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Opinion on the habilitation thesis by Dr. Sebastian Glatt

Dr. Sebastian Glatt is a structural biologist interested in posttranscriptional tRNA modifications. These modifications are an attractive research topic for many reasons. From a chemical perspective, the biosynthesis of exotic nucleobases presents challenges to understand the reaction mechanisms. From a biological perspective, the modifications are interesting, because they affect tRNA structure, the fidelity of tRNA charging (when close to the acylation site), and the codon-anticodon readout (when in the anticodon loop). The effect of nucleobase modifications can be different for the interaction with different codons that code for the same amino acid. Hence, there is the possibility that changes in the repertoire of tRNA modifications could influence ribosome translation of messenger RNAs differentially, thus providing another layer of protein homeostasis control. Finally, mutations in the genes for tRNA modification enzymes are associated with many diseases. Among these, neurological diseases are particularly highly represented, but it is completely unclear, at least to me, why non-tissue specific tRNA modifications can lead to such tissue-specific medical outcomes.

For his habilitation thesis, Dr. Glatt has chosen a particular modification of a uridine in the wobble position of the anticodon loop of a tRNA. The modified nucleobase is carboxy-methyl-uridine (cm^5U), which is of interest both in its own right and as an intermediate in the biosynthesis of other modified RNA bases. Carboxy-methyl-uridine can be enzymatically methylated (esterified) to methoxy-carbonyl-methyl-uridine (mcm^5U). Alternatively, it can be converted from the carboxylate to the carboxamide. This amidation, by an unidentified enzyme, then leads to the modified nucleobase carbamoyl-methyl-uridine (ncm^5U). Moreover, the modified bases can be hypermodified by thiolation elsewhere in the ring (in the 2-position).

The tRNAs that carry the modified bases are targeted by a nuclease from the fungus *Kluyveromyces lactis*, which cleaves only the modified tRNA. Strains that fail to introduce the modifications become resistant to the nuclease, thus creating the opportunity for a mutant screen for survivors of nuclease selection. The assay has been used by the field to identify the enzymes of the biosynthesis pathway, and –now that they are known– to test protein variants for the ability to sustain the reaction. From the screen, two key players in the biosynthesis of cm^5U were identified, the Kti11/Kti13 heteroduplex and the elongator complex. Together,

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these two protein complexes catalyze the formation of carboxymethyluridine, using acetyl-CoA as the source of both carbon atoms of the modification.

From a chemical perspective, the use of acetyl-CoA in cm^5U biosynthesis is interesting. Acetyl-CoA can act in two fundamentally different ways, as an electrophile in a nucleophilic displacement reaction (with CoA-SH as the leaving group), or as a nucleophile with an α-acidic carbon (in a reaction analogous to fatty acid synthesis, except that the carrier protein in this reaction is acyl carrier protein, ACP, not acetyl-CoA). The orientation of the carboxymethyl group in cm^5U shows that acetyl-CoA would have to react with the α-acidic carbon, hence as a nucleophile. However, there is a problem. The C5 of the pyrimidine is also nucleophilic, not electrophilic (see for example, by the standard C5 methylation mechanism, or consider the analogy between a pyrimidine base and a Michael acceptor). Hence, any two-electron reaction mechanism would violate basic chemical principles. In enzymatic reactions, conflicting polarities of reaction partners are typically resolved by a radical based mechanism. This is true also for cm^5U biosynthesis. The best evidence so far is the presence of a radical SAM domain in Elp3, which contains an iron-sulfur cluster. Radical SAM domains generate an adenosyl radical from SAM (by homoloytic cleavage of the C5'-S bond). The adenosyl radical must then attack either acetyl-CoA or uridine, generating a secondary radical, which then initiates the carbon-carbon bond formation. At some stage in the reaction, the thioester bond of acetyl-CoA (which is likely not involved in the C-C bond forming reaction) has to be hydrolyzed as well. How C-C bond formation and acetyl-CoA hydrolysis are coupled is unclear, at least to me. Dr. Glatt's structures suggest that acetyl-CoA thioester hydrolysis happens early in the catalytic reaction, but I am unsure whether this conclusion has been confirmed biochemically. Particularly for amidation of cm^5U, the CoA-SH would be a much better leaving group than H_2O, suggesting that thioester hydrolysis would actually complicate a downstream modification reaction. In summary, cm^5U biosynthesis is chemically a very interesting reaction that is not yet fully understood.

In the publications forming the material for his habilitation, Dr. Glatt provides a structural biology framework for the understanding of carboxymethylation.

- In the 1st publication, entitled "Structure of the Kti11/Kti13 heterodimer and its double role in modifications of tRNA and eukaryotic elongation factor 2", Dr. Glatt focuses on the Kti11 and Kti13 proteins. He shows that the two proteins form a stable complex, and presents a structure of the complex, as well as a structure of Kti13 alone. Interestingly, the Kti11/Kti13 complex is not only required for uridine carboxymethylation, but also for modification of a histidine residue to diphthamide, in elongation factor 2 (eEF2). Intriguingly, the biosynthesis of diphthamide has mechanistic similarities to carboxymethylation, in that it also involves a "radical SAM" domain, and is believed to also consume SAM as a co-substrate. On a chemical level, the reactions are similar

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(homolytic cleavage of a sulfur carbon bond). However, the outcome is different because a different bond is cleaved (between methionine Cγ and Sδ in the diphthamide biosynthesis, and between methionine Sδ and adenosine C5′ in carboxymethylation), and also because the methionine portion is transferred. With his crystal structures and biochemical assays, Dr. Glatt demonstrates that Kti11/Kti13 heterodimer formation is essential for the proteins to function, and identifies a metal cation binding site with four coordinating cysteine residues as the putative "active site" of the protein. He further suggests that the physiologically relevant metal cation bound in this site could be iron, which could alternate between Fe(II) and Fe(III) oxidation states and serve as an electron acceptor/donor for the cm^5U and diphthamide biosynthesis reactions. This is a mechanistically very reasonable suggestion, but from a structural perspective, it is surprising. To my knowledge, four cysteine cation binding sites would be expected to bind Zn^{2+} ions, not Fe^{2+} or Fe^{3+} ions. Dr. Glatt addresses this concern in his publication and demonstrates experimentally (with a radio-assay) that the protein can indeed bind iron, supporting his mechanistic proposal.

- In the 2nd publication, entitled "The Elongator subunit Elp3 is a non-canonical tRNA acetyltransferase", Dr. Glatt focuses on the catalytic subunit of the Elongator complex. Looking back, it is now almost taken for granted that the carboxymethyl group is derived from a CoA bound acetyl group. However, prior to Dr. Glatt's work, the hypothesis was primarily based on the presence of a GCN5-like acetyltransferase domain in the protein. The crystal structure confirmed the presence of an acetyl-CoA binding site in the KAT-domain (i.e. a GCN5-like domain), and identified residues that may play a role in catalysis. From a chemical perspective, thioester hydrolysis is facile, and hence a direct nucleophilic water attack may suffice. Consequently, the distinction between "catalytic" residues (histidines and lysines are main candidates) and residues that contribute "only" to the binding pocket is not easy to make based on activity data, and has been avoided. At least for me, the biggest surprise from the Elp3 structure is the large distance between the hydrolase active site, and the site of the iron-sulfur cluster. At the time, the latter could only be suspected to be close to the site of tRNA anti-codon binding where carboxymethylation takes place. Now –with hindsight from Dr. Glatt's next cryo-EM structures– this is definitely established. As radicals are very short-lived, the implication is that Acetyl-CoA is hydrolyzed at a distance, to generate acetate, which then travels to the region of the iron-sulfur cluster, where the reaction takes place. This creates two questions: How is the acetate channeled? And how is the carboxymethylation reaction coupled with thioester hydrolysis to avoid energetically wasteful hydrolysis of acetyl-CoA? Neither of these two questions is fully answered by the crystal structure, but Dr. Glatt's publication provides strong biochemical evidence that tRNA binding, and even the identity of the tRNA, are sensed by Elp3, and somehow communicated to the hydrolase active site to control its activity. It remains unclear, at least to me, whether Elp3 senses properties of the anticodon-region, and then communicates these over a distance to the hydrolysis site, or whether it is senses other features of the tRNA, that are spatially closer to the hydrolase active site and correlate with anti-codon loop properties.

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- In the 3rd publication, entitled "Molecular basis of tRNA recognition by the Elongator complex", Dr. Glatt and his team present a cryo-EM crystal structure of the entire Elongator Elp1/Elp2/Elp3 subcomplex, which includes the previously studied catalytic Elp3 subunit. The structure shows that dimerization is via Elp1, and that the catalytic Elp3 subunits are sandwiched in between Elp1 and Elp2. More importantly, the structure places the iron-sulfur cluster in close vicinity to the adenosyl, and shows some unidentified density in the immediate vicinity of the iron-sulfur cluster, which may derive from methionine in SAM. The structure also shows that the anticodon-loop of Elongator bound tRNA is in the vicinity of adenosyl, supporting the concept that an adenosyl radical is responsible for generating either an acetyl or a uridine radical in the next step. Finally, the work also positions approximately Elp4/5/6 at much lower resolution, providing an essentially complete picture. Despite the impressive structural work, some chemical questions remain unanswered. How is the reaction of acetyl with uridine coordinated with acetyl-Co thioester hydrolysis to generate acetyl from acetyl-CoA? And how is the radical reaction ultimately quenched at the end of the reaction? Why is the Kti11/Kti13 heterodimer required at all? A four-iron-four-sulfur cluster should be able to alternate between [$2Fe^{3+}$, $2Fe^{2+}$] ($Fe_4S_4^{2+}$) and [$3Fe^{3+}$, Fe^{2+}] ($Fe_4S_4^{3+}$) states.

- In the 4th paper, entitled "Charging the code — tRNA modification complexes", Dr. Glatt and his co-authors provide an overview of tRNA modifications, with special emphasis on methylation, pseudouridinylation, and carboxymethylation. The review provides not only an excellent overview of available structural data, but also puts the structural information nicely in context, and does a good job of explaining what effects modifications have on ribosomal protein synthesis.

On the whole, Dr. Glatt has presented an impressive and well-rounded body of work. In all four papers, his contribution to the paper is major. The presented publications are of high quality, and are published in very good journals. I am particularly impressed by the breath of techniques that have been used in this group of papers. The Kti11/Kti13 heterodimer and Elp3 structures were solved by conventional X-ray crystallography, but the Kti11/Kti13 protein interface is also interrogated by nuclear magnetic resonance. Finally, the most impressive, crowning achievement of the habilitation thesis, the structure of the 620 kDa dimeric Elp1/2/3 Elongator sub-complex with and without bound tRNA, at 3.3 and 4.4 Å, has been obtained using cryo-electron microscopy. This structure determination is an impressive tour de force, which goes far beyond routine cryo-EM analysis, with respect to both sample preparation and the data analysis (resolution improvement by local averaging when global averaging is impossible due to conformational heterogeneity). Aside from a broad repertoire of structural biology techniques, the presented work also required expertise in the assembly and preservation of large protein complexes, the ability to work with redox active proteins, and the expertise to prepare homogenous tRNA samples.

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Dr. Glatt's standing in the field is not only documented by his publications, and by the impact of these publications, but also by his collaborations with leading researchers in the field. Aside from his scientific achievements, Dr. Glatt has also proven himself to be an extremely effective organizer of science infrastructure. He was instrumental as the leader of a consortium that established state-of-the-art cryoelectron microscope in Poland, and does a very good job of running this infrastructure, to the benefit of the entire community. Very recently, Dr. Glatt was awarded an ERC Consolidator grant, confirming yet again his status as a leader in his field.

In summary, I am impressed by Dr. Glatt's achievements, summarized in the habilitation thesis and beyond, and **enthusiastically support bestowing the Dr. hab. title on him**. I also feel that the habilitation thesis is so strong that it **deserves a distinction**.

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