oceans. *Nature* 459: 185–192 (2009).

Transferrins are proteins with high affinity for Fe^{3+}

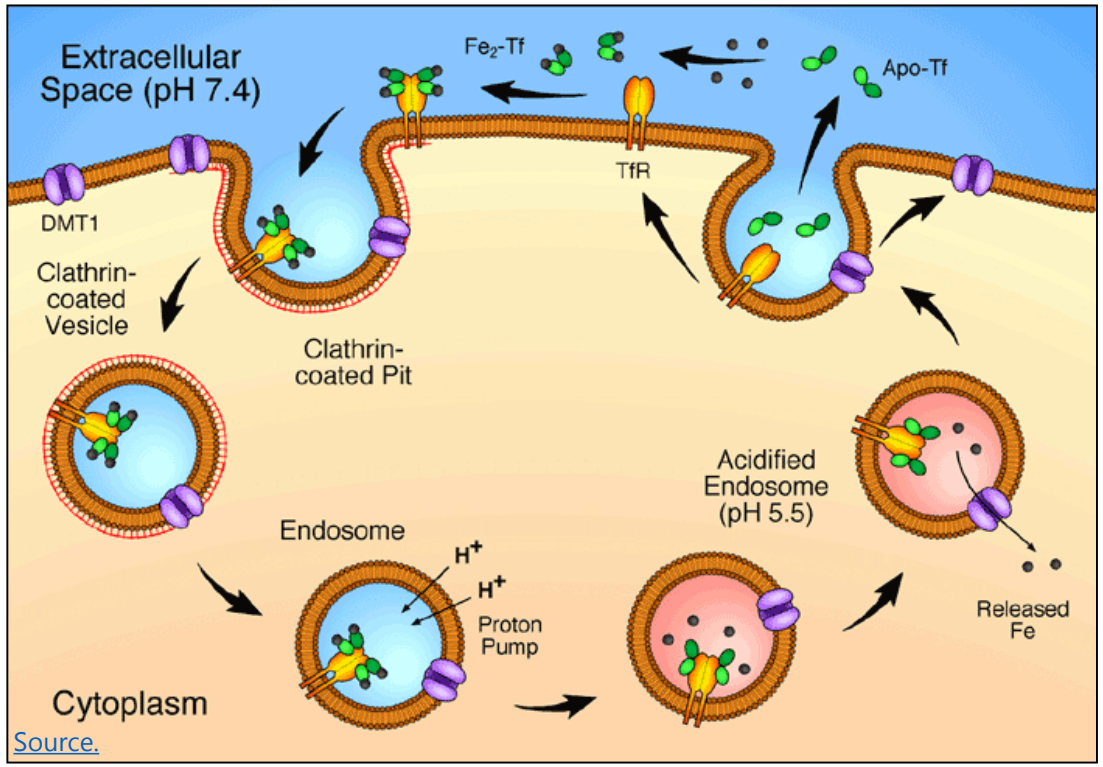


Bilobal proteins with iron-coordinating hinges.

Synergistic Fe^{3+} and CO_3^{2-} coordination.



Source



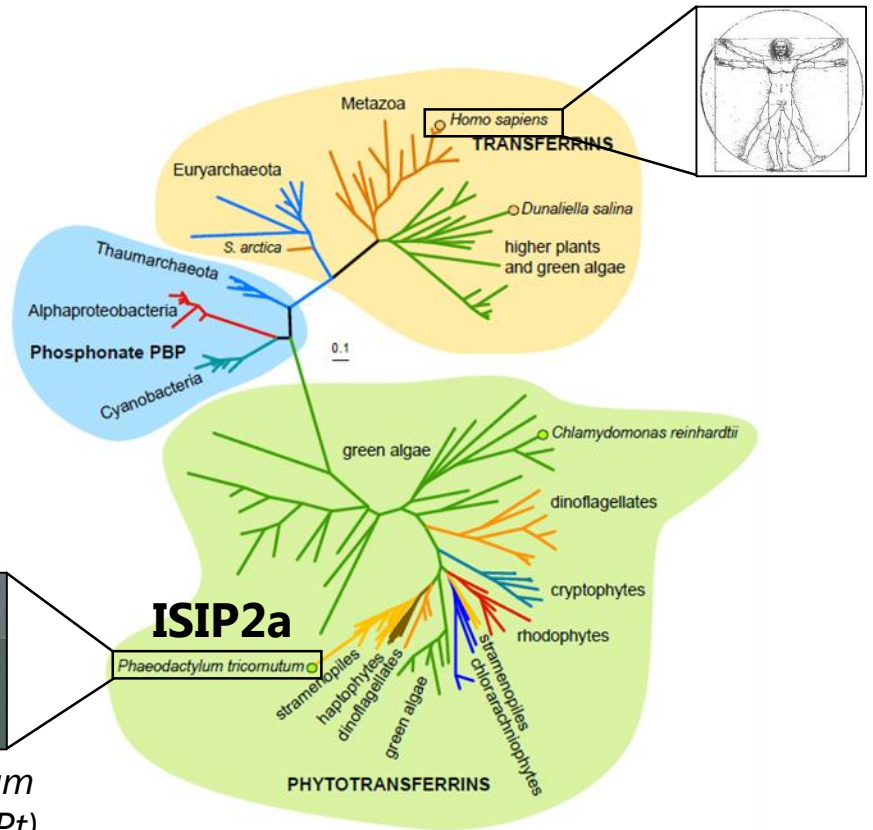
Source.

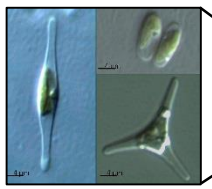
KEY: coordinating carbonate is also the release trigger:
- carbonate protonation
- transferrin conformational change

1. Aisen *et al.* Stoichiometric and Site Characteristics of the Binding of Iron to Human Transferrin. *The Journal of Biological Chemistry* 253, 6: 1930–1937 (1978)
2. Wally *et al.* The Crystal Structure of Iron-free Human Serum Transferrin Provides Insight into Inter-lobe Communication and Receptor Binding. *The Journal of Biological Chemistry* 281, 34: 24934 –24944 (2006).

Phytotransferrins are a new group of transferrins widespread among marine phytoplankton

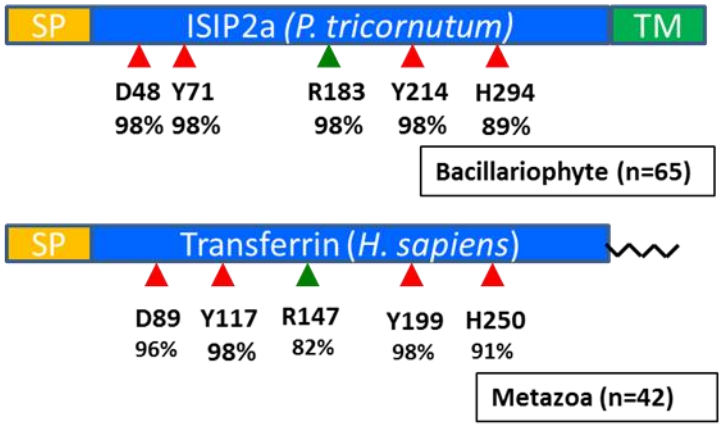
Phytotransferrins are a new group of transferrins.

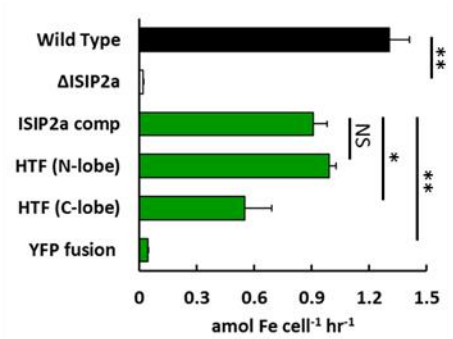


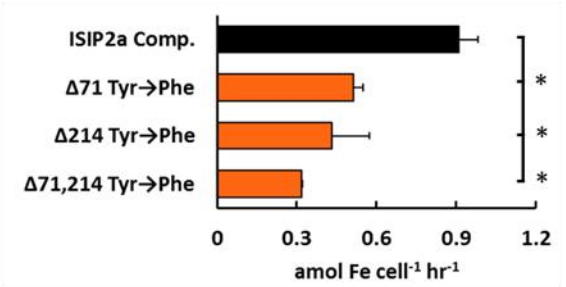


Phaeodactylum tricornutum (Pt)

ISIP2a KO and iron binding site mutants show reduced iron uptake rates.



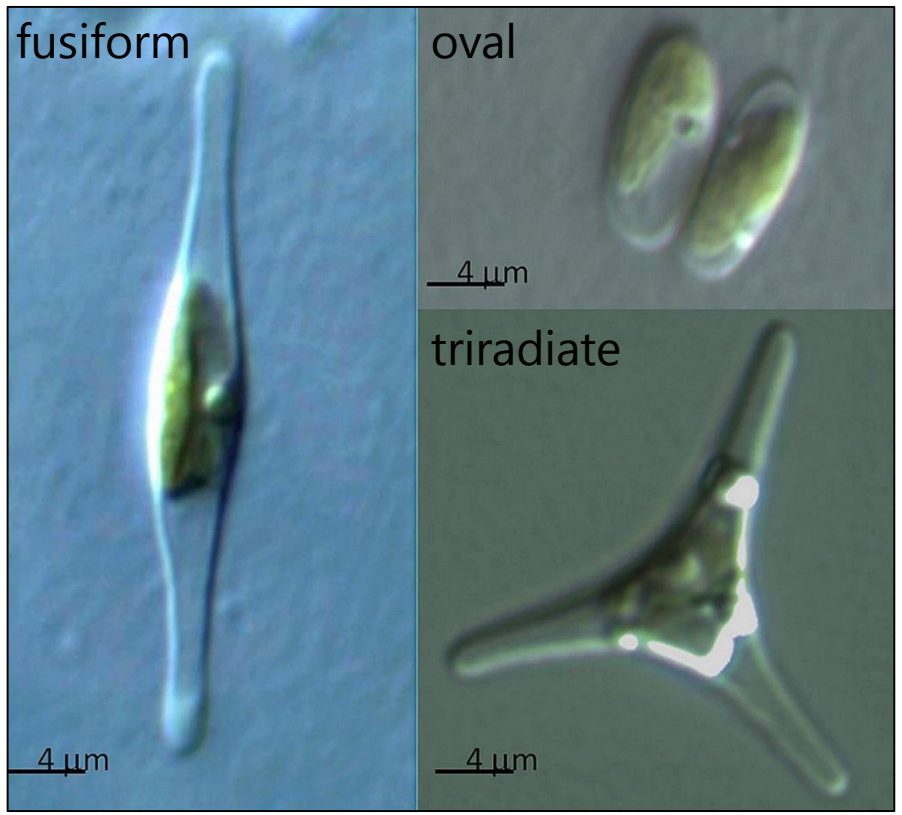




1. McQuaid *et al.* Carbonate sensitive phytotransferrin controls high affinity iron uptake in diatoms. *Nature* (in review).

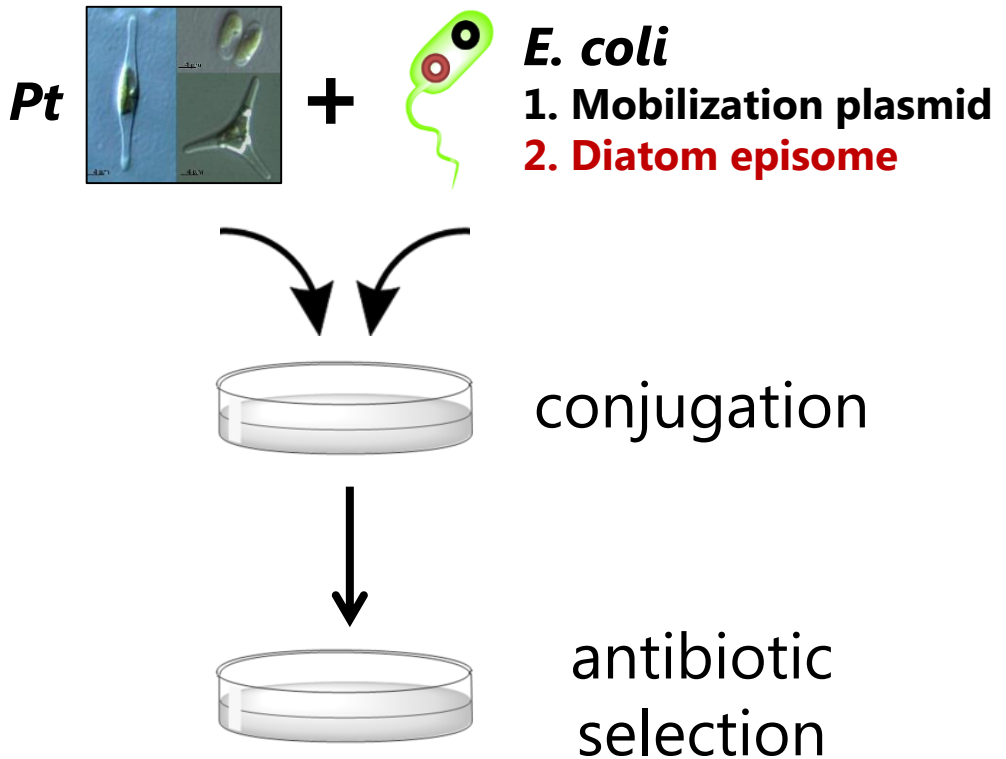
Phaeodactylum tricornutum (*Pt*) is a model diatom

***Pt* is a unicellular eukaryote**



Credit: Alessandra De Martino, Ecole Normale Superieure, Paris

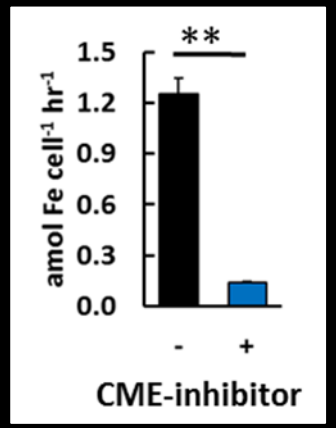
Bacterial conjugation of *Pt*



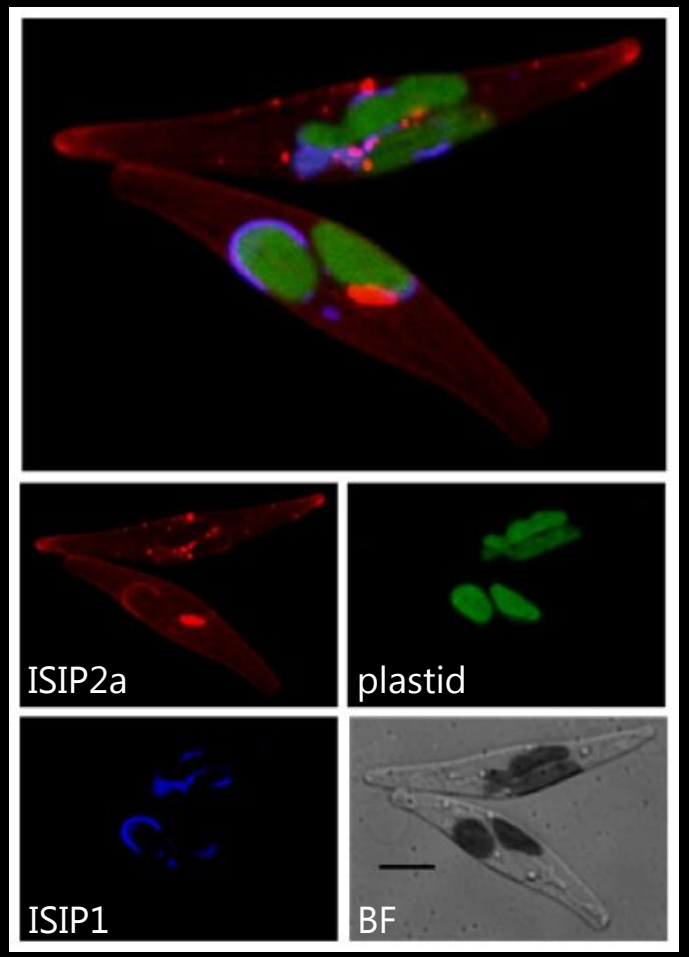
1. Bowler *et al.*, The *Phaeodactylum* genome reveals the evolutionary history of diatom genomes. *Nature* 456, 7219: 239–244 (2008).
2. Karas *et al.*, Designer diatom episomes delivered by bacterial conjugation. *Nature Communications* 6, 6925 (2015).
3. Diner *et al.* Refinement of the Diatom Episome Maintenance Sequence and Improvement of Conjugation-Based DNA Delivery Methods. Frontiers in Bioengineering and Biotechnology 4, 65 (2016).

ISIP2a is endocytosed and localized to intracellular vesicles

Iron-limited cells post iron addition internalize parts of the cell membrane.



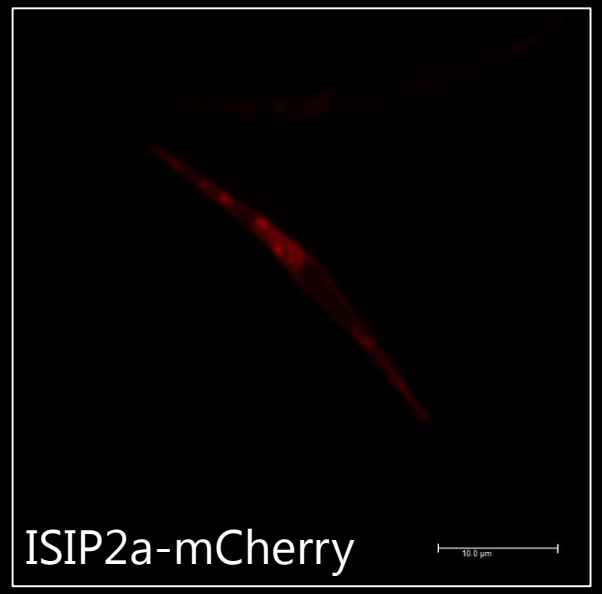
Iron uptake is inhibited in the presence of clathrin-mediated endocytosis (CME) inhibitor.

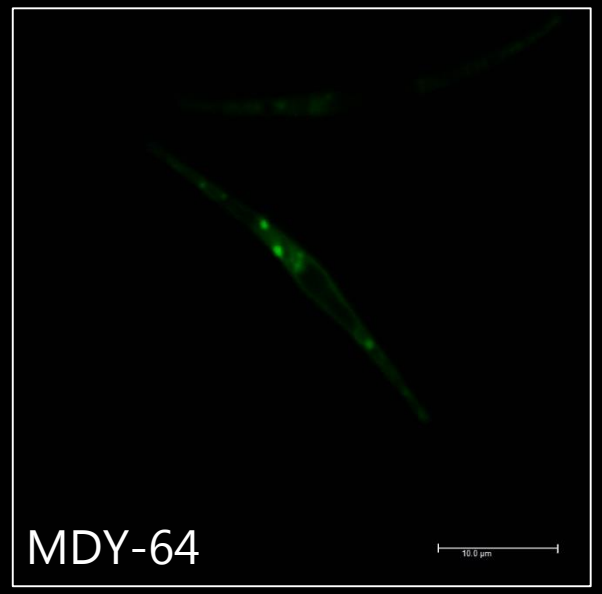


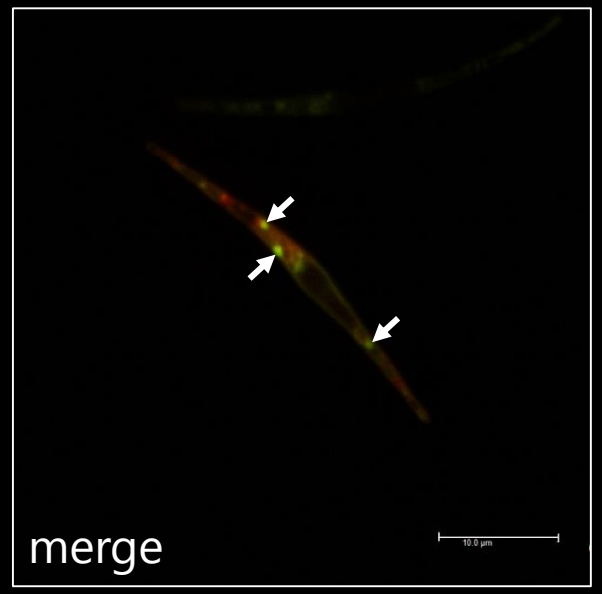
scale bar is 5 µm

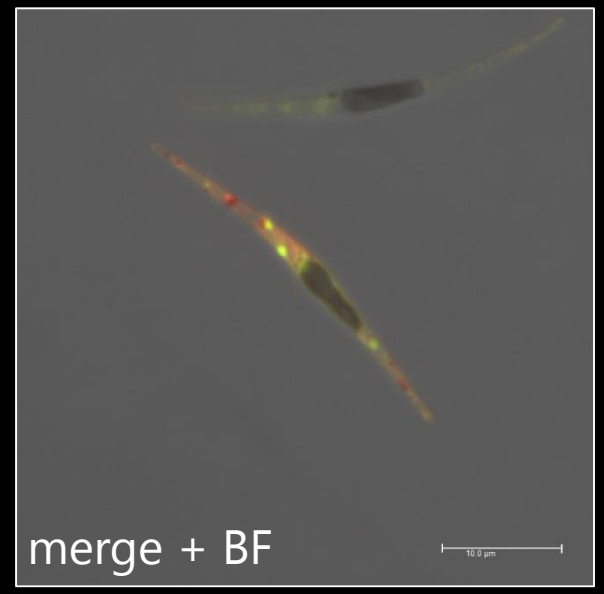
1. McQuaid *et al.* Carbonate sensitive phytotransferrin controls high affinity iron uptake in diatoms. *Nature* (in review).

ISIP2a is co-localized with a membrane stain MDY-64



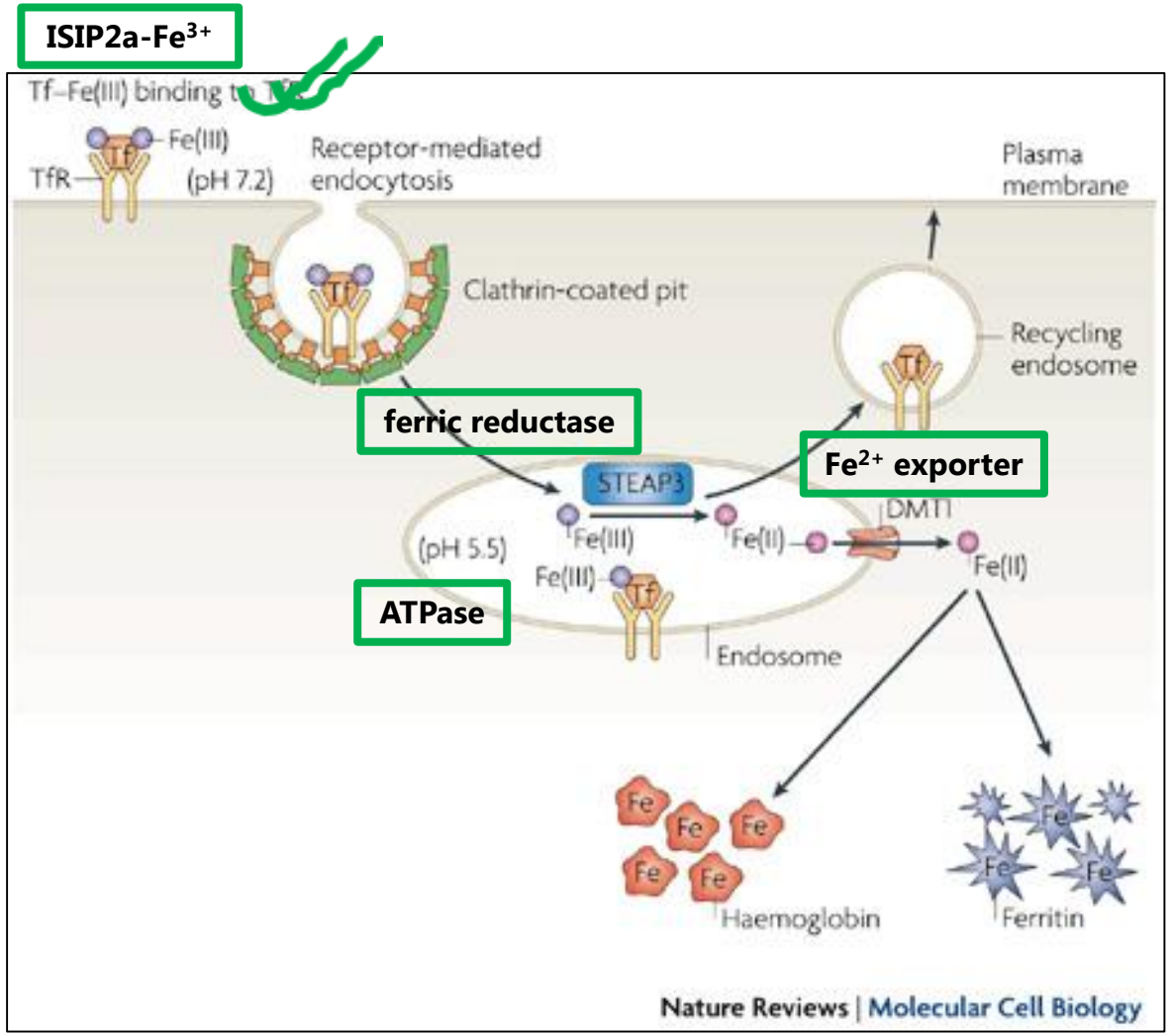






scale bar is 10 µm

Phytotransferrin cycle proteome?



1. "Iron proteins"

- ISIP1 ("receptor-like")
- ISIP3 ("ferritin-like")
- ferric reductase (Fe^{3+} → Fe^{2+})
- Fe^{2+} exporter
- metallochaperons

2. Vesicular proteins

- eisosome complex?
- clathrin (formation of coated vesicles)
- dynamin (vesicle scission GTPase)
- v-SNARE (vesicle fusion)
- ATPase (vesicle acidification)

3. Cytoskeletal proteins

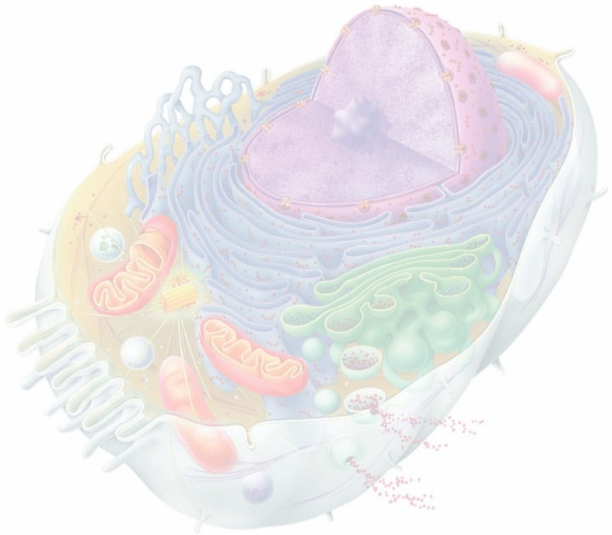
- actin, myosin
- tubulin, dynein, dynactin

1. De Domenico *et al.*, Regulation of iron acquisition and storage: consequences for iron-linked disorders. *Nature Reviews Molecular Cell Biology* 9, 1: 72–81 (2008).

Part 2: Technology

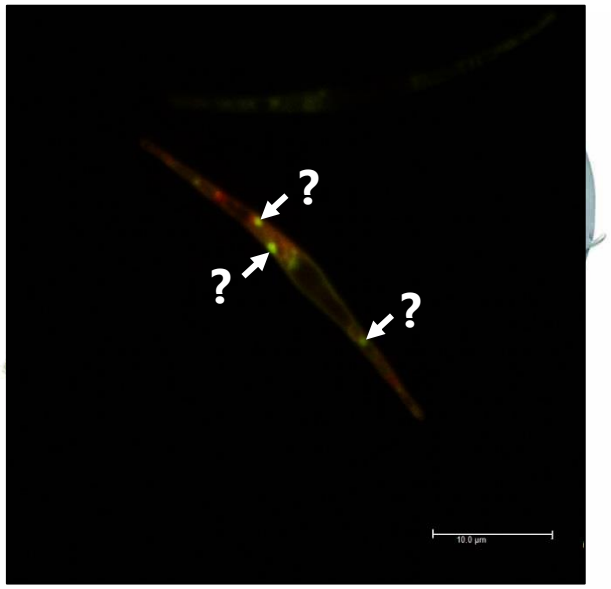
Subcellular proteomics

Whole cell

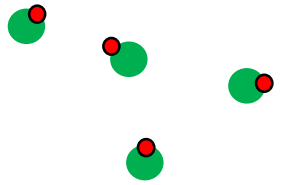


unmodified total cellular proteome

Compartment



chemically modified subcellular proteome

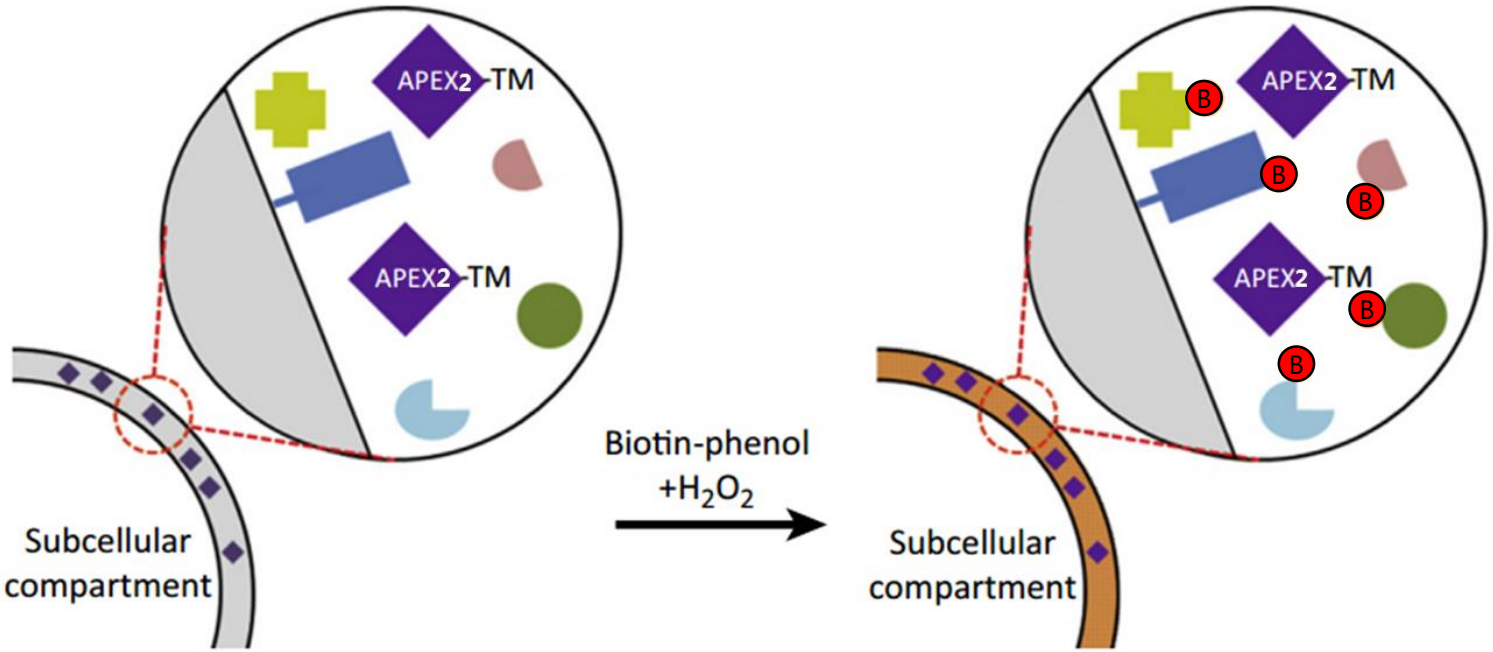


● small molecule tag

Source

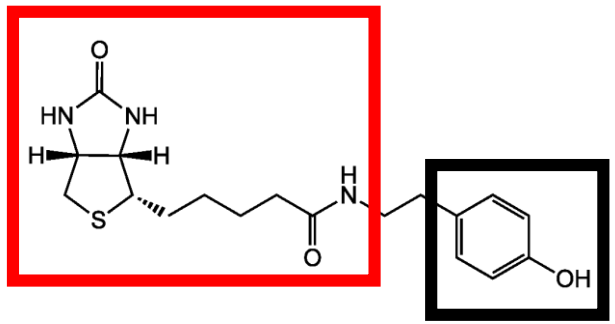
Subcellular proteomics with APEX2

(A)



(B)

Biotin-phenol



binding to streptavidin beads

covalent protein modification (Tyr)

APEX2 | ~27 kDa engineered soybean ascorbate peroxidase
TM | targeting motif (your favorite protein or its localization sequence)
B | biotin

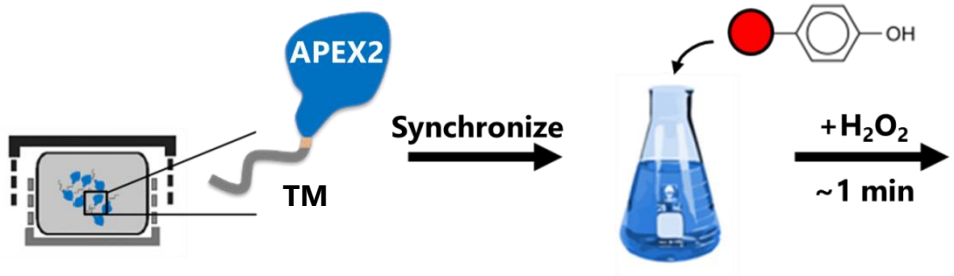
1. Lam *et al.*, Directed evolution of APEX2 for electron microscopy and proximity labeling. *Nature Methods* 12: 51–54 (2015).
2. Kim, D.I. & Roux, K.J. Filling the Void: Proximity-Based Labeling of Proteins in Living Cells. *Trends in Cell Biology* 26: 804–817 (2016).

APEX2 is a bifunctional probe

Proteomics functionality

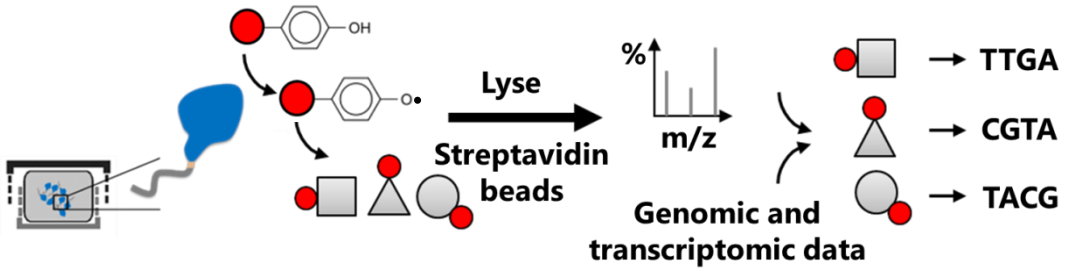
(A) Express APEX2-tagged TM.

(B) Incubate with biotin-phenol.

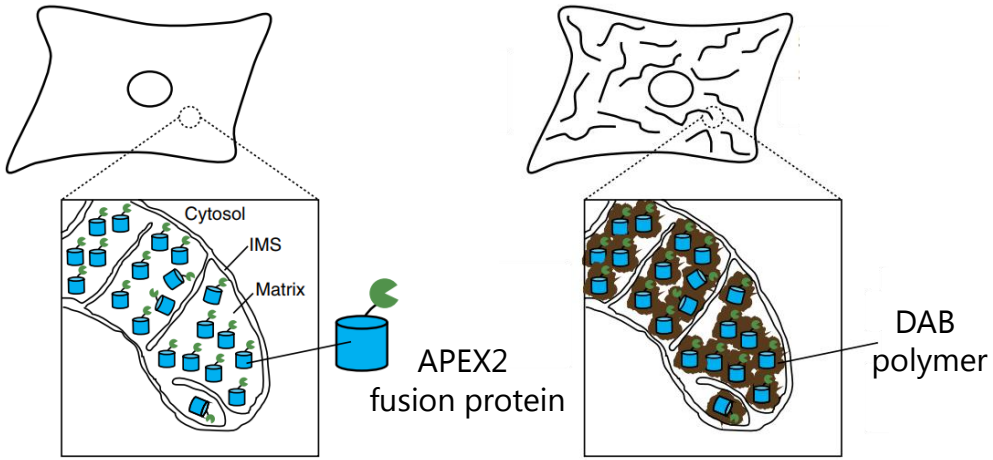


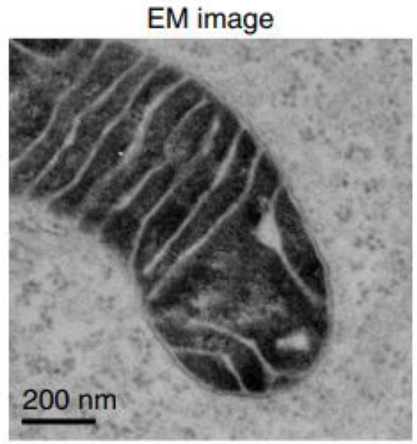
(C) *In vivo* protein biotinylation.

(D) Mass spectrometry and proteogenomic analysis.



Electron microscopy functionality





1. Adapted from Martell *et al.*, Electron microscopy using the genetically encoded APEX2 tag in cultured mammalian cells. *Nature Methods* 12, 9: 1792–1816 (2017).

APEX2 has been used in a variety of model systems

Proteomic Mapping of the Human

Proteomics of Primary Cilia by Proximity Labeling

Proteomic Analysis of Unbounded

Proximity-dependent biotin labelling in yeast using the engineered

Proteomic mapping in live *Drosophila* tissues using an

In vivo mapping of tissue- and subcellular-specific

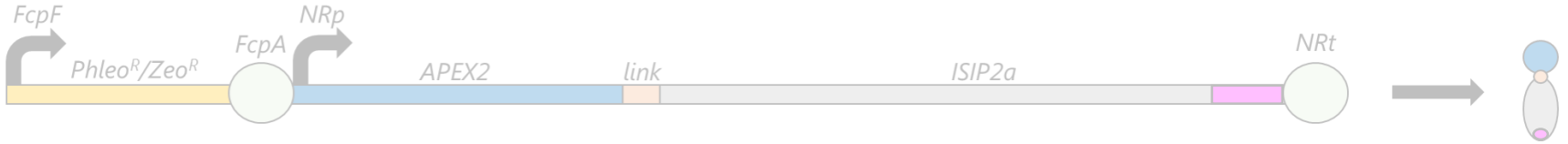
Expanding the inorganic iron acquisition proteome in *Phaeodactylum tricornutum* using subcellular proteomics.

Jernej Turnšek *et al.* (2018?)

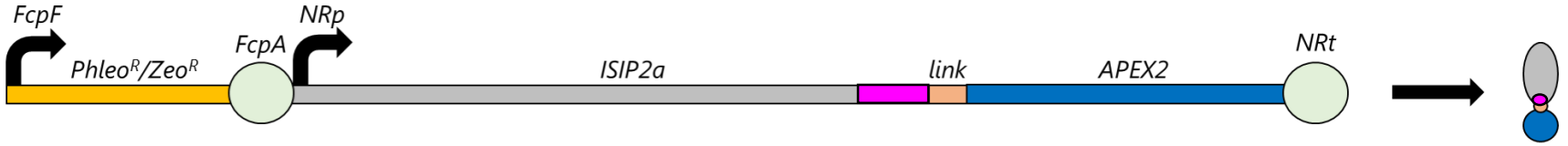
Part 3: Technology to illuminate biology

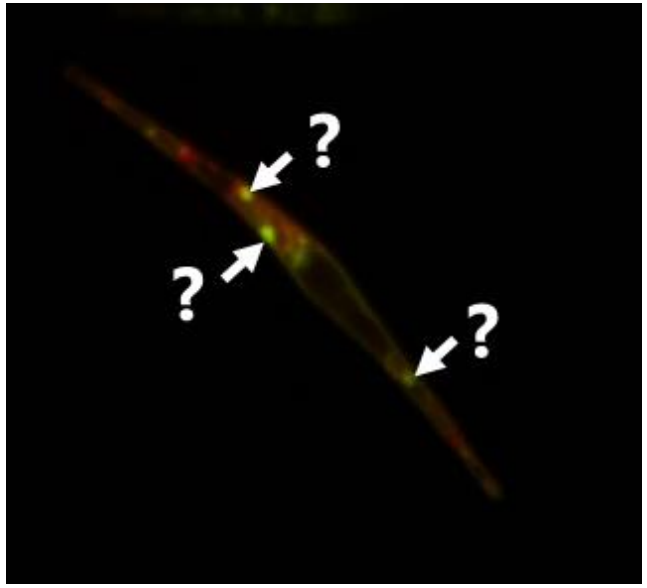
Using APEX2 for proteomic characterization of ISIP2a-containing vesicles

N-terminal APEX2

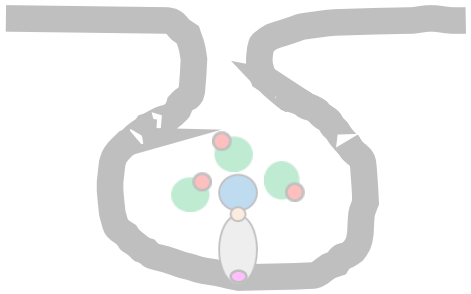


C-terminal APEX2



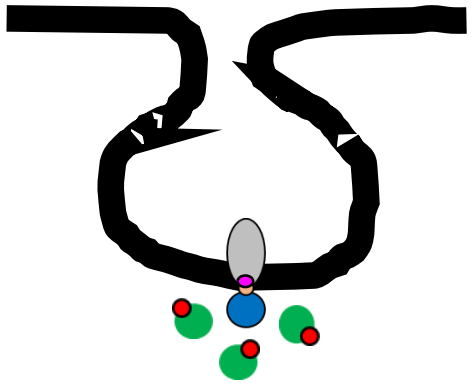


N-terminal APEX2



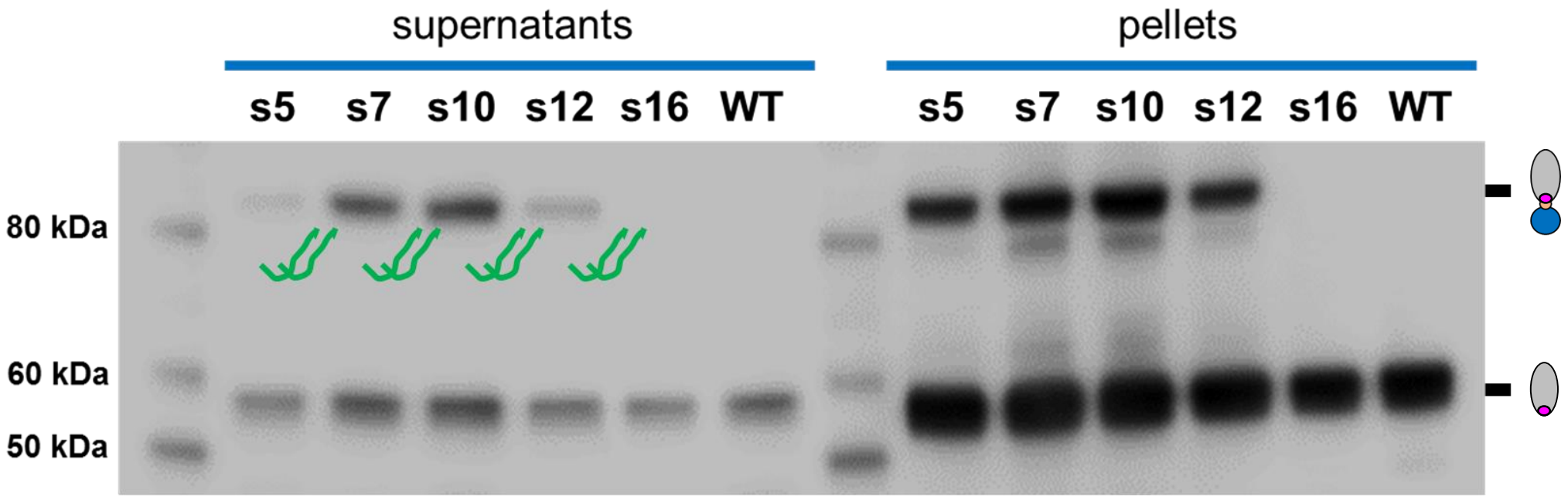
vesicle interior

C-terminal APEX2



vesicle surface

Successful heterologous expression of ISIP2a-APEX2 fusion proteins

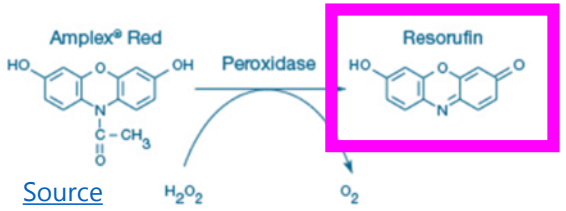


α ISIP2a blot | ~1 µg total protein (supernatants)

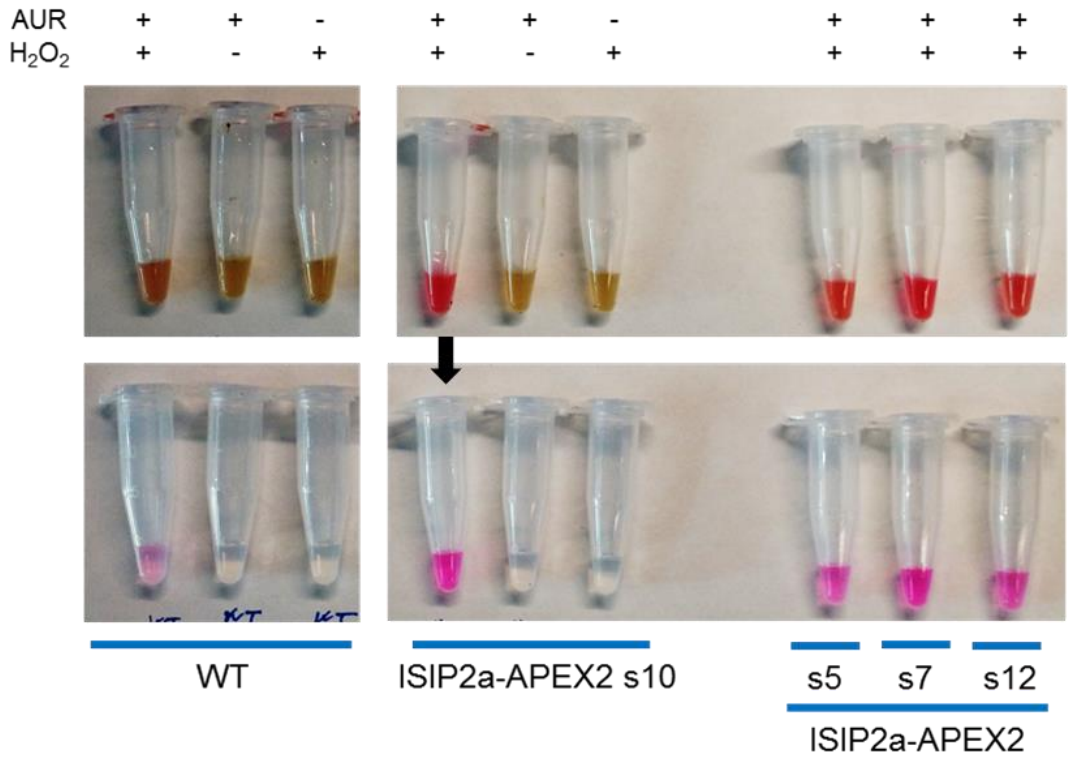
ISIP2a: ~57 kDa | ISIP2a-APEX2: ~85 kDa

Detection of APEX2 activity in ISIP2a-APEX2+ strains using Amplex UltraRed assay

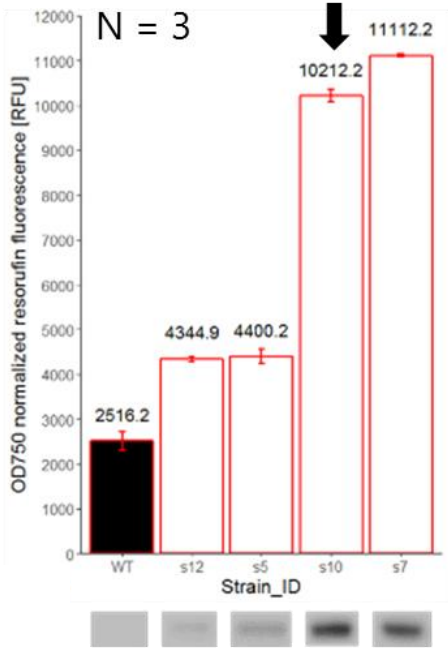
(A)



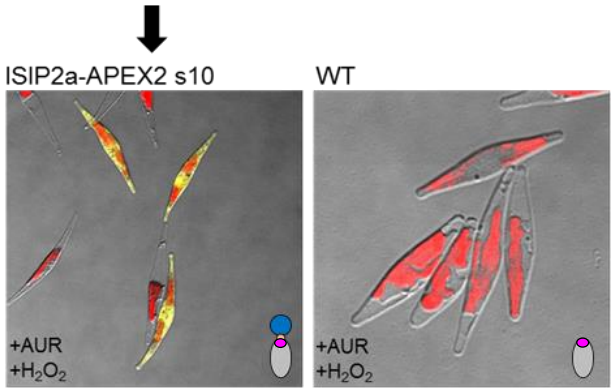
Source



(B)



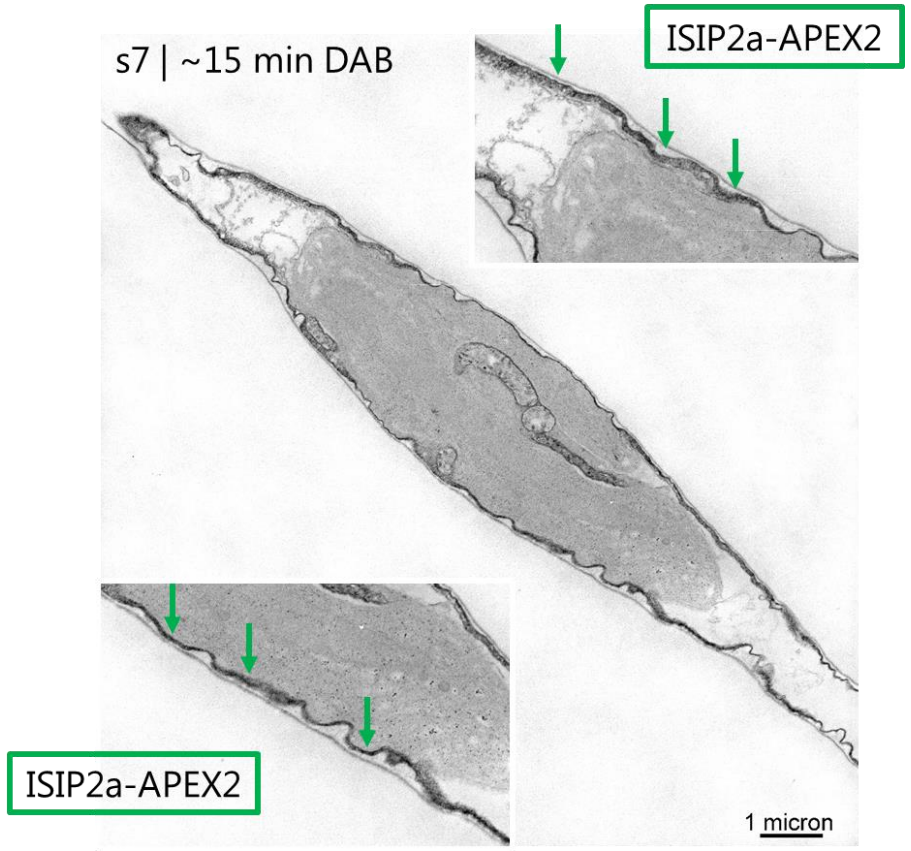
(C)



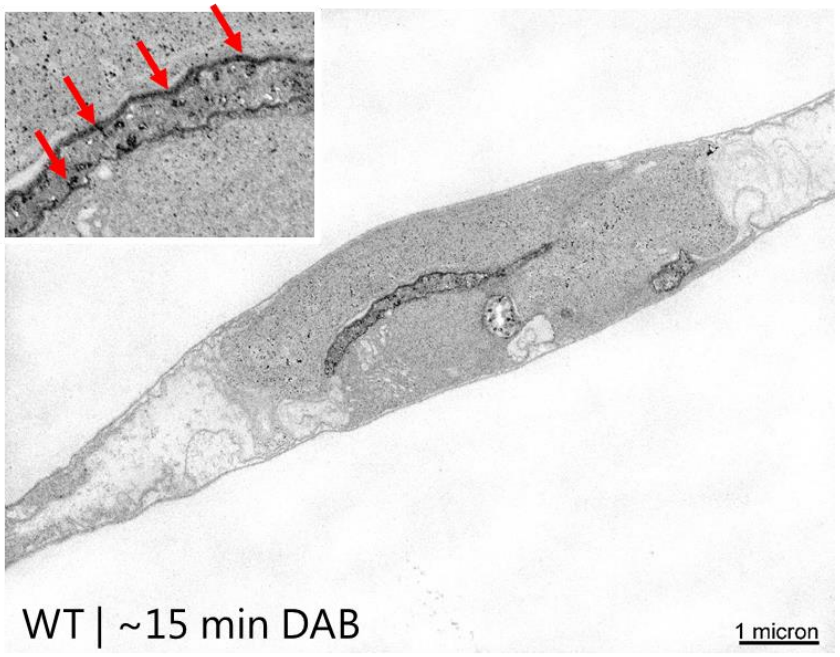
Yellow: resorufin
Red: autofluorescence

APEX2 enables high resolution subcellular localization studies of diatom proteins using TEM

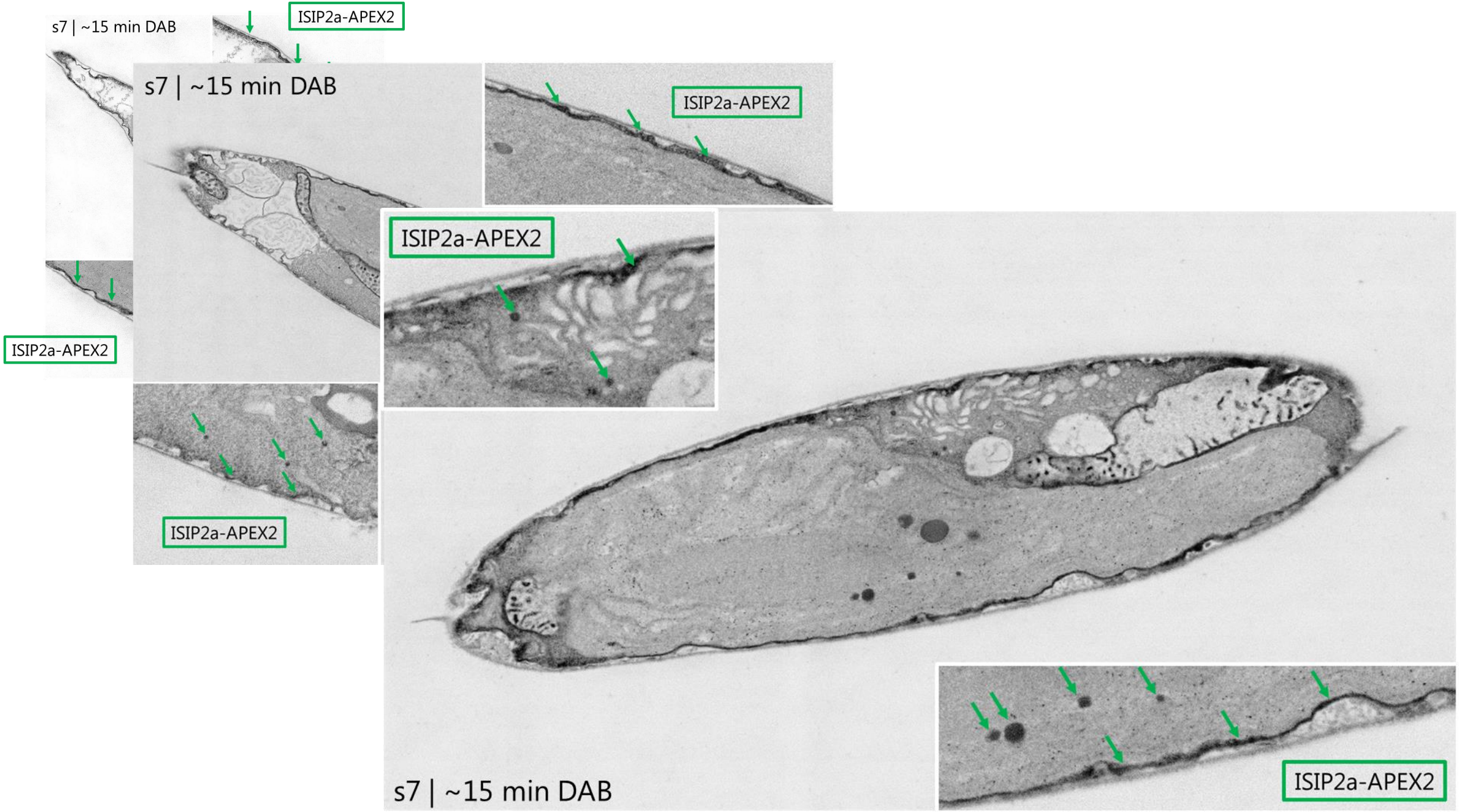
ISIP2a-APEX2



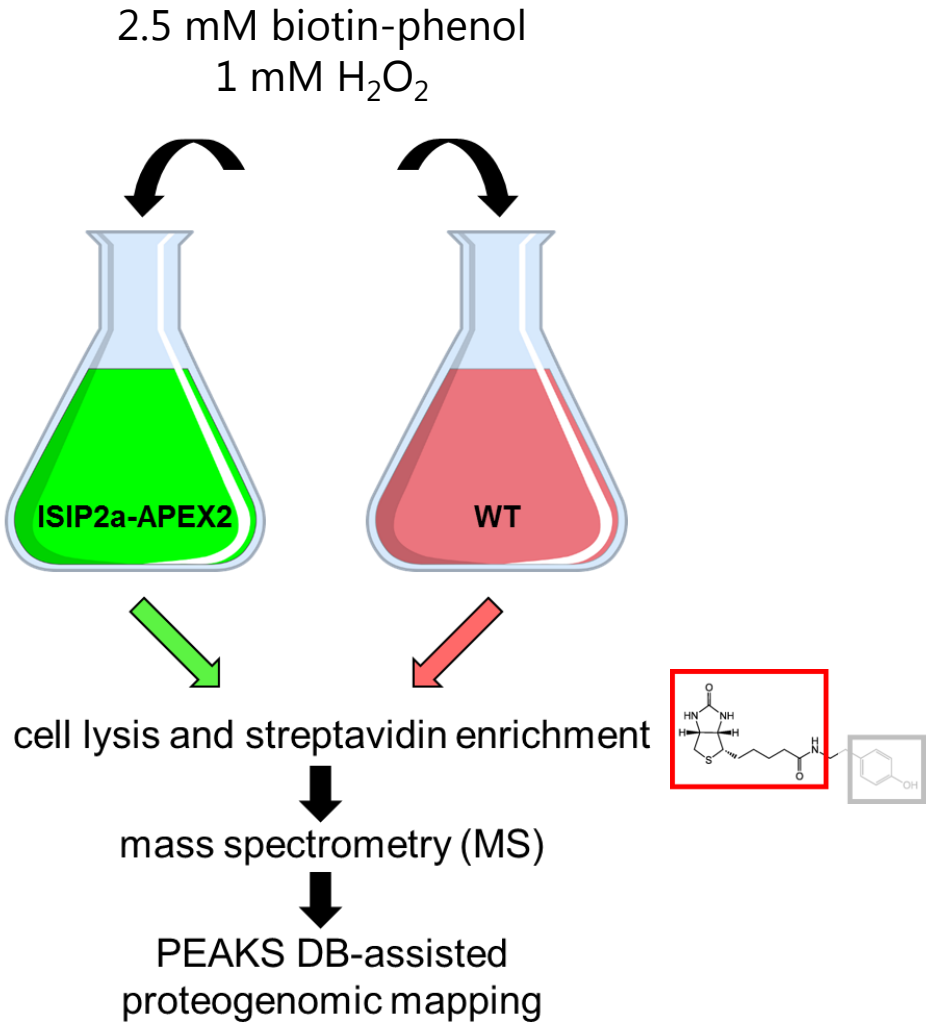
WT control

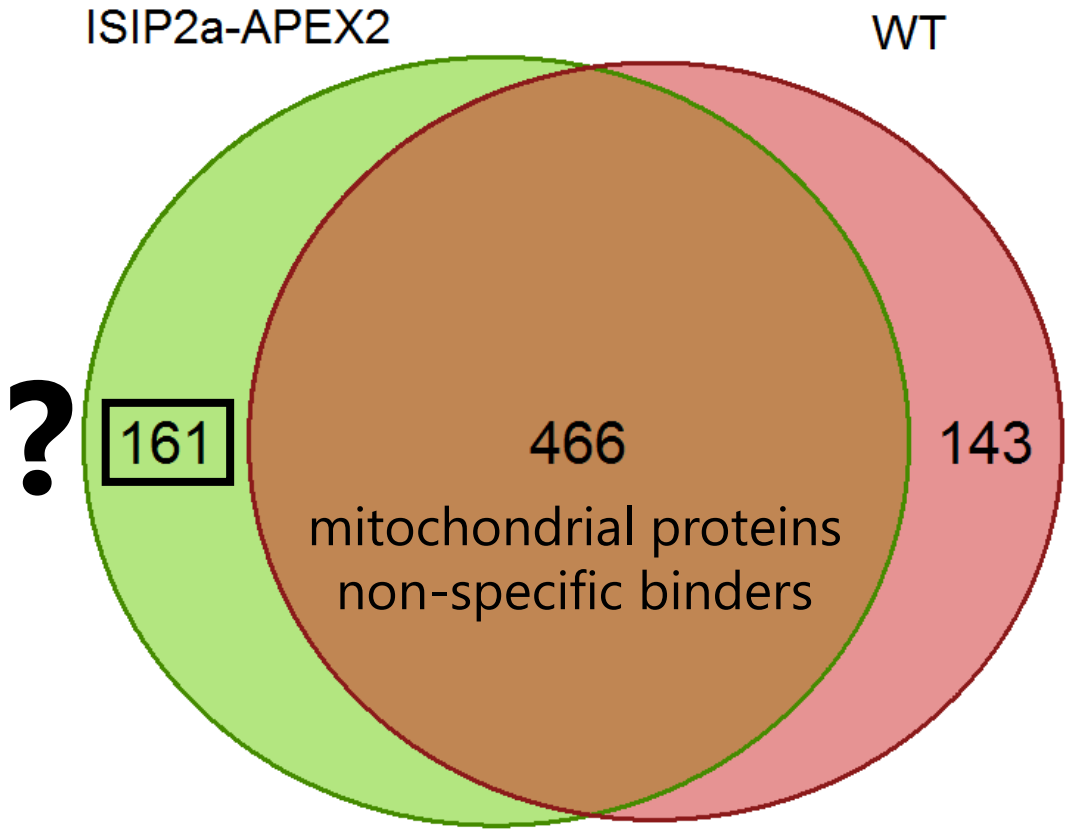


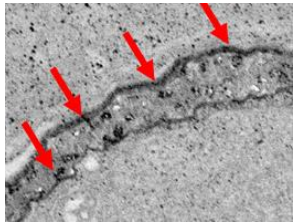
Putative mitochondrial and APEX2-like ascorbate peroxidases identified in *Pt*.


s7 | ~15 min DAB
ISIP2a-APEX2
s7 | ~15 min DAB
ISIP2a-APEX2
ISIP2a-APEX2
ISIP2a-APEX2
ISIP2a-APEX2
s7 | ~15 min DAB
ISIP2a-APEX2

Overview of our preliminary proteomics experiment

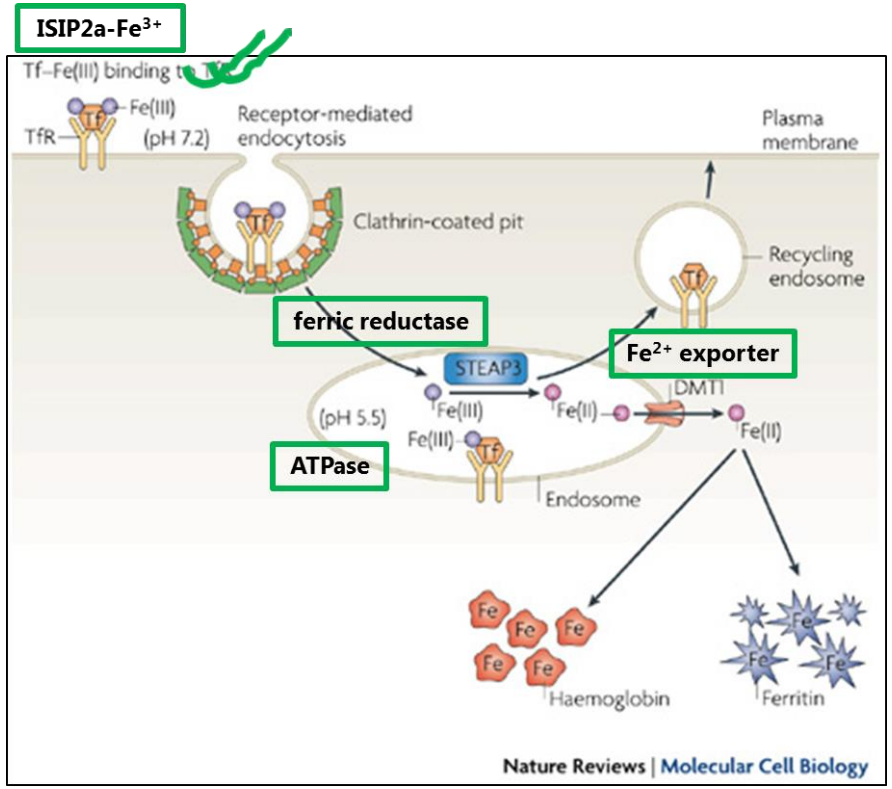






Proteomics data reveal a number of endocytosis-related proteins

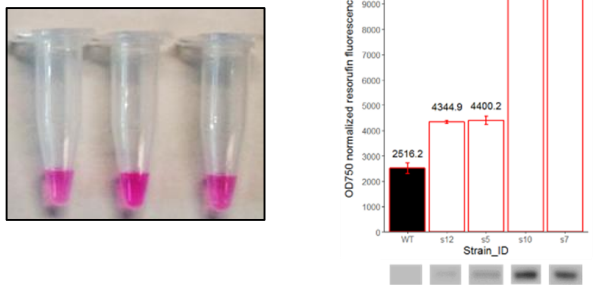
Phatr3 ID	Function and/or location
Phatr3_J30139 | SEC4	exocytosis
Phatr3_J21535 | RAB6	Golgi to ER trafficking
Phatr3_EG02235 | RAB7	**late endosomes**, vacuole
Phatr3_J22713 | RAB1A	Golgi
Phatr3_J54987 | ISIP2B	**similar to ISIP2a?**
Phatr3_EG02335 | Myosin 29	**vesicle transport?**
Phatr3_J41031 | Sec61 α	ER protein translocator
Phatr3_J44474 | ATPase	**vesicle acidification?**



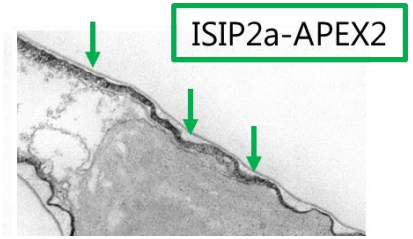
Only proteins with clear functional annotations are listed.

Summary

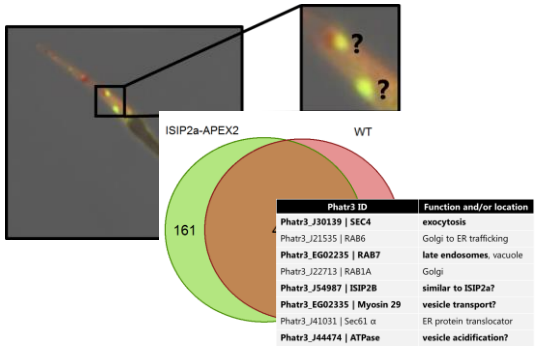
1. Diatom proteins can be expressed from an episome as fusions with APEX2 while retaining APEX2 activity.



2. APEX2 enables high resolution imaging of diatom proteins using electron microscopy.



3. Endocytosis-related proteins identified in our preliminary subcellular proteomics experiment with ISIP2a-APEX2.



Next steps and future directions

1. Repeating experiments in ***ΔISIP2a* genetic background** with **native promoter/terminator** and **iron-deplete conditions**.

2. Performing traditional **pull-down assays**.

3. Measuring **vesicle pH** with a pH-dependent photoconvertible fluorescent protein **mKeima**.

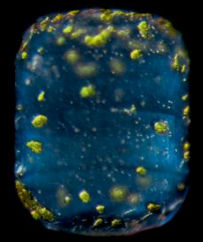
4. **Time-lapse imaging** to capture and visualize endocytic events.

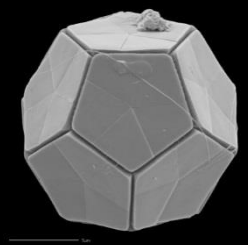
5. APEX2-independent discovery of a putative **ferrous iron transporter** (subcellular localization determined, KO generation and functional studies in yeast happening).

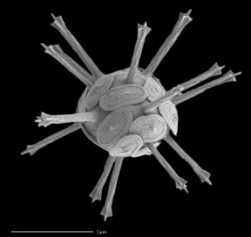
APEX2 vision for diatom biology and beyond

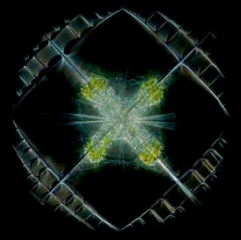
Diatom biology

Genetic control of biomineralization

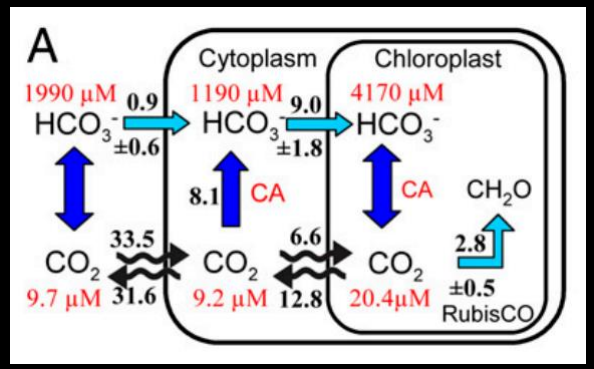

SiO_2


$CaCO_3$


$CaCO_3$


$SrSO_4$

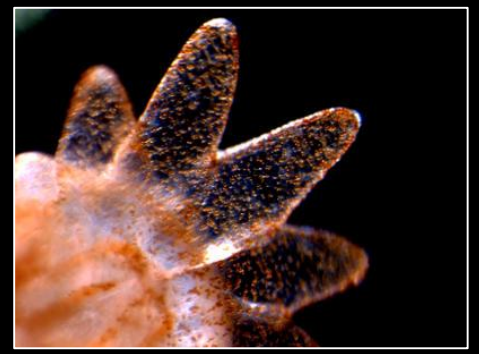
Carbon concentration mechanism



Other marine systems

(Cyano)bacteria

Coral biology


Credit: Emily Howells

Sea urchin biology


Source

1. Hopkinson *et al.*, Efficiency of the CO_2-concentrating mechanism of diatoms. *PNAS* 108, 10: 3830–3837 (2011).

Sharing means progress

OPEN ACCESS protocols.io

Conjugation of Thalassiosira pseudonana Version 3

Jernej

OPEN ACCESS protocols.io

Simple & rapid genotyping of marine microeukaryotes

Jernej

OPEN ACCESS protocols.io

Pour plating of Thalassiosira pseudonana (Tp) Version 3

Jernej

OPEN ACCESS protocols.io

Guidelines for highly efficient construction of diatom episomes using Gibson Assembly

Jernej

OPEN ACCESS protocols.io

HA tag enables highly efficient detection of heterologous proteins in Phaeodactylum tricornutum (Pt) exconjugants

Version 2

Jernej Turnsek

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protocols.io
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BBS PhD Program in Biological and Biomedical Sciences

Are transcriptomic data holding any clues?

"iron" proteins (10)

cytoskeletal and vesicular proteins (3)

proteolytic enzymes (3)

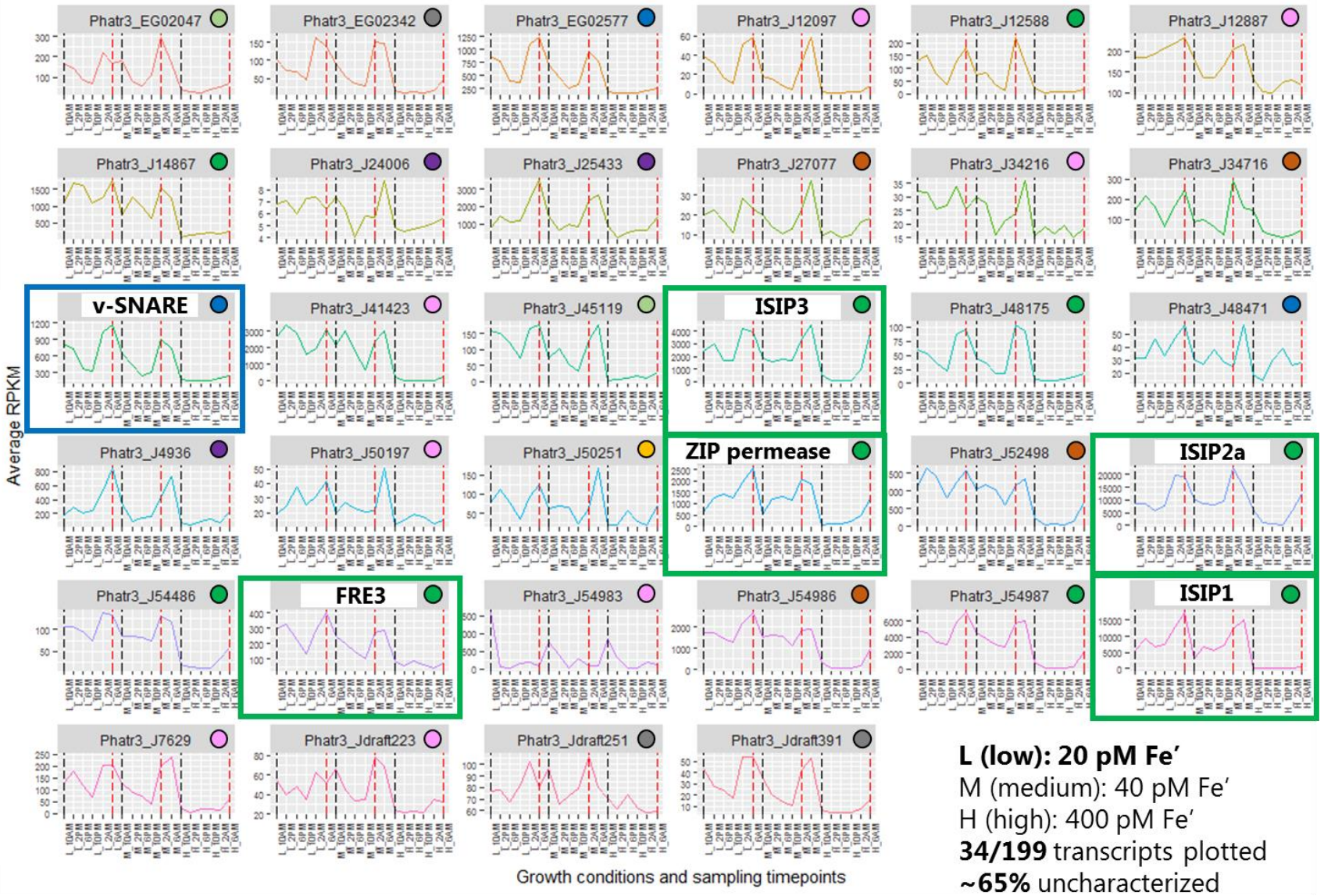
cell wall proteins (4)

cell cycle proteins (1)

ion transporters (2)

ATPases (3)

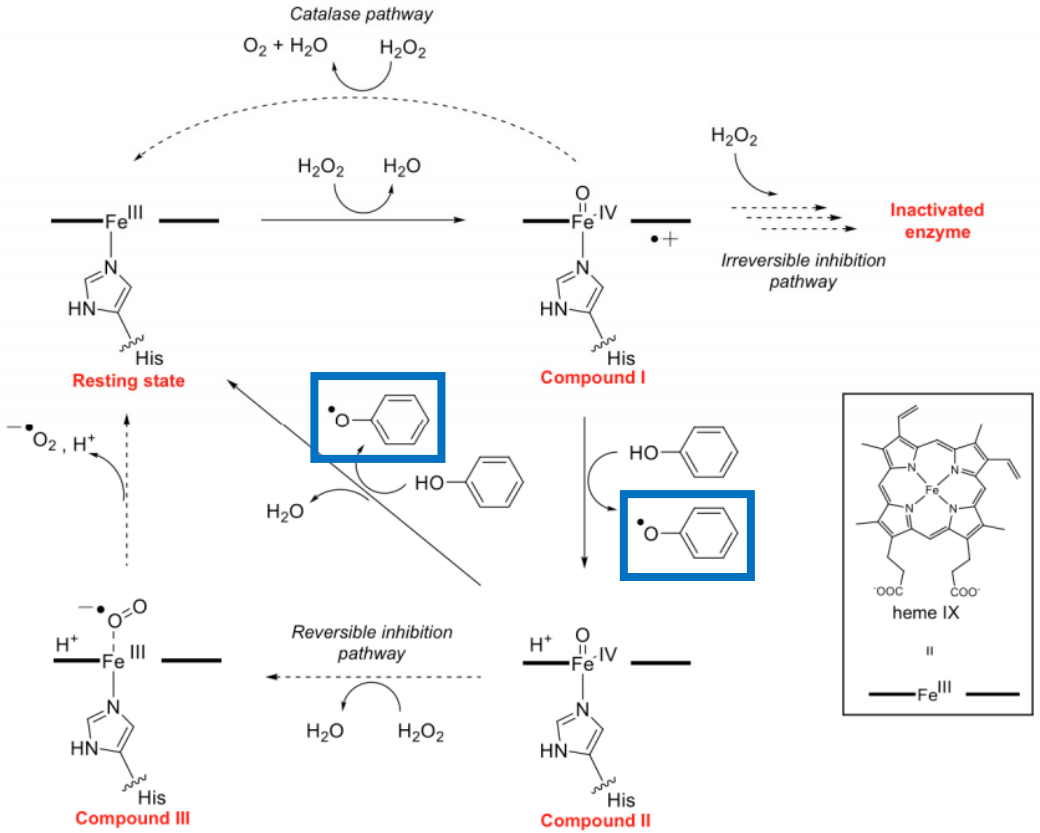
other (TFs …) (8)



L (low): 20 pM Fe'
M (medium): 40 pM Fe'
H (high): 400 pM Fe'
34/199 transcripts plotted
~65% uncharacterized

From: Smith *et al.* Transcriptional Orchestration of the Global Cellular Response of a Model Pennate Diatom to Diel Light Cycling under Iron Limitation. *PLoS Genetics* 12, e1006490 (2016).

APEX2 catalytic cycle for *in vivo* biotinylation of endogenous proteins



Short lived (<1 ms) biotin-phenoxyl radicals released from APEX2 active site.

Covalent adducts to Tyr side chains, possibly other electron-rich amino acids (Trp, Cys, His).

Biotinylation radius ~10-20 nm, but "better to think of it as a **contour map** where biotinylation efficiency falls down with distance from APEX2-tagged TM."

Have **surface exposed tyrosines**? You will be biotinylated!

1. Lam *et al.*, Directed evolution of APEX2 for electron microscopy and proximity labeling. *Nature Methods* 12: 51–54 (2015).