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## Reply to: Acetyl-CoA is produced by the Citrate Synthase Homology module of ATP-citrate lyase

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In their Matters Arising, Verstraete *et al.*<sup>1</sup> assert that the conclusions we draw in our manuscript (Wei *et al.*<sup>2</sup>) are not supported by the data presented. Based on seven cryo-EM structures of ATP Citrate Lyase (ACLY) in different liganded states, we concluded that both the reaction of ATP, citrate and CoA substrates and the formation of acetyl-CoA and OAA products occur in the ASH domain of ACLY. We proposed the following model for ACLY catalysis: CoA first binds to the ACLY CSH domain, which induces a structural rearrangement of ACLY, resulting in CoA translocation to the ASH domain. Once bound to the ASH domain, CoA reacts with ASH-bound citrate and ATP to form acetyl-CoA and OAA products. Once acetyl-CoA product is formed in the ASH domain, another structural rearrangement of ACLY unloads acetyl-CoA through binding to the CSH domain before acetyl-CoA leaves ACLY. Instead, Verstraete *et al.*<sup>1</sup> contend that, as previously proposed in Verschueren *et al.*<sup>3</sup>, the citryl-CoA intermediate is formed in the ASH domain, followed by a ~35 Å movement of the intermediate into the CSH module where cleavage to acetyl-CoA and OAA products occurs. Although we cannot exclude the model for ACLY catalysis proposed by Verstraete *et al.*<sup>1</sup>, we believe that our alternative model is also consistent with the data reported by Verschueren *et al.*<sup>3</sup>, Verstraete *et al.*<sup>1</sup> and Wei *et al.*<sup>2</sup>.

The specific concerns raised Verstraete *et al.*<sup>1</sup> are (1) The reduced catalytic activity of ACLY mutants within the CSH module (H976, R976, D1026 and R1065) supports the catalytic roles of these residues, (2) The data reported in Wei *et al.*<sup>2</sup> are not sufficient to claim that formation of acetyl-CoA and OAA products occurs in the ASH domain, (3) Even though we observed CoA bound to the CSH domain in the reported structure for ACLY in complex with CoA (PDB 6POE), a role of the CSH module in citryl-CoA cleavage is not considered,

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Author contribution

Conceptualization, X.W. and R.M.; Methodology, X.W. and R.M.; Investigation, X.W.; Formal Analysis, X.W.; Writing – Original Draft, R.M.; Visualization, X.W.; Writing – X.W. and R.M.; Funding Acquisition; R.M.; Resources, R.M.; Supervision, R.M.

Competing interests

The authors declare no conflict of interests.

METHODS

A description of the methods is available as Supplementary Note.

REPORTING SUMMARY

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

and (4 and 5) the phospho-citryl-CoA intermediate in the structure of the ACLY-E599Q mutant (PDB 6UUW) is incorrectly modeled.

### Response to (1)

The mutational sensitivity of residues H975, R976, D1026 and R1065 within the human ACLY CSH module indeed argues strongly for their functional importance. However, based on the D2-symmetric 3.0 Å ACLY product structure with acetyl-CoA and OAA reported in Wei *et al.*<sup>2</sup> (PDB 6UI9), we propose that these residues participate in binding of regulatory OAA and CoA molecules rather than play direct catalytic roles.

### Response to (2)

Verstraete *et al.*<sup>1</sup> argue that observing acetyl-CoA and OAA products in the ASH domain of ACLY does not demonstrate that their cleavage occurs at this site. Furthermore, the authors contend that the experimental density for the acetyl-thioester moieties of acetyl-CoA in the ASH active site is poorly defined. We believe that our D2-symmetric 3.0 Å ACLY product structure with acetyl-CoA and OAA (PDB 6UI9) shows compelling density for acetyl-CoA in the ASH domain (Figure 1a) and that the more compromised density for the mercapto group of acetyl-CoA bound to the ASH domain likely reflects the structural variability of the ASH domain relative to the CSH domain<sup>2</sup>. Indeed, the mercapto group of CoA and the citrate molecule in the ASH domain of the crystal structure reported in Verschueren *et al.*<sup>3</sup> (PDB 6QFB) is also poorly resolved in the electron density map (Extended Data Figure 1a). Nonetheless, to increase confidence that acetyl-CoA and OAA are correctly modeled in the ASH domain, we carried out non-uniform refinement together with particle symmetry expansion, particle subtraction and local refinement with Cryosparc (Extended Data Figure 2, Supplementary Table 1) on the cryo-EM density corresponding to the ACLY ASH domain of our ACLY–OAA–acetyl-CoA–D2 structure (PDB 6UI9)(Figure 1)<sup>4,5</sup>. This resulted in improved cryo-EM density for acetyl-CoA and OAA products in the ASH domain (Figure 1b). We have therefore updated the deposited atomic model and EM density map in the wwPDB and EMDB, which can be accessed via accession codes PDB-7LJ9 and EMBD-23389, respectively. While we also observe cryo-EM density in the CSH module, we believe this density corresponds to a regulatory OAA molecule. To note, we do not observe compelling density corresponding to acetyl-CoA in the CSH module, despite the significantly higher local resolution of the CSH module. In our view, these data are consistent with formation of acetyl-CoA and OAA in the ASH domain of ACLY as we originally proposed<sup>2</sup>, although we agree that they are not sufficient to prove this model.

### Response to (3)

Our model for ACLY catalysis involves the initial binding of CoA to the CSH domain prior to its translocation to the ASH domain for product formation, consistent with CoA binding to both the ASH and CSH domains as observed in our ACLY–citrate–CoA structure (PDB 6POE)<sup>2</sup> and by Verschueren *et al.*<sup>3</sup>. The use of regulatory or allosteric sites to load substrates prior to translocation to catalytic sites in metabolic enzymes is seen in other metabolic enzymes, for example with NADP<sup>+</sup> in human glucose-6-phosphatedehydrogenase<sup>6</sup> and we

therefore favor our model over the one proposed by Verstraete *et al.*<sup>1</sup>, since it rationalizes how CoA could bind to two distinct sites of ACLY.

## Response to (4) and (5)

To address the concern that the phospho-citryl-CoA intermediate (ligand QHD) in the structure of the ACLY-E599Q mutant is incorrectly modeled, we carried out non-uniform and local refinement on the cryo-EM density corresponding to the ACLY ASH domain of the ACLY-E599Q-ATP-citrate-CoA-D2 structure (PDB-6UUW)<sup>4,5</sup> (Extended Data Figure 2, Supplementary Table 1). This resulted in improved ligand density in the ASH active site, which could be best modeled as CoA + phospho-citrate, although at the current resolution we cannot exclude the additional presence of other reaction intermediates, such as phosphate + (3S)-citryl-CoA, and phospho-(3S)-citryl-CoA (Figure 2)<sup>7</sup>. We were also able to correct the stereochemistry of the modeled ligand QHD. The updated atomic model and EM density map have been deposited in the wwPDB and EMDB, respectively, with accession codes PDB-7LIW and EMBD-23387.

Verstraete *et al.*<sup>1</sup> also report new data to support their model for ACLY catalysis including (1) Biochemical data that an isolated CSH module added in trans can complete the ACLY reaction of a catalytically inactive ACLY-D026A mutant, (2) A crystal structure of a CSH module incubated with acetyl-CoA and OAA resulting in one of four CSH modules containing a modeled (3S)-citryl-CoA molecule, and (3) A crystal structure of an archaeal ACLY Asp541 mutant incubated with substrates, resulting in CSH modules containing additional electron density modeled as (3S)-citryl-CoA.

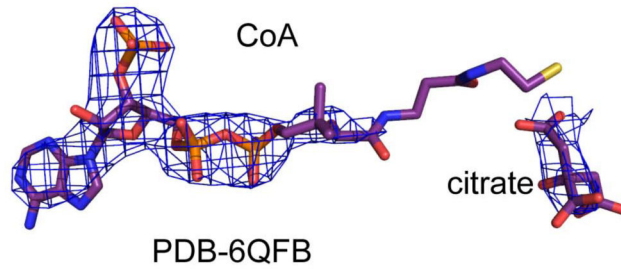
## Response to additional data

(1) While the isolated CSH module is able to catalyze cleavage of citryl-CoA to OAA and acetyl-CoA, in our view that does not necessarily demonstrate that the CSH module carries out the same chemistry in the context of the intact ACLY enzyme. (2) The observation that the isolated CSH module is able to form a stable complex with citryl-CoA is not surprising given that the CSH module can also bind citrate, OAA, CoA and acetyl-CoA<sup>2,3</sup>. The observation that the CSH module can synthesize citryl-CoA from acetyl-CoA and OAA is consistent with the reversibility of enzymes and the demonstration by Verstraete *et al.*<sup>1</sup> that the CSH module can convert citryl-CoA to OAA and acetyl-CoA. (3) The crystal structure of an archaeal ACLY Asp541 mutant incubated with substrates showing citryl-CoA bound to the CSH module is consistent with a defect of the mutant in producing acetyl-CoA and OAA products. However, this result does not prove that the CSH domain of the archaeal ACLY directly participates in chemistry to produce OAA and acetyl-CoA products. For example, citryl-CoA could be formed in the ASH domain and then translocated to the CSH module for release. It is also possible that the archaeal and human ACLY enzymes have diverged in their molecular mechanisms of product formation. In line with this idea, we previously reported that orthologous N-terminal acetyltransferase enzymes in archaea and human differ in their mechanism of N-terminal acetylation<sup>8</sup>.

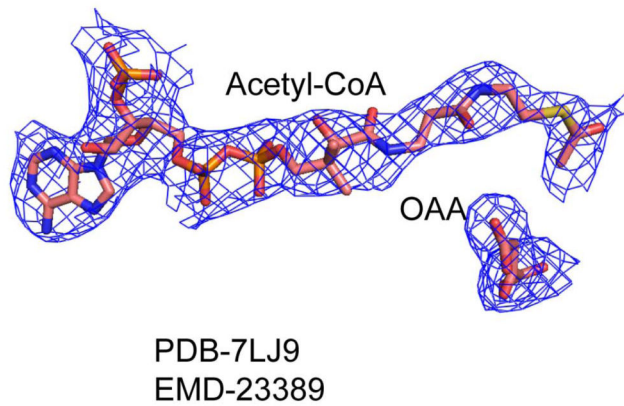
Overall, we think that further studies are required to unambiguously resolve the catalytic mechanism of ACLY.

### Extended Data

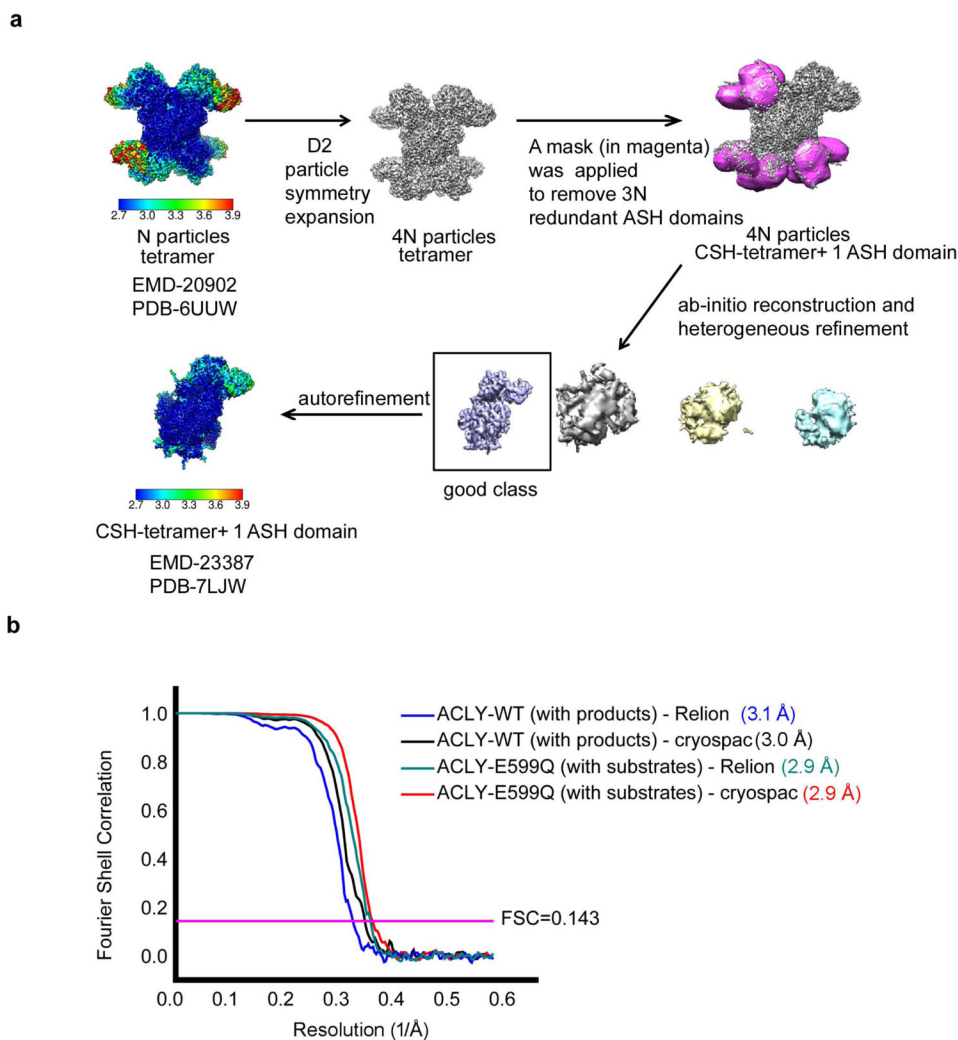
**a**



**b**



**Extended Data Fig. 1.**  
Revised Cryo-EM density for ACLY with products.



**Extended Data Fig. 2.**  
Workflow of data analysis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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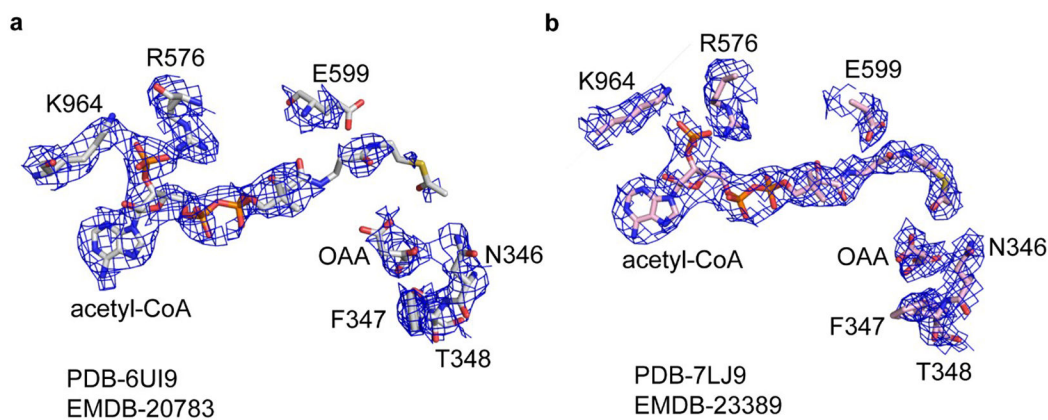
## DATA AVAILIBLTY

A revised cryo-EM map of the ACLY ASH domain of the ACLY–OAA–acetyl-CoA complex and the associated coordinate were generated from micrographs corresponding to ACLY–OAA–acetyl-CoA-D2 (PDB-6UI9) and EMBD-20783 reported in Wei *et al.*<sup>2</sup>, and were deposited with local refinement as accession codes PDB-7LJ9 and EMBD-23389 to

the wwPDB and EMDB, respectively; and without local refinement as accession codes PDB-7LLA and EMBD-23413 to the wwPDB and EMDB, respectively. A revised cryo-EM map of the ACLY ASH domain of the ACLY-E599Q–ATP–citrate–CoA complex and the associated coordinate were generated from micrographs corresponding to ACLY-E599Q–ATP–citrate–CoA-D2 (PDB-6UUW) and EMBD-20902 reported in Wei *et al.*<sup>2</sup> and deposited with accession codes PDB-7LIW and EMBD-23387 to the wwPDB and EMDB, respectively.

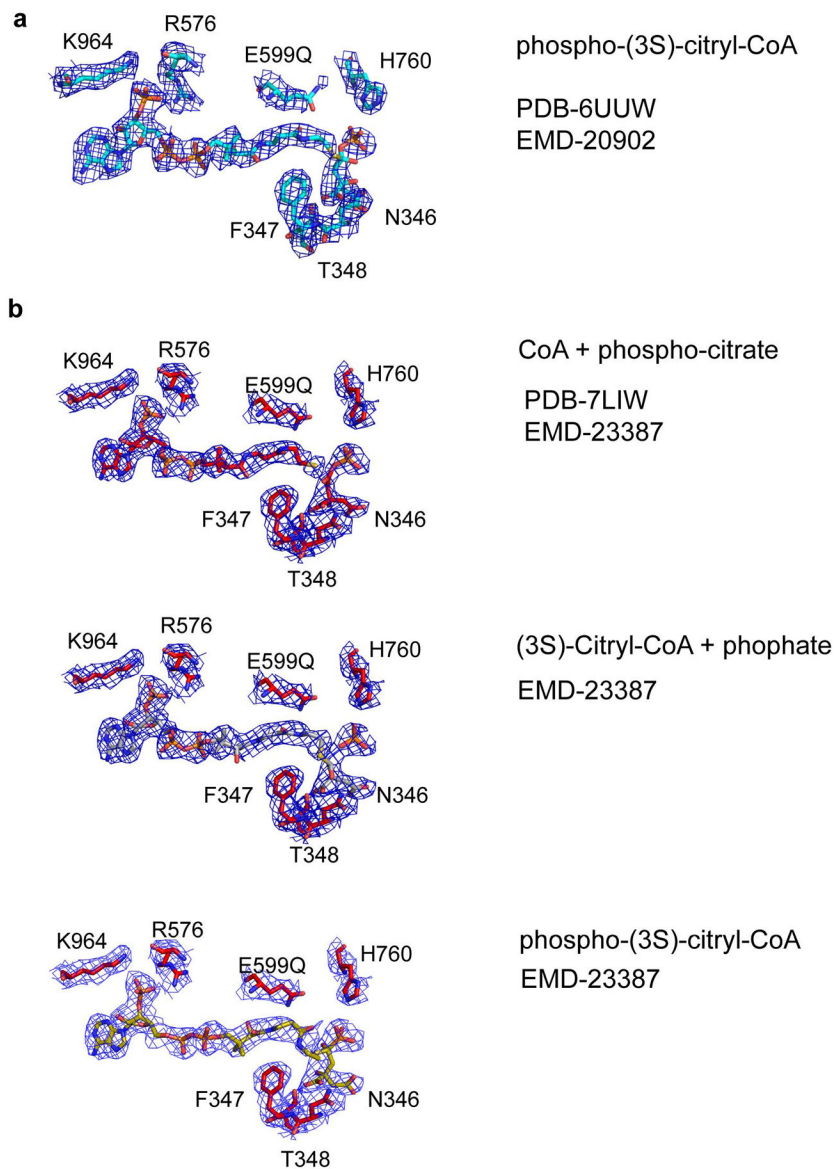
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**Figure 1.**

Cryo-EM density of acetyl-CoA and OAA in the ACLY active site. (a) Cryo-EM density corresponding to acetyl-CoA and OAA in the ASH domain of the ACLY–OAA–acetyl-CoA-D2 structure (PDB- 6UI9/EMD-20783) reported in Wei *et al.*<sup>2</sup>. (b) Cryo-EM density corresponding to acetyl-CoA and OAA in the ASH domain after non-uniform refinement together with particle symmetry expansion, particle subtraction and local refinement in Cryosparc (PDB-7LJ9/EMD-23389)<sup>4,5</sup>. The figure was made in Pymol with a contour level of  $2.0 \sigma$  applied. Important acetyl-CoA and OAA contacting residues, and catalytic E599, with their corresponding cryo-EM densities are shown for reference.

**Figure 2.**

Cryo-EM density of a reaction intermediate in the ACLY active site. (a) Cryo-EM density ( $2.5 \sigma$ ) corresponding to modeled phospho-(3S)-citryl-CoA in the ASH domain of the ACLY-E599Q–ATP–citrate–CoA–D2 structure (PDB- 6UUW/EMD-20902) reported in Wei *et al.*<sup>2</sup>. (b) Cryo-EM density ( $3.5 \sigma$ ) corresponding to the reaction intermediate in the ASH domain after non-uniform refinement together with particle symmetry expansion, particle subtraction and local refinement in Cryosparc (PDB-7LIW/EMD-23387)<sup>4,5</sup>. While the cryo-EM density can be best modeled as CoA + phospho-citrate (top, PDB-7LIW), at the current resolution we cannot exclude the additional presence of phosphate + (3S)-citryl-CoA (middle), and phospho-(3S)-citryl-CoA (bottom). The figure was made in Pymol. Important contacting residues, and catalytic E599, with their corresponding cryo-EM densities are shown for reference.