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Additional Comment on Three X-ray Crystal Structure Papers

We appreciate the opportunity to comment on Dr. Salunke's response. Regrettably, we find the response unsatisfactory, as he and his colleagues suggest that it is acceptable to project arbitrary structural models into electron density noise.

Technical facts related to proper electron density reconstruction

All of the seven structure models in question are plagued by the same issues, so we will illustrate this with only one example. Figure 2E in Dr. Salunke's response (showing the difference density map for the 4H0H model) shows a choppy pattern of electron density contoured at 2.5 σ level that to some extent covers the proposed peptide model.

We have repeated map calculations using identical software (the Crystallography and NMR system [CNS] 1.3). The positive difference density presented by Dr. Salunke matches our calculations. However, a difference density map is universally presented in publications with both positive (green) and negative (red) contoured levels. The image shown in Fig. 1 reveals why this is important.

Whereas some positive electron density is present at the 2.5 σ contouring level, so is an equal amount of negative density. If the claim is that the positive density at this level reflects the physical presence of the peptide molecule, then what is the meaning of the negative density? Does it indicate that some elements of the structure model are contradicting the experimental data? The much more plausible explanation is that both positive and negative electron density features at that level simply represent noise in the experimental data.

So what would be a correct cutoff level for interpretable electron density? Luckily, protein crystal structures provide an internal standard. If we remove a single amino acid from the 4H0H scFv model, it is expected that the recalculated omit difference maps will contain a clear indication for that residue in the form of positive omit electron density. We show the results of such a calculation in Fig. 2 (the omitted residue is the tyrosine [Y240] in the center of the image). The resulting difference electron density is equally uninterpretable to that in Fig. 1, because at the 2.5 σ level the reliable evidence in the form of positive difference density for the tyrosine is obscured by noise. If we increase the map contouring level to 6 σ, the resulting density, shown in Fig. 3, is clarified.

It is obvious that a single amino acid not included in the model yet present in the structure is clearly detectable even

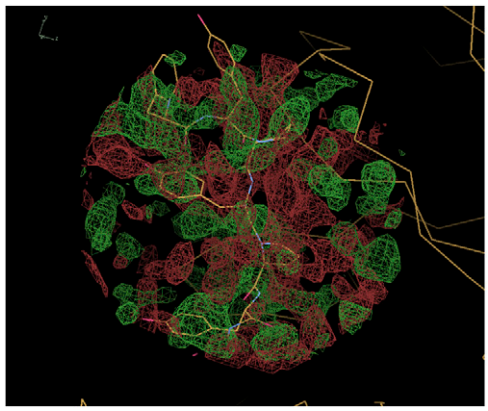

FIGURE 1. Positive omit electron density (green) and negative omit electron density (red) for chain P in PDB entry 4H0H generated by CNS. 2.5 σ level for both contour levels. This figure and Figs. 2–4 were rendered using Coot (1).

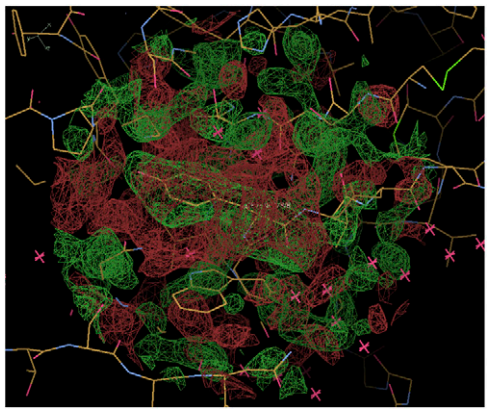

FIGURE 2. Positive omit electron density (green) and negative omit electron density (red) of the Ab fragment in PDB entry 4H0H, generated by CNS. 2.5 σ level for both contours. The tyrosine (Y240) in the center was omitted from the model.

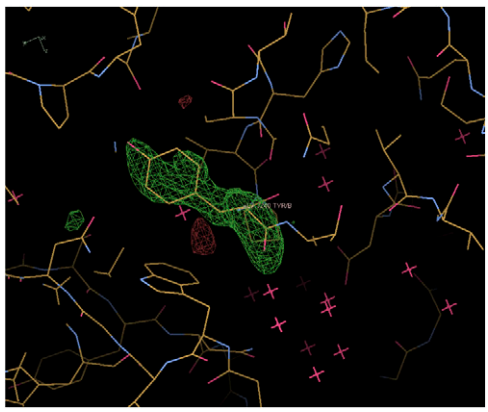

FIGURE 3. Positive omit electron density (green) and negative omit electron density (red) of the Ab fragment in PDB entry 4H0H, generated by CNS. 6 σ contour level. The tyrosine in the center (Y240) was omitted from the model.

FIGURE 4. Positive omit electron density (green) and negative omit electron density (red, none visible at this level) for chain P in PDB entry 4H0H generated by CNS. 6 σ level for both contours.

at the high 6 σ difference in electron density level. If peptides were present in the crystals, it would be expected that a similar positive difference density could be observed for them as well. The area where peptide was placed in the 4H0H model is shown in Fig. 4, this time contoured at 6 σ.

Perhaps there are solvent or buffer molecules present (green spherical density features) but no density for peptide residues is visible. Features similar to those suggested by Salunke et al. to represent the peptides can be found in positive density at the 2.5 σ level in many other places throughout this structure, and it would be inaccurate to suggest that the scFv molecule binds multiple peptides. If this level of scientific proof was acceptable, many wonderful discoveries could be made from faint bands on Western blots, within margin of error changes in enzymatic activity, and in many other forms of inconclusive data.

Demonstration of highly implausible structural and real space fit properties of the purported peptide models

To strengthen our argument that there is no ambiguity in the interpretation of these structural data as to the absence of peptides, we are submitting a summary (Table I) of the Protein Data Bank (PDB) validation reports for the structure models published by Dr. Salunke, and for his and our cited reference structures.

These validation results are public record (PDB validation reports, http://www.ebi.ac.uk/pdbe), and are produced by PDB. They provide the most damaging evidence by showing clearly that the peptides are structurally not reasonable, largely because they have no supporting evidence in the form of electron density.

1) Based on the number of Ramachandran outliers Dr. Salunke's peptides fall into the zeroth percentile of expected backbone geometry, whereas only a small percent of outliers is considered acceptable in a protein–peptide structure model. These peptides are in a distorted, strained high energy conformation that is simply physically improbable and would require exceptionally strong proof for their existence. We list in Table I but do not comment on the peptide models Dr. Salunke has selected as examples to defend his improbable peptides.
2) The real space *R*-value (RSR), measuring the fit of the model to electron density, shows that there is only spurious fit and in essence 100% of the peptide residues are density outliers (RSR Z-score [RSRZ] > 2), which is again strong evidence that the implausible models were fitted into spurious density (noise).

In conclusion, these structure models, resulting from highly speculative interpretation of spurious noise, contradict the

Table I. Summary of PDB validation reports for structure models and cited reference structures

PDB ID	Chain	Ramachandran Favored (%)	Ramachandran Allowed (%)	Ramachandran Outliers (%)	Ramachandran Percentile	Rotamer Percentile	Real Space *R* Value *Z*-Score (RSRZ)	RSRZ >2 Outliers (%)	Author
1pww	C	44%	11%	44%	0(0)	0(2)	3.1	72%	Liddington
	D	44%	11%	44%	0(0)	0(0)	3.3	72%	Liddington
1uvi	D	RNA	-	-	-	-	2.9	75%	Grimes
	E	RNA	-	-	-	-	3.9	100%	Grimes
	F	RNA	-	-	-	-	3.2	75%	Grimes
2xzq	P	29%	0%	71%	0(0)	0(0)	5.3	100%	Salunke
2y06	P	0%	38%	62%	0(0)	5(8)	4.3	100%	Salunke
2y07	P	25%	0%	75%	0(0)	0(0)	3.3	100%	Salunke
2y36	P	11%	22%	67%	0(0)	0(0)	5.9	100%	Salunke
4bh7	P	29%	29%	43%	0(0)	100(100)	3.2	55%	Salunke
4bh8	P	14%	14%	71%	0(0)	1(1)	6.3	100%	Salunke
4h0h	D	20%	20%	40%	0(0)	0(0)	9.7	100%	Salunke
3fn0	P	86%	14%	0%	100(100)	100(100)	-0.1	11%	Wilson
3ggw	E	100%	0%	0%	100(100)	100(100)	0.3	9%	Bentley
	F	100%	0%	0%	100(100)	16(3)	0.6	8%	Bentley

basic principles of protein structure. We do not believe that any reviewer with access to the PDB validation reports would have accepted these peptide structure models for publication. Presenting this situation as ambiguous and open to interpretation would be a scientific mistake.

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Abbreviations used in this article: CNS, Crystallography and NMR system; PDB, Protein Data Bank; RSR, real space *R*-value; RSRZ, RSR *Z*-score.

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Response to the Second Letter by Pozharski et al.

We did not intend to infer that it is acceptable to project arbitrary structural models into electron density noise. What we emphasized, however, is that it is possible to see meaningful structural information from relatively weak electron density. We provided several examples in support of our argument from published work in our previous response.

Presence of negative density in some region does not necessarily mean that positive density in the ligand binding region is also noise, especially when this positive density is also supported by the presence of densities in simulated annealing 2Fo–Fc composite omit maps. After deletion of Y240 from Protein Data Bank (PDB): 4H0H, the suggestion that electron density for Y240 is not interpretable at contour level 2.5 σ is not correct, as one can clearly make out boundaries of the hydrophobic ring and other features of Y240 in figure 2 of the response by Pozharski et al. to our comments.

The main argument of Pozharski et al. has been concerning the presence of experimental electron density for building the model in the map. In this context, it has to be noted that the Ramachandran parameters are not directly linked to the presence or absence of the electron density. Guidelines to define fit of model to the electron density used by the Uppsala Electron Density Server, on the basis of which the graphs providing real space *R*-value *Z* scores are calculated, are meant only for the relative quality of a PDB entry, but not for its absolute authenticity as indicated by the report of the Task Force of the Worldwide Protein Data Bank (1). It is important to note that a significant number of PDB entries associated with published structures have model fits similar to those in our structures. Incidentally, such data were included in the PDB subsequent to the acceptance of our papers for publication. We disagree with the contention of Pozharski et al. about the interpretations of our electron density maps.

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Abbreviation used in this article: PDB, Protein Data Bank.

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Comment on "Cutting Edge: Inhibiting TBK1 by Compound II Ameliorates Autoimmune Disease in Mice"

With interest, we read the paper by Hasan et al. (1), who showed that inhibition of TANK-binding kinase 1 (TBK1) by Compound II has beneficial effects in a mouse model of autoimmune disease. Similarly, TBK1 inhibition by the small molecule drug amlexanox has been shown to mediate favorable metabolic and anti-inflammatory effects in mouse models of diabetes, obesity (2, 3) and multiple sclerosis (4).

We and others recently showed that haploinsufficiency of *TBK1*, i.e., *TBK1* gene dosage reduction from two to one allele by heterozygous loss-of-function mutations, can cause the genetically related neurodegenerative diseases amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (5–7). TBK1 protein levels were reduced to 50% in cell lines derived from patients with heterozygous *TBK1* loss-of-function mutations (5).

Consequently, it is plausible that pharmacological reduction of cellular TBK1 activity to 50% or less might have similar cellular effects as *TBK1* haploinsufficiency. Long-term pharmacological TBK1 inhibition, as proposed for the treatment of autoimmune diseases (1, 4), could thus pose a risk of causing ALS or FTD. Also, in terms of a two-hit-model, chronic TBK1 inhibition may be able to trigger ALS/FTD in individuals exposed to an environmental insult or with a genetic predisposition who would otherwise not have developed ALS or FTD during their lifetime.

Experimental proof that pharmacological TBK1 inhibitors increase the risk for neurodegenerative diseases is currently lacking. Nevertheless, the existing unequivocal genetic evidence that *TBK1* haploinsufficiency causes ALS/FTD with high penetrance, in our opinion, strongly suggests the need to critically reconsider currently ongoing or planned clinical trials that are based on pharmacological TBK1 inhibition.