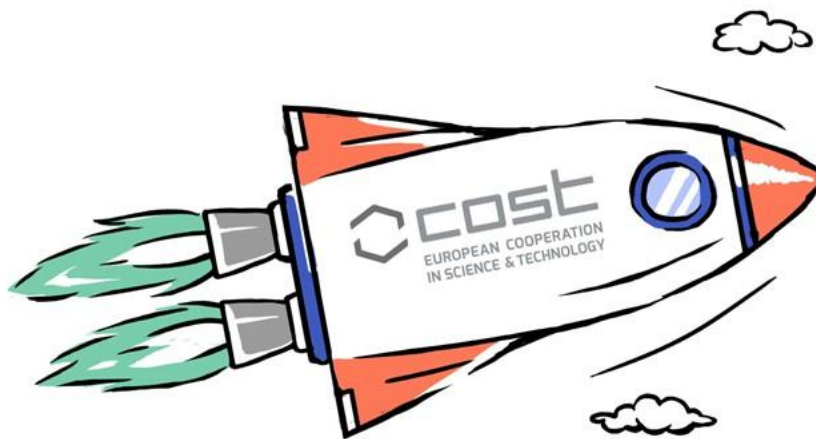




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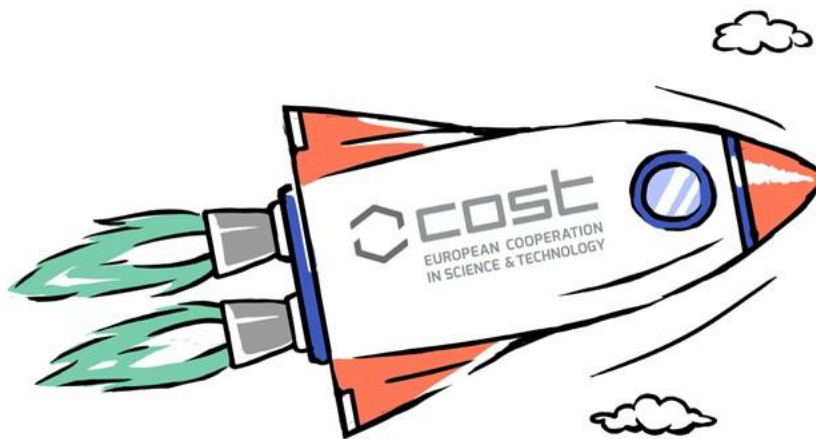
Report date: October 2026

WG3. Mechanistic studies of FeS biogenesis

Deliverables:

- Deliverable 9. Report on spectroscopic characterisation of FeS clusters in different components of FeS biogenesis.
- Deliverable 10. Report on structural studies of enzymatic reactions using wild-type and variants.
- Deliverable 11. Report on structural studies of enzymatic reactions using wild-type and variants.
- Deliverable 12. Report on structural studies of the interaction of components of FeS biogenesis machinery.
- Deliverable 13. Scientific papers on the catalytic function of members of radical-SAM enzymes.

Period: Nov 2022 – Nov 2026



Deliverables 9-12

Executive Summary

Working Group 3 (WP3) has made transformative advances in understanding the molecular mechanisms governing iron-sulfur (Fe-S) cluster biogenesis, maturation, and transfer. Through an integrated approach combining structural biology, biochemistry, spectroscopy, and protein engineering, WP3 has elucidated fundamental principles that span from bacterial model systems to human disease relevance and eukaryotic cellular machinery. Key achievements include: (1) the first step-by-step dissection of the complete [2Fe-2S] cluster assembly pathway in the *Escherichia coli* ISC system; (2) structural characterization of the human IBA57-ISCA2 complex revealing the molecular basis of a severe mitochondrial disease; (3) elucidation of sulfur trafficking hierarchy and competition at the IscS hub; (4) structural and functional characterization of the *Mycobacterium tuberculosis* SufS-SufU sulfur transfer mechanism; (5) rational engineering of the [FeFe]-hydrogenase maturation pathway achieving 41-fold rate enhancement; (6) structural insights into electron transfer partnerships in radical-SAM enzymes; and (7) discovery of a conserved tripeptide motif governing client recognition in eukaryotic Fe-S cluster delivery. These outputs collectively establish WP3 as a leading force in mechanistic Fe-S enzymology with implications spanning from fundamental biochemistry to therapeutic development and synthetic biology.

1. Structural Studies of Enzymatic Reactions Using Wild-Type and Variants

1.1. Complete Dissection of [2Fe-2S] Cluster Assembly by the ISC Machinery (Output 3)

A fundamental question in Fe-S biology has been the precise sequence of molecular events leading to cluster assembly on scaffold proteins. WP3 researchers have now provided the first comprehensive, step-by-step description of this process.

- **Experimental Approach:** Using an in vitro reconstituted system with purified components of the *E. coli* ISC machinery, combined with biochemical and spectroscopic techniques, the study tracked cluster assembly intermediates in real-time.
- **Sequential Mechanism Elucidated:**
 1. **Iron binding to IscU** initiates the process, triggering a conformational state competent for interaction with IscS.
 2. **Persulfide insertion** occurs via IscS cysteine desulfurase activity upon IscS-IscU complex formation.
 3. **Persulfide cleavage to sulfide** is catalyzed by ferredoxin (Fdx), generating a novel **[1Fe-1S] precursor** intermediate.
 4. **IscU dissociation and dimerization** enables fusion of two [1Fe-1S] precursors to generate a bridging [2Fe-2S] cluster.
 5. **Dimer dissociation** releases monomeric holo-IscU ready for cluster transfer to acceptor proteins.
- **Action Impact:** This work provides the definitive mechanistic framework for the ISC pathway, identifying key intermediates that were previously hypothetical. The discovery of the [1Fe-1S] precursor represents a paradigm shift in understanding cluster assembly chemistry and provides new targets for mechanistic interrogation.

1.2. Structural Basis of MMDS3: The IBA57 Gly104Cys Variant (Output 4)

Multiple mitochondrial dysfunctions syndrome type 3 (MMDS3) is a severe leukoencephalopathy caused by mutations in *IBA57*, a protein of previously unknown function in Fe-S biogenesis. WP3 has now defined the molecular consequences of a pathogenic variant.

- **Finding:** The study investigated the p.Gly104Cys (c.310G>T) variant associated with severe MMDS3, characterized by defects in respiratory complex activity and lipoic acid-dependent enzymes.
- **Biophysical Characterization:** Using size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS), NMR, circular dichroism, and fluorescence spectroscopy, the study demonstrated that:
 - The Gly104Cys variant **does not impair** the conversion of the homo-dimeric [2Fe-2S]-ISCA2 complex into the functional hetero-dimeric IBA57-[2Fe-2S]-ISCA2 complex.
 - The variant **significantly reduces the stability** of IBA57, both in its isolated form and in complex with ISCA2.
- **Mechanistic Insight:** These findings establish that IBA57 functions as a stabilizing partner for ISCA2 during [4Fe-4S] protein maturation. The pathogenic variant

compromises this stabilizing function, leading to defective [4Fe-4S] cluster delivery to respiratory complexes and lipoic acid synthase.

- **Action Impact:** This work provides the first structural rationale for MMDS3 pathology, linking protein stability to disease severity. The findings open avenues for therapeutic strategies aimed at stabilizing IBA57 variants.

1.3. Sulfur Transfer Mechanism in *Mycobacterium tuberculosis* SufS-SufU (Output 6)

Tuberculosis remains a global health threat, and the essential SUF system in *M. tuberculosis* (Mtb) represents an attractive drug target. WP3 has now provided the first structural and functional characterization of the Mtb SUF sulfur mobilization module.

- **Finding:** The study characterized Rv1464 (SufS) and Rv1465 (SufU) from Mtb, revealing their coordinated mechanism of sulfur transfer.
- **Structural and Mechanistic Insights:**
 - **Rv1464 (SufS)** was confirmed as a type II cysteine desulfurase.
 - **Rv1465 (SufU)** is a **zinc-dependent protein** that directly interacts with SufS and possesses sulfurtransferase activity.
 - SufU enhances SufS desulfurase activity by accepting the persulfide sulfur onto its conserved **Cys40** residue.
 - The **zinc ion in SufU is essential** for the sulfur transfer reaction.
 - **His354 in SufS** plays a critical role in the sulfur transfer to SufU.
- **Differential Regulation:** The Mtb SufS-SufU system exhibits **enhanced resistance to oxidative stress** compared to the *E. coli* SufS-SufE system, attributable to the presence of zinc in SufU.
- **Action Impact:** This structural and mechanistic characterization of the Mtb SUF system provides a foundation for rational drug design targeting essential Fe-S biogenesis in this pathogen.

1.4. Rational Engineering of [FeFe]-Hydrogenase Maturation (Output 2)

The [FeFe]-hydrogenase from *Desulfovibrio desulfuricans* (DdHydAB) is a promising biocatalyst for sustainable H₂ oxidation/production, but its industrial application is limited by slow in vitro maturation with synthetic cofactor mimics. WP3 has addressed this bottleneck through structural analysis and protein engineering.

- **Finding:** Structural and sequence analysis identified that slow maturation results from inefficient rearrangement of a **critical "locking element"** near the active site after cofactor uptake.
- **Engineering Solution:** The enzyme was redesigned via **subunit fusion**, inserting short peptide linkers near the active site to facilitate the required conformational change.
- **Outcome:** The modification increased the **maturation rate by up to 41-fold** without compromising catalytic performance.

- **Mechanistic Contribution:** The findings elucidate a key step in the "plug-lock-lid" mechanism underlying [FeFe]-hydrogenase maturation, providing a general framework for engineering other metalloenzyme maturation pathways.
 - **Action Impact:** This work establishes a blueprint for the scalable production of holo-[FeFe]-hydrogenases, advancing their industrial applicability for green energy applications.
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2. Structural Studies of Interactions Between FeS Biogenesis Machinery Components

2.1. Hierarchy and Competition at the IscS Sulfur Trafficking Hub (Output 5)

The cysteine desulfurase IscS serves as a central sulfur donor for multiple biosynthetic pathways in *E. coli*, yet the mechanisms governing sulfur allocation remain poorly understood. WP3 has now dissected the competitive interactions governing this critical hub.

- **Finding:** The study demonstrated that **TusA** (required for molybdenum cofactor biosynthesis and tRNA thiolation) and **IscU** (the Fe-S scaffold) **compete for binding to IscS**, with IscU exhibiting slightly higher affinity.
- **Heterocomplex Formation:** Heterocomplexes involving the IscS dimer with single IscU and TusA molecules readily form, indicating that these partner proteins can occupy different protomers of the IscS dimer simultaneously.
- **Activity Modulation:** Binding of both TusA and IscU to IscS **modulates cysteine desulfurase activity**, suggesting an allosteric communication mechanism.
- **Proposed Model:** A regulatory model was proposed in which sulfur delivery to different pathways is controlled by:
 1. Relative cellular levels of sulfur acceptor proteins.
 2. Differential binding affinities for IscS.
 3. Acceptor protein-mediated modulation of IscS desulfurase activity.
- **Action Impact:** This work establishes a quantitative framework for understanding how cells prioritize sulfur allocation during stress, infection, and metabolic adaptation. The competitive hierarchy between Fe-S biogenesis and tRNA modification pathways represents a critical regulatory node.

2.2. Structural Basis of Viperin-CYB5R3 Electron Transfer Partnership (Output 1)

The antiviral radical-SAM enzyme Viperin (RSAD2) requires an electron donor for catalytic activity, yet the identity and structural basis of this partnership have remained elusive. WP3 has solved this long-standing mechanistic question.

- **Finding:** The study identified **cytochrome b5 reductase 3 (CYB5R3)** as the physiological electron donor for the ER-anchored antiviral enzyme Viperin.
- **Structural Insights:** Structural characterization of the **RSAD2:CYB5R3 complex** revealed that their functional partnership is strictly dependent on **co-localization at the endoplasmic reticulum membrane**.
- **Mutagenesis Analysis:** Targeted mutagenesis identified the **specific structural elements required for electron transfer** between the two proteins, providing atomic-level understanding of the redox partnership.
- **Engineering Application:** This structural knowledge enabled the rational design of **iCAREs (immune-silent, catalytically self-sufficient antiviral restriction factor enzymes)** that autonomously generate antiviral nucleotide analogues without requiring endogenous redox partners.
- **Action Impact:** This work resolves a key mechanistic question in radical-SAM enzymology regarding the necessity of ER localization for Viperin activity. The structural insights establish a general strategy for engineering self-sufficient radical-SAM enzymes and open new avenues for antiviral therapeutic development.

2.3. Molecular Recognition in Eukaryotic Fe-S Cluster Delivery: The TCR Motif (Output 12)

The eukaryotic cytosolic iron-sulfur protein assembly (CIA) pathway must deliver clusters to more than 30 different client proteins, raising fundamental questions about how specificity is encoded. WP3 has now identified the molecular determinant governing client recognition.

- **Finding:** A conserved **tripeptide motif [LIM]-[DES]-[WF]-COO⁻** is present at the C-terminus of more than a quarter of CIA clients or their adaptors.
- **Functional Validation:** This **targeting complex recognition (TCR) motif** is **necessary and sufficient** for:
 - Binding to the CIA-targeting complex (CTC) in vitro.
 - Directing Fe-S cluster delivery in vivo.
- **Bioengineering Application:** Fusion of the TCR signal to a nonnative protein **enabled engineering of cluster maturation** via recruitment of the CIA machinery.
- **Mechanistic Insight:** Unlike other metalloprotein maturation systems with one-to-one correspondence between metallochaperone and apo-client, the CIA pathway uses a **common recognition motif** to identify diverse clients, providing an elegant solution to the specificity challenge.
- **Action Impact:** This discovery provides fundamental insights into the biochemistry of Fe-S protein maturation and unlocks the potential for bioengineering Fe-S cluster delivery to nonnative enzymes—a critical bottleneck in synthetic biology applications involving these versatile metalloproteins.

2.4. ATP-Dependent Fe(CN)₂CO Transfer in [NiFe]-Hydrogenase Maturation (Output 10)

The maturation of [NiFe]-hydrogenases requires the HypCD complex to assemble and transfer the $\text{Fe}(\text{CN})_2\text{CO}$ subfragment, but the role of its ATPase activity has remained mysterious. WP3 has now uncovered unexpected mechanistic details.

- **Finding:** Comprehensive spectroscopic characterization using infrared, Mössbauer, and nuclear resonance vibrational spectroscopy (NRVS) revealed **molecular details of $\text{Fe}(\text{CN})_2\text{CO}$ coordination** to the HypCD complex.
 - **ATP Requirement Deciphered:** Combining biochemical assays, spectroscopy, AlphaFold structure predictions, protein-ligand docking, and crosslinking MS revealed that **ATP hydrolysis triggers the transfer of the $\text{Fe}(\text{CN})_2\text{CO}$ fragment** to the apo-hydrogenase subunit.
 - **Mechanistic Insight:** The ATPase activity of HypCD does not function in cluster assembly per se but rather in **driving the conformational change or energetically unfavorable transfer step** required for cofactor delivery.
 - **Action Impact:** This work resolves a long-standing mechanistic question in [NiFe]-hydrogenase maturation and establishes a general principle for ATP-dependent cofactor transfer in metalloenzyme biogenesis.
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3. Synthesis and Future Directions for WP3

The outputs from this reporting period demonstrate WP3's comprehensive approach to understanding Fe-S biogenesis mechanisms across biological scales—from individual protein-protein interactions to complete assembly pathways, and from bacterial model systems to eukaryotic cellular machinery.

Key Mechanistic Themes Emerging:

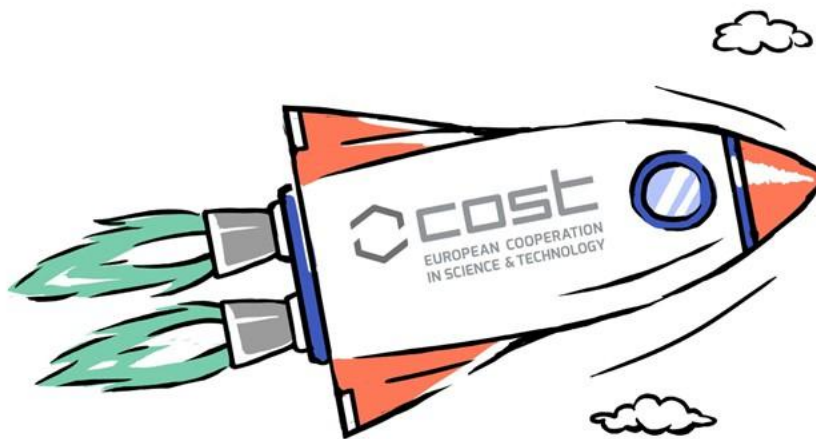
1. **Sequential Assembly with Defined Intermediates:** The ISC pathway dissection reveals that Fe-S cluster assembly proceeds through discrete, spectroscopically identifiable intermediates ($[1\text{Fe-1S}] \rightarrow [2\text{Fe-2S}]$) with tight coordination between partner proteins.
2. **Conformational Dynamics in Cluster Transfer:** The IBA57-ISCA2, hydrogenase maturation, and HypCD studies highlight the critical importance of protein conformational changes and stability in successful cluster delivery and cofactor insertion.
3. **Competitive Sulfur Allocation:** The IscS hub study reveals that Fe-S biogenesis competes with other essential sulfur-requiring pathways, establishing a regulatory hierarchy responsive to cellular demands.
4. **Structural Determinants of Electron Transfer:** The Viperin-CYB5R3 complex structure provides a template for understanding how redox partners are selected and how electron transfer can be engineered.

5. **Molecular Recognition Codes:** The discovery of the TCR motif establishes that specific sequence determinants encode client recognition in the eukaryotic CIA pathway, analogous to signal sequences in protein trafficking.
6. **ATP-Dependent Cofactor Transfer:** The HypCD mechanism reveals that ATP hydrolysis can drive energetically unfavorable cofactor transfer steps, establishing a general principle in metalloenzyme maturation.

Proposed Next Steps for WP3:

1. **Intermediate Trapping in SUF Pathway:** Following the success with the ISC system, WP3 will prioritize the development of experimental strategies to trap and characterize intermediates in the *M. tuberculosis* SUF pathway for drug targeting.
2. **Time-Resolved Structural Studies:** We will pursue time-resolved crystallography and cryo-EM approaches to capture conformational transitions during cluster transfer from IscU to acceptor proteins.
3. **Expanding the IscS Interaction Network:** Building on the TusA-IscU competition study, WP3 will systematically characterize the full hierarchy of IscS partner interactions to understand sulfur allocation under stress conditions relevant to infection.
4. **TCR Motif Structural Characterization:** WP3 will pursue high-resolution structural studies of the CTC-TCR peptide complex to understand the molecular basis of client recognition and enable rational design of novel targeting signals.
5. **Cross-WP Collaboration with WP2:** WP3 will collaborate with WP2 to apply mechanistic insights from SufS-SufU characterization toward the development of novel antitubercular agents targeting the SUF system.
6. **Hydrogenase Maturation Optimization:** The successful subunit fusion strategy will be extended to other [FeFe]-hydrogenases and metalloenzymes with complex maturation requirements.
7. **Bioengineering Fe-S Enzymes:** The TCR motif discovery will be leveraged to engineer novel Fe-S enzymes for synthetic biology applications, with initial focus on nonnative hydrogenase and nitrogenase expression in eukaryotic systems.

Disclaimer: *This report is a synthesis document prepared for COST Action Working Group 3 based on the provided abstracts. Specific grant numbers and author lists are omitted for brevity but are available in the original publications.*



Deliverable 13

Publications

1. Dark proteome ensemble superposition gating calibrates biosynthesis of antiviral cocktail, BioRxiv DOI: <https://doi.org/10.64898/2026.09.10.750538>
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4. The ISC machinery assembles [2Fe–2S] clusters by formation and fusion of [1Fe–1S] precursors. *Nature Chemical Biology* (2025) volume 21, pages767–778. DOI: <https://doi.org/10.1038/s41589-024-01818-8>
5. Cross-regulation of [2Fe–2S] cluster synthesis by ferredoxin-2 and frataxin. *Nature* (2026) **649**, pages721–728
6. Unraveling the Complexity of the Suf-Based Fe–S Biogenesis, (Book Chapter), *Iron-Sulfur Clusters: Biogenesis and Biochemistry* (2025). DOI: <https://doi.org/10.1002/9783527843596.ch2>
7. Fe-S biogenesis by SMS and SUF pathways: A focus on the assembly step, *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* (2024) Volume 1871, Issue 7, 119772. DOI: <https://doi.org/10.1016/j.bbamcr.2024.119772>
8. Ancestral [Fe-S] biogenesis system SMS has a unique mechanism of cluster assembly and sulfur utilization, *PLoS Biol* (2025) 23(6): e3003223. <https://doi.org/10.1371/journal.pbio.3003223>
9. CyaY and TusA regulate ISC- and SUF-mediated l-cysteine desulfurase activity, *RSC Chem. Biol.* (2024) 5 (11): 1165–1176. DOI: <https://doi.org/10.1039/d4cb00225c>
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11. Binding of IscU and TusA to different but competing sites of IscS influences the activity of IscS and directs sulfur to the respective biomolecular synthesis pathway, *Journal of Bacteriology* (2024) DOI: <https://doi.org/10.1128/spectrum.00949-24>

12. Unraveling the molecular determinants of a rare human mitochondrial disorder caused by the P144L mutation of FDX2, Protein Science, (2024) Volume33, e5197. DOI: <https://doi.org/10.1002/pro.5197>
13. Cytosolic iron–sulfur protein assembly system identifies clients by a C-terminal tripeptide, PNAS (2023) 120 (44) e2311057120, DOI: <https://doi.org/10.1073/pnas.2311057120>
14. Structural and Biochemical Characterization of Mycobacterium tuberculosis Zinc SufU-SufS Complex, Biomolecules 2023, 13(5), 732; DOI: <https://doi.org/10.3390/biom13050732>