

Peer Review File

Manuscript Title: Structural basis of cytokine-mediated activation of ALK family receptors

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee #1 (Remarks to the Author):

The manuscript submitted by De Munck et al., "Structure and mechanism of cytokine-mediated activation of ALK and LTK receptor tyrosine kinases", describes the structure of a portion of the ALK and LTK extracellular domain with the ALKAL ligands. While it would be great to see the full length ALK and LTK receptor ECD (or more) complexes, this work still represents a huge step forward. The authors show that the TNF-like and Glycine-rich domains of these receptors function together as a supradomain, rather than as separate domains, to form a tent like structure that encompass a single ligand molecule. This is a very fascinating finding and really provides valuable insight into ALK/LTK receptor-ligand biology. The structural work appears to be very rigorous and this work provides an important step forward in our understanding of the ALK family of RTKs and their activation. Their work brings together the work of many groups over the years, studying ALK function, identifying the ALKAL (FAM150/AUG) ligands and their role in neuroblastoma, and provides a mechanistic explanation for many of the biological findings. The manuscript is very well written and presented, and generally of a very high standard.

This reviewer has some points that should be addressed that would strengthen the manuscript, please see below (most are rather minor, and they are listed in order of going through the manuscript):

1. Fig 1a. Please define MAM and TG in the legend to make it easier for the reader.
2. On page 3, line 104, the authors discuss N-glycosylated linkers. It seems Extended data Fig 2d might be a better choice here than 2e. Also on page 3, line 104-105, they mention three more N-linked glycans 709,808,886 (Fig 1b), so not seem to all be clearly marked, or perhaps there is a mix up with numbers. The authors should clearly mark these in Fig 1 and Extended data 2 so that the reader can follow.
3. Fig 1e. and the pGII helix arrangements, also alignment in Extended Data 8b we first see the importance of the Glycine-rich domain. This domain in ALK has long been enigmatic and it is great to finally visualize a structure here. Interestingly, the Glycine-rich domain has been shown to be critical for function in *Drosophila* (Lorén, EMBO Reports, 2003), where four loss-of-function Alk mutations (Alk4, Alk5, Alk6 and Alk7) all have a mutation of glycine to an acidic amino acid. Later in Fig 2, when they are looking at the ALK-ALKAL and LTK-ALKAL complexes (Fig 2) they discuss that the Glycine-rich domain might be important for critical receptor-receptor contacts. This would indeed make sense with these *Drosophila* mutants where the ligand is not conserved, which are:
 - Alk4 (*Drosophila* G788D/equivalent to human 741 in pGII a)
 - Alk5 (*Drosophila* G876D/equivalent to human 824 in pGII d)
 - Alk6 (*Drosophila* G958E/equivalent to human 893 in pGII h)
 - Alk7 (*Drosophila* G1003D/equivalent to human 937 in pGII l)This may be worth commenting on in their discussion of this.
4. Fig 1c. Can the authors comment on why it was so difficult to make the LTKTG-EGFL domain? What is the similarity with the same region in ALK – can they comment more?
5. Fig 1f,g. The close up here is of the boxed area in b and c, right. Please clarify in the Fig legend

for the reader.

6. The finding that the full length ALKAL1 ligand is monomeric is interesting, and somewhat in contradiction to previously published data (this reviewer is thinking of Reshetnyak, PNAS 2018). There the authors reported that full length ALKAL is a dimer (in this case they were looking at ALKAL2), but that the smaller conserved domain alone was a monomer. Do the authors have any idea why they do not see this. Is it possible that this is different with ALKAL1 versus ALKAL2?

7. In Extended data 4 the authors show mass determination of ALKAL-ALK and ALKAL—LTK complexes. Have they tried mixing ALK and LTK TG or TG-EGFL domains? Do they think that the ALKAL ligands may be able to support an ALK:LTK heterodimer?

8. In Extended data 3c it seems like full length ALKAL1 is able to support the growth of Ba/F3 cells at higher concentrations. The shorter variants seem unable to do this. Can the authors please comment on this.

9. In Fig 4g and 4h. Here the authors should include an ALK/pALK (either pY-1604 or pY-1278) blot with the Ba/F3 analysis.

10. Extended data 9. Here the authors select some somatic mutations in the ALK and LTK TG domains that they test for oncogenic potential. As comment, it is nice to see some explanation for H694R. Here the authors could be a bit more convincing in looking at ALK activity. At the very least they should include pALK (either pY-1604 or pY-1278) – in ED9, Panel C.

11. There is a small typo in ED3, Panel b.

12. Final comment. There is obviously many experiments and mutant variants that the authors could generate and test to provide more insight and understanding of their structures. However, this reviewer finds that the range of experiments and mutational analysis provided here is enough to support their interpretations.

Referee #2 (Remarks to the Author):

This study by Munck and colleagues focuses on structural investigation of receptor tyrosine kinases, ALK and LTK, and their activation by the extracellular ligands, which until recently (2014) have not even been discovered. The discovery of these ligands as secreted cytokines FAM150A and FAM150B (here referred to as ALKAL1 and ALKAL2) did not offer much insight into the mechanism of receptor activation, and the structures of both ligands and the extracellular ligand-binding domains of the receptor have been missing. This is despite emerging importance of ALK and LTK receptors as targets in cancer and auto-immune disorders, among other diseases.

This milestone paper unravels the structures of the extracellular portions of ALK and LTK alone, and bound to their cytokine ligands ALKAL1 and ALKAL2, at last. These structures are important, not only because this is the first time when they are determined, but also because they are very unusual and contradict their earlier predictions by existing domain fold databases and homology modeling. The intertwining of two domains – a permuted TNF-like module with the Glycine-rich subdomain, results in a unique chimeric domain, previously not seen in other proteins. The 2:1 receptor:ligand ratio in the active complex, which is more common to the cytokine receptors than classical receptor tyrosine kinases, is non-canonical for receptor tyrosine kinases. The resulting C2-symmetry of receptor assembly despite asymmetric engagement of the ligand is fascinating. These new structures, together with thorough analysis of the ligand binding, do constitute a landmark study in the signaling field. My enthusiasm for publishing this study in Nature is high, pending the authors addressing the comments listed below.

Major Points

1. While the authors show convincingly the importance of receptor site 1 and site 2 engagement for binding the ALKAL ligands by mutating the interfacial residues in the ALKAL ligands, reciprocal analysis in which receptors are mutated is not done. This is particularly important for functional validation of site 3, which is contributed by two receptors.
2. The analysis of disease mutations in the TG domains of ALK and LTK receptors, and their potential relevance to modulation of ligand/receptor interactions is not satisfactory. While the authors group the mutations into two categories (core and periphery), it is unclear what conclusion can be drawn from this. Objectively, mutations map everywhere on the TG domains, and the authors do not provide a convincing analysis (even by simple structural modelling) what effects any of these mutations would make on ligand/receptor interactions. For example, H694R and G747R mutations are proposed to promote receptor-receptor contacts. But it is never explained how. A statement of that kind needs some structural analysis, ideally molecular dynamics simulations, but even showing potential new contacts made by introducing these new arginine residues at the receptor/receptor interface would be informative. Likewise, it is unclear how the F856S and R753Q mutations because no clear structural analysis of their potential mode of action is described.
3. It is puzzling that there is no decrease in binding affinity between ALKAL1/2 ligands and ALKTG-EGFL receptor fragment, comparing to binding to ALKTG? The structural analysis shows that the ligand is fully encapsulated in the ALKTG-EGFL construct in contrast to the ALKTG fragment in which ligand binding pocket seems quite open. It sounds that such encapsulation would certainly affect an on and/or off rates of ligand binding.

Minor points

1. The authors mention that they were able to purify full-length ALKAL2, but never present any data on how this construct interacts with ALK and LTK receptors, and entirely focus on the conserved C-terminal domain of ALKAL2. It is unclear what is the reason for focusing on the shorter fragment (except for the fact that only a shorter fragment of ALKAL1 was purified), and what could be learnt from studying the longer fragment.
2. It is unclear if the final ALKTG-ALKAL2 crystal structure was obtained in the presence of Fab or its absence. Unless I misunderstood, lines 172 and 173 provide conflicting information.
3. The colorful labels in the figures do not always work. For example, in Figure 1c – pale yellow is barely visible. Likewise, in Figure 1f and 1g, the residue numbering does not contrast well enough with the structure and blends in, becoming hard to read.
4. The analysis could use a broader perspective. The authors mention parallels between ligand/receptor ALK and LTK complexes with known structures of hematopoietic helical cytokines and their receptors. Is there something interesting there to be learnt by making this comparison? A supplemental figure showing these parallels would be a great addition.
5. Please mark relevant hydrogen bonds in Extended Data Figure 6. Like mentioned before, light colors used to mark residues make the numbers hard to read.
6. It is unclear why R115 residue in ALKAL1 was chosen for charge reversal mutation (R115E) in site 1, as described in line 258. It is not marked in Extended Data Figure 6a or Extended Data Figure 5b where it does not seem to be at that site at all.

7. There is a typo in Figure 4h, in the label. R1115E should be R115E.

8. Can the authors speculate on the potential of ALK and LTK to form ALKAL1 or ALKAL2-driven heterodimers? Would that be of physiological importance?

Referee #3 (Remarks to the Author):

De Munck et al. report biochemical, biophysical, and X-ray crystallographic studies of ligand binding fragments of the ALK and LTK receptor tyrosine kinases (RTKs) both alone and complexed with their cognate ligands ALKAL1 or ALKAL2. These structures represent to my knowledge the first atomic resolution views of the ALK/LTK ligand regions, ALKAL cytokines, and the nature of their interaction. The structures reveal an interesting modification of a TNF-like domain with glycine-rich regions that are inserted in distinct loops but combine to form a poly-glycine II rich structure with hexagonally-packed strands. The authors validate their structures by showing mutations in ligand-receptor interfaces decrease binding and activity.

These results are important and will form the basis of all future studies and understanding of this important class of RTKs. I thus believe these results merit publication in a high-profile journal like Nature. That said, the authors seem so immersed in their topic that they have prepared a manuscript that in many places is confusing if not misleading for a general reader. After 3 readings of the MS I still do not have a clear idea of several key issues and had to spend considerable time puzzling over what is shown and meant in several figures.

I admire this work and commend the authors, but I note below several aspects of the presentation that I found confusing or frustrating in case the authors find them helpful in revising the MS for clarity.

1. The authors note that SEC-MALS experiments demonstrate 2:1 LTK:ALKAL and 1:1 ALK:ALKAL stoichiometries in solution, but never explicitly state the stoichiometries found in the crystal structures of receptor-ligand complexes. Discussions later in the paper imply it is 2:1, but the solution results don't necessarily translate to crystal results because, as the authors point out, the high concentrations of components of crystals can overcome weak binding affinities. Thus, when looking at Figure 2c and 2f, it is not immediately clear whether the two blue (2c) or green (2f) patches represent independent binding of different ligands to similar sites on receptor dimer subunits or whether one ligand contacts both sites. I believe it is the latter, but this point seems only implied later in the MS.

Assuming that the stoichiometry in the crystals is 2:1 receptor:ligand, could that be an artifact of the molar ratio of receptor and ligand in the crystallization trials? Could 2 receptors form the Site 3 interaction with an ALKAL molecule bound to Site 1 of each receptor? If ligand binding to Site 1 of each receptor is incompatible with receptor dimers do high amounts of ligand inhibit receptor activation?

The authors hint that there are some structural rearrangements in the receptor that may underlie dimerization (lines 238-9; see also Extended Figure 6). Is this correct? How do the structures of apo and ligand-bound receptors compare? I.e. are there conserved structural changes in the receptors when ligands are bound that could affect dimerization? Or are the differences ascribable to differences between ALK and LTK? Does ligand binding promote dimerization simply through crosslinking receptors?

Also, in Figures 2d and 2e what does the dotted outline represent? What do $\alpha 1$ and $\alpha 2$ mean? Throughout Figure 2, which light colored domains belongs to which dark colored domains? What is the relationship of Sites 1&2 with Site 3? Do they overlap? Are they contiguous? I presume the DE,

H" designations refer to strands in the underlying receptor, but that should be made clear. In general, any abbreviation or demarcation shown in a figure should be defined in the legend.

2. The schematic in Figure 1a is a little hard to follow. The "TG" portions look like a ligand docked on the receptor; TG is not defined in the legend. Figures 1d and 1e are very helpful, however.
3. In Figure 3, which portion of the cytokine contacts the receptor? The hydrophobic region?

Author Rebuttals to Initial Comments:

Point-by-point Response to Reviewer's remarks

Referee #1 (Remarks to the Author):

The manuscript submitted by De Munck et al., "Structure and mechanism of cytokine-mediated activation of ALK and LTK receptor tyrosine kinases", describes the structure of a portion of the ALK and LTK extracellular domain with the ALKAL ligands. While it would be great to see the full length ALK and LTK receptor ECD (or more) complexes, this work still represents a huge step forward. The authors show that the TNF-like and Glycine-rich domains of these receptors function together as a supradomain, rather than as separate domains, to form a tent like structure that encompass a single ligand molecule. This is a very fascinating finding and really provides valuable insight into ALK/LTK receptor-ligand biology. The structural work appears to be very rigorous and this work provides an important step forward in our understanding of the ALK family of RTKs and their activation. Their work brings together the work of many groups over the years, studying ALK function, identifying the ALKAL (FAM150/AUG) ligands and their role in neuroblastoma, and provides a mechanistic explanation for many of the biological findings. The manuscript is very well written and presented, and generally of a very high standard.

We thank the reviewer for the expressed appreciation of the novelty, importance, and quality of our reported findings. It is tremendously motivating and rewarding to read such remarks.

This reviewer has some points that should be addressed that would strengthen the manuscript, please see below (most are rather minor, and they are listed in order of going through the manuscript):

1. Fig 1a. Please define MAM and TG in the legend to make it easier for the reader.

We have added the definition for each abbreviation in the figure legend.

2. On page 3, line 104, the authors discuss N-glycosylated linkers. It seems Extended data Fig 2d might be a better choice here than 2e. Also on page 3, line 104-105, they mention three more N-linked glycans 709,808,886 (Fig 1b), so not seem to all be clearly marked, or perhaps there is a mix up with numbers. The authors should clearly mark these in Fig 1 and Extended data 2 so that the reader can follow.

We apologize for the confusion. We have experimental evidence in our structures regarding four N-glycosylation sites. We have now simplified the main text and have added a description of the N-glycans as part of the legend of Figure 1b,c as follows:

"The four experimentally observed N-linked glycans in ALK at positions N808, N863, N864 within the TG supradomain, and N986 in the linker adjoining the TG supradomain and the EGF-like domain are depicted as transparent spheres embedded with stick representations of the modelled glycans."

3. Fig 1e. and the pGII helix arrangements, also alignment in Extended Data 8b we first see the importance of the Glycine-rich domain. This domain in ALK has long been enigmatic and it is great to finally visualize a structure here. Interestingly, the Glycine-rich domain has been shown to be critical for function in Drosophila (Lorén, EMBO Reports, 2003), where four loss-of-function Alk mutations (Alk4, Alk5, Alk6 and Alk7) all have a mutation of glycine to an acidic amino acid.

Later in Fig 2, when they are looking at the ALK-ALKAL and LTK-ALKAL complexes (Fig 2) they discuss that the Glycine-rich domain might be important for critical receptor-receptor contacts. This would indeed make sense with these Drosophila mutants where the ligand is not conserved, which are:

- Alk4 (Drosophila G788D/equivalent to human 741 in pGII a)
- Alk5 (Drosophila G876D/equivalent to human 824 in pGII d)
- Alk6 (Drosophila G958E/equivalent to human 893 in pGII h)
- Alk7 (Drosophila G1003D/equivalent to human 937 in pGII l)

This may be worth commenting on in their discussion of this.

We thank the reviewer for raising this important point. Given the strict conservation of these glycine residues, aspartate or glutamate residues at these positions would indeed be detrimental to the fold of the Glycine-rich subdomain. Disturbing the integrity of the Glycine-rich subdomain in such a dramatic way can indeed abrogate important site 3 interactions. We have now included a concluding statement at the end of the section entitled "Structure of ALK/LTK cytokine-binding domains" that also cites these earlier findings in the context of our reported structural insights.

lines 94-97: “Such tight packing appears to restrict the amino acid composition of these central pGII-helices, offering a rationale for the exquisite conservation of the participating poly-glycine sequences (*Extended Data Fig. 2i*) and for loss-of-function mutants in this region of *Drosophila* ALK²⁹.”

4. Fig 1c. Can the authors comment on why it was so difficult to make the LTKTG-EGFL domain? What is the similarity with the same region in ALK – can they comment more?

While LTK_{TG-EGFL} could be produced and purified for biophysical studies such as SEC-MALLS and ITC, we indeed had difficulties obtaining crystals of this LTK ectodomain version. For this reason, we expressed and purified a minimized LTK ectodomain lacking the EGFL domain, which proved amenable to crystallization yielding crystals diffracting to 1.3 Å.

The EGF-like domains of ALK and LTK share 36.5% sequence identity. Moreover, the EGFL domain of LTK carries an N-linked glycosylation site at Asn411. The combination of such important differences might have contributed to our inability to crystallize LTK_{TG-EGFL}.

5. Fig 1f,g. The close up here is of the boxed area in b and c, right. Please clarify in the Fig legend for the reader.

We have added the following text to the figure legend of Fig. 1f,g:

“Close-up view of the hydrophobic interface between the H’HGFCB sheet in the TNFL subdomain and the glycine-rich subdomain of ALK (f) and LTK (g).”

6. The finding that the full length ALKAL1 ligand is monomeric is interesting, and somewhat in contradiction to previously published data (this reviewer is thinking of Reshetnyak, PNAS 2018). There the authors reported that full length ALKAL is a dimer (in this case they were looking at ALKAL2), but that the smaller conserved domain alone was a monomer. Do the authors have any idea why they do not see this. Is it possible that this is different with ALKAL1 versus ALKAL2?

Indeed, Reshetnyak *et al.* report full-length ALKAL2 as a disulfide-linked homodimer. This disulfide appears to be formed between Cys66 of two ALKAL2 protomers. This cysteine is located in the N-terminal variable region, which is a unique region in primate ALKAL2. ALKAL1 only shares 13% sequence identity with ALKAL2 in this variable region and does not contain this extra cysteine. Thus, the N-terminal region in ALKAL1 may not contain the same dimerization capacity as the ALKAL2 variable region. Clearly, more work lies ahead in order to more fully unravel the structure-function landscape of the ALKAL cytokines.

7. In Extended data 4 the authors show mass determination of ALKAL-ALK and ALKAL—LTK complexes. Have they tried mixing ALK and LTK TG or TG-EGFL domains? Do they think that the ALKAL ligands may be able to support an ALK:LTK heterodimer?

ALK and LTK co-expression has been observed in the hippocampus as described by Weiss *et al.* 2012;

<https://doi.org/10.1016/j.pbb.2011.10.024>. Furthermore, Guan *et al.*

(<https://doi.org/10.7554/eLife.09811.001>) showed that upon addition of ALKAL1, ALK could be

immunoprecipitated together with LTK. These results may point to a possible physiological role for ALK-LTK heterodimers. Our structures show that the TG supradomains of both receptors are very similar in overall shape suggesting that they might be able to engage in heterodimerization. While we did discuss this possibility once we realized how similar the two ectodomain regions are, we decided against pursuing any additional experimental undertakings in order to allow consolidation of our already very voluminous set of findings. Nevertheless, we envisage that investigation of such mechanistic scenarios will be the type of work that our reported findings could inspire in order to move the field forward.

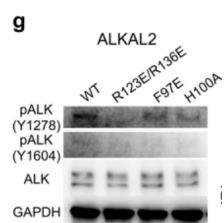
8. In Extended data 3c it seems like full length ALKAL1 is able to support the growth of Ba/F3 cells at higher concentrations. The shorter variants seem unable to do this. Can the authors please comment on this.

As reported via our detailed binding studies (Fig. 5 ; Ext. Data. Fig. 7 ; Ext. Data. Fig. 10) ALKAL1 is not the preferred cytokine ligand for ALK. Our cellular studies in Ba/F3 cells illustrate this as well, and clearly establish ALKAL2 as the preferred ligand in this cellular setting. Moreover, our data shows that ALKAL1 and ALKAL1_{FL} are very similar in their capacity to signal through ALK.

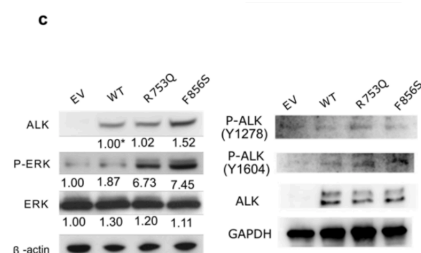
9. In Fig 4g and 4h. Here the authors should include an ALK/pALK (either pY-1604 or pY-1278) blot with the Ba/F3 analysis.

We have addressed this remark together with remark 10 by providing Western blot analysis of pY1604 and pY1278 in ALK in Extended Data Figure 7g and Extended Data Figure 9c. In this way we could demonstrate that ALK does indeed become phosphorylated in a ligand-dependent manner, in full agreement with the data we provide in Figure 4 and Extended Data Figure 9e where cellular proliferation for all three receptors (WT, R753Q, F856S) is drastically abrogated in the presence of the ALK phosphorylation inhibitor Crizotinib. Admittedly, it has proven very challenging to obtain Western blots with high signal/background ratios for pY1604 and pY1278 in Ba/F3 cell lysates despite the use of antibodies from different providers and adopting many variations of protocols. We note that the only other study reporting Western blotting of pALK from Ba/F3 cells (Reshetnyak *et al.* 2015) employed an ALK-immunoprecipitation step using an unreported anti-ALK antibody.

Extended Data Figure 7g



Extended Data Figure 9c



10. Extended data 9. Here the authors select some somatic mutations in the ALK and LTK TG domains that they test for oncogenic potential. As comment, it is nice to see some explanation for H694R.

The reviewer's remark made us realize that our presentation of this analysis could be improved. We have now drastically revised the relevant section as shown below, which also entailed replacing panel a in Ext. Data Fig. 9:

lines 240-256: Mapping of somatic mutations in ALK

"To expand structure-function insights on ALK and LTK activation we structurally mapped mutations in ALK_{TG} and LTK_{TG} combining documented oncogenic potential and frequently occurring missense single-nucleotide polymorphisms^{44,45} (Extended Data Fig. 9a,b). Mutations leading to constitutive receptor activation or that may enhance receptor–receptor contacts or stabilize active receptor states are widely studied to evaluate oncogenic potential. LTK variant R243Q and ALK variants G685R, G747R, and H694R locate at positions that would be compatible with such roles. Interestingly, H694R is a gain-of-function mutation in lung adenocarcinoma leading to constitutive activation of ALK⁴⁶. On the other hand, mutations that may impact cytokine binding, e.g. by increasing affinity, are less common. Here, we identify two such candidate mutations: LTK variant P363L and ALK variant S737L mapping to interaction sites 2 and 1, respectively. ALK^{F856S}, a gain-of-function mutation linked to acute myeloid leukemia⁴⁷, and ALK^{R753Q} identified in histiocytic neoplasms⁴⁸, are roughly equidistant from the start of αC of bound ALKAL2 (Extended Data Fig. 9a). To gain insight into their possible mechanism, we established Ba/F3 cell lines expressing the two mutant ALK variants and found that while their expression levels were comparable to ALK^{WT}, they both drastically increased cytokine-dependent cell proliferation (Extended Data Fig. 9c,d,e,f). Unlike previously reported⁴⁷, ALK^{F856S} was

not constitutively active. In light of their structural context, these mutations might facilitate the reorganization of key regions of the cytokine-receptor interfaces.”

Here the authors could be a bit more convincing in looking at ALK activity. At the very least they should include pALK (either pY-1604 or pY-1278) – in ED9, Panel C.

Please see our response to point 9 above.

11. There is a small typo in ED3, Panel b.

We have fixed the typo by changing the label “WT+ALK2AL2+DMSO” to “WT+ALKAL2+DMSO”

12. Final comment. There is obviously many experiments and mutant variants that the authors could generate and test to provide more insight and understanding of their structures. However, this reviewer finds that the range of experiments and mutational analysis provided here is enough to support their interpretations.

We thank the reviewer for the expressed appreciation of the volume, breadth, and conclusive nature of our experimental undertakings.

Referee #2 (Remarks to the Author):

This study by Munck and colleagues focuses on structural investigation of receptor tyrosine kinases, ALK and LTK, and their activation by the extracellular ligands, which until recently (2014) have not even been discovered. The discovery of these ligands as secreted cytokines FAM150A and FAM150B (here referred to as ALKAL1 and ALKAL2) did not offer much insight into the mechanism of receptor activation, and the structures of both ligands and the extracellular ligand-binding domains of the receptor have been missing. This is despite emerging importance of ALK and LTK receptors as targets in cancer and auto-immune disorders, among other diseases.

This milestone paper unravels the structures of the extracellular portions of ALK and LTK alone, and bound to their cytokine ligands ALKAL1 and ALKAL2, at last. These structures are important, not only because this is the first time when they are determined, but also because they are very unusual and contradict their earlier predictions by existing domain fold databases and homology modeling. The intertwining of two domains – a permuted TNF-like module with the Glycine-rich subdomain, results in a unique chimeric domain, previously not seen in other proteins. The 2:1 receptor:ligand ratio in the active complex, which is more common to the cytokine receptors than classical receptor tyrosine kinases, is non-canonical for receptor tyrosine kinases. The resulting C2-symmetry of receptor assembly despite asymmetric engagement of the ligand is fascinating. These new structures, together with thorough analysis of the ligand binding, do constitute a landmark study in the signaling field. My enthusiasm for publishing this study in Nature is high, pending the authors addressing the comments listed below.

We thank the reviewer for the expressed appreciation of the novelty, importance, and quality of our reported findings. It is tremendously motivating and rewarding to read such remarks.

Major Points

1. While the authors show convincingly the importance of receptor site 1 and site 2 engagement for binding the ALKAL ligands by mutating the interfacial residues in the ALKAL ligands, reciprocal analysis in which receptors are mutated is not done. This is particularly important for functional validation of site 3, which is contributed by two receptors.

We agree with the reviewer that functional validation of site 3 could strengthen our manuscript. To this end, we have performed biochemical/biophysical analysis of site 3 mutants in LTK and cellular assays employing an ALK mutant.

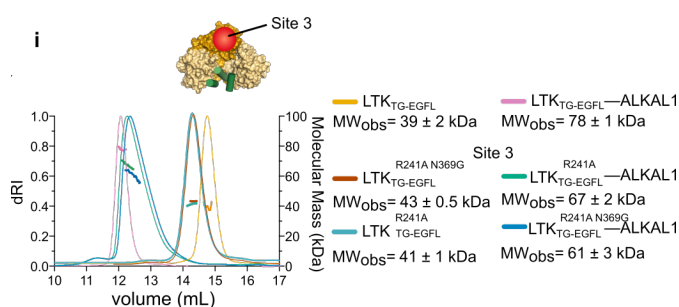
We expressed and purified two mutant versions of LTK_{TG-EGFL} carrying as a R241A single mutant or a R241A/N369G double mutant and characterized their complex formation with ALKAL1 via SEC-MALLS.

For ALK we created a M751T mutant and tested its functional effects in our Ba/F3 cellular proliferation assay.

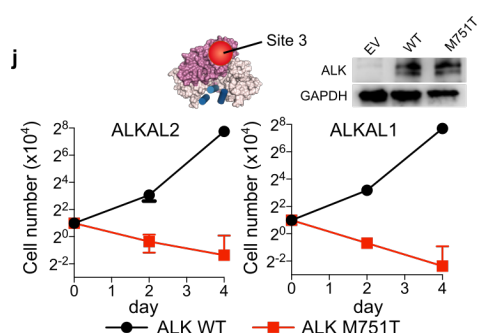
The corresponding data are introduced in Extended Data Fig. 7i and 7j and are reported in the main text as follows (lines 219-225):

“Finally, we interrogated the functional importance of site 3 in LTK and ALK. For LTK, mutants LTK^{R241A} and LTK^{R241A/N369G} had a decreased propensity to form the native 2:1 stoichiometric complexes with ALKAL1 (Extended Data Fig. 7i). Given the resistance of ALK ectodomains to establish 2:1 stoichiometric complexes with cytokines at concentrations applicable in chromatographic methods, we established a Ba/F3 cell line expressing ALK^{M751T} hypothesizing that this mutation would introduce an N-linked glycosylation site at Asn749. We found that ALK^{M751T} was unable to drive cytokine-dependent cellular proliferation while it expresses very similarly to wildtype ALK (Extended Data Fig. 7j).”

Extended Data Fig. 7i



Extended Data Fig. 7j



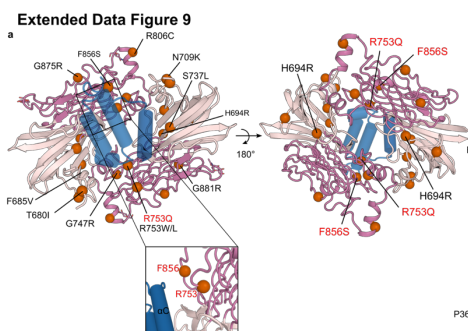
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We agree with the reviewer that categorizing the mutations in our original manuscript as stated was confusing. We have now drastically revised the relevant section as shown below, which also entailed replacing panel a in Ext. Data Fig. 9:

lines 240-256: Mapping of somatic mutations in ALK

“To expand structure-function insights on ALK and LTK activation we structurally mapped mutations in ALK_{TG} and LTK_{TG} combining documented oncogenic potential and frequently occurring missense single-nucleotide polymorphisms^{44,45} (Extended Data Fig. 9a,b). Mutations leading to constitutive receptor activation or that may enhance receptor-receptor contacts or stabilize active receptor states are widely studied to evaluate oncogenic potential. LTK variant R243Q and ALK variants G685R, G747R, and H694R locate at positions that

would be compatible with such roles. Interestingly, H694R is a gain-of-function mutation in lung adenocarcinoma leading to constitutive activation of ALK⁴⁶. On the other hand, mutations that may impact cytokine binding, e.g. by increasing affinity, are less common. Here, we identify two such candidate mutations: LTK variant P363L and ALK variant S737L mapping to interaction sites 2 and 1, respectively. ALK^{F856S}, a gain-of-function mutation linked to acute myeloid leukemia⁴⁷, and ALK^{R753Q} identified in histiocytic neoplasms⁴⁸, are roughly equidistant from the start of α C of bound ALKAL2 (**Extended Data Fig. 9a**). To gain insight into their possible mechanism, we established Ba/F3 cell lines expressing the two mutant ALK variants and found that while their expression levels were comparable to ALK^{WT}, they both drastically increased cytokine-dependent cell proliferation (**Extended Data Fig. 9c,d,e,f**). Unlike previously reported⁴⁷, ALK^{F856S} was not constitutively active. In light of their structural context, these mutations might facilitate the reorganization of key regions of the cytokine-receptor interfaces.”



3. It is puzzling that there is no decrease in binding affinity between ALKAL1/2 ligands and ALK_{TG}-EGFL receptor fragment, comparing to binding to ALK_{TG}? The structural analysis shows that the ligand is fully encapsulated in the ALK_{TG}-EGFL construct in contrast to the ALK_{TG} fragment in which ligand binding pocket seems quite open. It sounds that such encapsulation would certainly affect an on and/or off rates of ligand binding.

It is likely that the reviewer may have mixed up LTK with ALK, which is likely due to the lack of clear labeling from our side in Figure 5 and Extended Data Figure 10. We have rectified this now.

While we did not measure binding kinetics involving ALK_{TG}-EGFL and LTK_{TG}-EGFL, our comparative binding studies by isothermal titration calorimetry (ITC) have shown that the EGFL domain dictates the ligand preference of ALK for ALKAL2 over ALKAL1 (Figure 5). A reciprocal analysis for LTK showed no contribution by its EGFL domain to ligand binding affinity (Extended Data Figure 10). ALK and LTK share 36.5% sequence identity in their EGF-like domains. Moreover, the LTK EGFL domain contains a N-linked glycosylation site at Asn411. Glycosylation at this site may hamper the interaction of LTK_{EGFL} with the ALKALs. Finally, it is difficult to predict kinetics of binding at the cell surface. Our binding studies were intended to provide a comparative study in order to benchmark a first delivery of structure-function insights.

Minor points

1. The authors mention that they were able to purify full-length ALKAL2, but never present any data on how this construct interacts with ALK and LTK receptors, and entirely focus on the conserved C-terminal domain of ALKAL2. It is unclear what is the reason for focusing on the shorter fragment (except for the fact that only a shorter fragment of ALKAL1 was purified), and what could be learnt from studying the longer fragment.

Unfortunately, we could not purify full-length ALKAL2. On the other hand, we could produce the full-length version of ALKAL1 to yields that enabled some experimental undertakings. We reported the full array of our experimental work with ALKAL1_{FL} as follows:

Binding studies by ITC studies of ALKAL1_{FL} against the high-affinity receptor fragments LTK_{TG}-EGFL and LTK_{TG} (Extended Data Fig. 10 d and h). The low affinity of ALKAL1_{FL} for ALK_{TG}-EGFL note that such studies using ALK receptor fragments were limited by the low affinity

2. It is unclear if the final ALK_{TG}-ALKAL2 crystal structure was obtained in the presence of Fab or its absence. Unless I misunderstood, lines 172 and 173 provide conflicting information.

We agree with the reviewer that this was unclear. **We now provide clarity after having revised our text in lines 131-135 to:**

“After failing to obtain diffraction-quality crystals from purified LTK_{TG}-EGFL-ALKAL1 and ALK_{TG}-EGFL-ALKAL2 complexes, we leveraged ALK/LTK-cytokine complexes lacking the EGFL domain. In the case of LTK, we additionally employed a non-neutralizing single-domain VHH antibody (Nb3.16) fragment. Crystals of ALK_{TG}-ALKAL2 and a tripartite complex comprising LTK_{TG}-ALKAL1-Nb3.16 (Extended Data Fig. 4f,g) led to structures to 4.2 Å and 1.9 Å, respectively (Fig.2a,b, Extended Data Table 1).

”

3. The colorful labels in the figures do not always work. For example, in Figure 1c – pale yellow is barely visible. Likewise, in Figure 1f and 1g, the residue numbering does not contrast well enough with the structure and blends in, becoming hard to read.

We thank the reviewer for the comment. Figure 1 has been revised to remedy such issues and more, including removing redundant labels and improving labeling annotations and clarity throughout.

4. The analysis could use a broader perspective. The authors mention parallels between ligand/receptor ALK and LTK complexes with known structures of hematopoietic helical cytokines and their receptors. Is there something interesting there to be learnt by making this comparison? A supplemental figure showing these parallels would be a great addition. We thank the reviewer for this remark, which prompted us to fully revise the last subsection under the new title “Mechanistic considerations”. This allowed us to better formally address mechanistic considerations in relation to other RTK.

To this end we have introduced Extended Data Figure 10k, which we now call out in this section together with Fig. 5f.

Mechanistic considerations

Whereas cytokine-mediated dimerization of ALK and LTK leads to structurally similar ternary complexes, the mechanistic requirements for their assembly appear to be distinct. LTK-cytokine complexes are fully cytokine-driven whereas ALK-cytokine complexes might synergize with glycosaminoglycans (Fig. 5f). Our reported ALK/LTK-cytokine complexes are likely representative in all vertebrates because of the strong sequence conservation in receptors and ligands, and their species cross-reactivity^{6,43}. However, invertebrate ALK ligands are unable to activate human ALK and are structurally distinct from ALKAL1/2⁵⁰. The currently known modes of ligand-induced activation of RTKs display a broad array of structural principles^{36,37}, ranging from dimerized receptor assemblies exclusively driven by the activating ligands (e.g. TrkA-NGF)⁵¹ to complexes mediated fully via receptor contacts (e.g. EGFR-EGF)⁵². However, several cytokine-RTK assemblies feature an amalgamation of ligand- and receptor-mediated contributions (e.g. CSF-1/IL-34-CSF-1R)^{53,54} including the involvement of accessory molecules (e.g. FGF-FGFR)⁵⁵ or co-receptors^{56,57}, and the use of multiple cytokine copies to (e.g. EGFR and Insulin receptor)^{52,58}. The utilization of a single copy of a monomeric cytokine by ALK family receptors to undergo dimerization with twofold symmetry introduces a novel cytokine-driven assembly mechanism among RTK (Extended Data Fig. 10k).

It is now clear that key differences in the cytokine-binding regions of ALK and LTK dictate cytokine specificity and that receptor-receptor contacts are also important differentiating factors. The resistance of isolated ALK ectodomains towards cytokine-induced dimerization suggests that the reduced dimensionality of their membrane-proximal engagement and additional interactions of its N-terminal domains with glycosaminoglycans or proteoglycans⁸ might be important for productive cytokine-receptor assemblies, much like bound heparin bridging receptors in FGF-FGFR complexes. In this context, the reported proteolytic shedding of the N-terminal segment of ALK's ectodomain presents with a physiological conundrum⁵⁹. Intriguingly, the EGFL module of ALK, but not LTK, appears to dictate cytokine specificity, such that the mode of its engagement in ALK-cytokine complexes may impact the orientation of the membrane-proximal domains (and their connected transmembrane helices) to fine tune signaling assemblies. We envisage application of our findings to further interrogate ALK/LTK signaling in physiology and disease, and in the therapeutic targeting of the ALK/LTK ectodomains¹⁷ and their cognate cytokines¹⁶.

k, Overview of the different ligand-mediated extracellular assembly mechanisms across RTKs. TrkA—NGF (PDB: 2IFG), EGFR—EGF (PDB: NIVO), INSR—INS (PDB: 6PXW), CSF1R—CSF1 (PDB: 4WRM), FGFR—heparin—FGF (PDB: 1FQ9), ALK_{TG}—ALKAL2 (PDB: 7NWZ).

8. Can the authors speculate on the potential of ALK and LTK to form ALKAL1 or ALKAL2-driven heterodimers? Would that be of physiological importance?

ALK and LTK co-expression has been observed in the hippocampus as described by Weiss et al. Furthermore, Guan et al. showed that upon addition of ALKAL1 ALK could be immunoprecipitated with LTK. These results may point to a possible physiological role for ALK-LTK heterodimers. Our structures show the TG domains of both receptors are very similar in overall shape and likely allow for heterodimer formation. The investigation of these heterodimers via biophysical methods will be an interesting aspect of future research undertakings.

Referee #3 (Remarks to the Author):

De Munck et al. report biochemical, biophysical, and X-ray crystallographic studies of ligand binding fragments of the ALK and LTK receptor tyrosine kinases (RTKs) both alone and complexed with their cognate ligands ALKAL1 or ALKAL2. These structures represent to my knowledge the first atomic resolution views of the ALK/LTK ligand regions, ALKAL cytokines, and the nature of their interaction. The structures reveal an interesting modification of a TNF-like domain with glycine-rich regions that are inserted in distinct loops but combine to form a poly-glycine II rich structure with hexagonally-packed strands. The authors validate their structures by showing mutations in ligand-receptor interfaces decrease binding and activity. These results are important and will form the basis of all future studies and understanding of this important class of RTKs. I thus believe these results merit publication in a high-profile journal like Nature.

We thank the reviewer for the expressed appreciation of the novelty, importance, and quality of our reported findings. It is tremendously motivating and rewarding to read such remarks.

That said, the authors seem so immersed in their topic that they have prepared a manuscript that in many places is confusing if not misleading for a general reader. After 3 readings of the MS I still do not have a clear idea of several key issues and had to spend considerable time puzzling over what is shown and meant in several figures.

I admire this work and commend the authors, but I note below several aspects of the presentation that I found confusing or frustrating in case the authors find them helpful in revising the MS for clarity.

We do realize that our manuscript reports a very voluminous package of research results. For this inaugural paper on the structural biology of ALK/LTK we felt that we needed to cover as much ground as possible in order to help the field move forward.

Our revised manuscript reflects scores of very substantial improvements in terms of text (5100 to 3700 words), structure (rearranged subsections), and display items (revised figures etc). We very much hope that this has had the desired effect.

1. The authors note that SEC-MALS experiments demonstrate 2:1 LTK:ALKAL and 1:1 ALK:ALKAL stoichiometries in solution, but never explicitly state the stoichiometries found in the crystal structures of receptor-ligand complexes. Discussions later in the paper imply it is 2:1, but the solution results don't necessarily translate to crystal results because, as the authors point out, the high concentrations of components of crystals can overcome weak binding affinities.

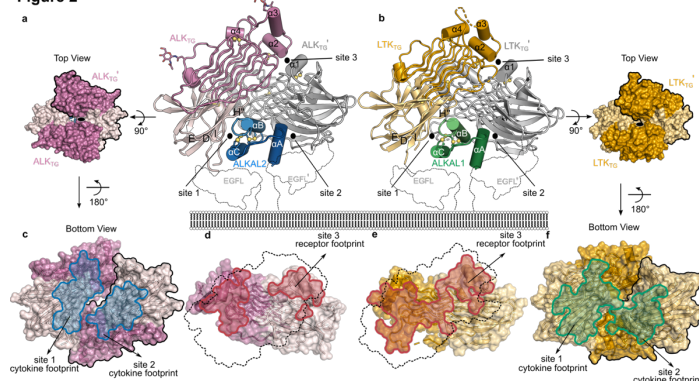
Both of our crystal structures of the ALK-ALKAL2 and LTK-ALKAL1 reveal 2:1 stoichiometric complexes. We have now made this clear in the main text as follows (lines 136-138):

"Our structures revealed that the overarching assembly mode of ALK/LTK-cytokine complexes entails a 2:1 stoichiometric assembly where a single cytokine molecule is cradled proximal to the membrane by two copies of the receptor TG supradomains resulting in receptor dimerization (Fig. 2a,b)."

Thus, when looking at Figure 2c and 2f, it is not immediately clear whether the two blue (2c) or green (2f) patches represent independent binding of different ligands to similar sites on receptor dimer subunits or whether one ligand contacts both sites. I believe it is the latter, but this point seems only implied later in the MS.

We thank the reviewer for addressing a potential point of confusion. This remark has prompted us to revise Figure 2 substantially by creating appropriate contrast between the different structural representations including changing fully to ribbon-diagrams for the centerfold structures and with particular emphasis on highlighting/labeling special features.

Figure 2



Assuming that the stoichiometry in the crystals is 2:1 receptor:ligand, could that be an artifact of the molar ratio of receptor and ligand in the crystallization trials? Could 2 receptors form the Site 3 interaction with an ALKAL molecule bound to Site 1 of each receptor? If ligand binding to Site 1 of each receptor is incompatible with receptor dimers do high amounts of ligand inhibit receptor activation?

The site 3 contacts between the two receptor molecules in the 2:1 complex require the GR subdomain of one protomer to contact the TNFL subdomain of a second receptor molecule. One ALKAL molecule bringing together two receptor molecules via site 1 and site 2 interactions allows for two receptors to reach out to each other at a 45° angle. We have illustrated this in Figure 2. If each receptor would bind an ALKAL molecule via site 1 interactions the receptor molecules would be spaced too far apart to form their site 3 interactions. The fact that we observe two compositionally different complexes (LTK-ALKAL1 vs ALK-ALKAL2) adopting such highly similar assemblies provides very strong support for a common assembly mode with 2:1 stoichiometry. Finally, we have provided many lines of biochemical evidence that these stoichiometries are observable in solution and can be interrogated by structure-driven mutagenesis of all three interaction sites. The regulatory mechanisms of ALKAL-mediated ALK/LTK signalling require further research but given the unique assembly of these complexes, it might be possible that saturating ALKAL concentrations might inhibit signalling activation by occupying all receptors via site 1 interactions. Nevertheless, this is a general scenario that should be applicable to many cytokine-receptor pairs with high- and low-affinity binding sites.

The authors hint that there are some structural rearrangements in the receptor that may underlie dimerization (lines 238-9; see also Extended Figure 6). Is this correct? How do the structures of apo and ligand-bound receptors compare? I.e. are there conserved structural changes in the receptors when ligands are bound that could affect dimerization? Or are the differences ascribable to differences between ALK and LTK? Does ligand binding promote dimerization simply through crosslinking receptors?

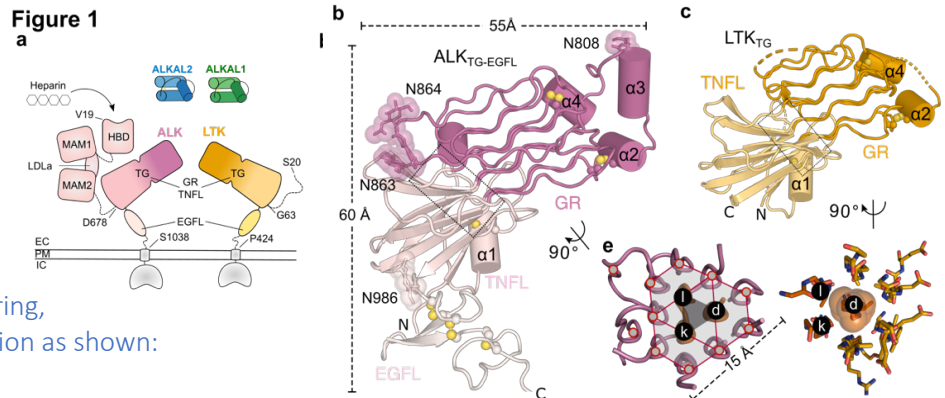
There are no large conformational changes in the ALK and LTK TG supradomains upon complex formation. However, the high-resolution structures of both LTK_{TG} and LTK_{TG}—ALKAL1 did allow us to identify the change in rotamer for L364 between unbound LTK and LTK engaging ALKAL1 via site 1 or site 2. We have included a panel in Extended Data Figure 6 to indicate this structural change.

Also, in Figures 2d and 2e what does the dotted outline represent? What do $\alpha 1$ and $\alpha 2$ mean? Throughout Figure 2, which light colored domains belongs to which dark colored domains? What is the relationship of Sites 1&2 with Site 3? Do they overlap? Are they contiguous? I presume the DE, H" designations refer to strands in the underlying receptor, but that should be made clear. In general, any abbreviation or demarcation shown in a figure should be defined in the legend.

We have adapted main Figure 2 to improve clarity (see above). More specifically to address this remark, we have now indicated one ALK and LTK protomer with a black outline in order to make it easier for the reader to link the light and dark-coloured domains. Additionally, we now indicated the location of the different interaction sites on the structures shown in panels a and b.

2. The schematic in Figure 1a is a little hard to follow. The "TG" portions look like a ligand docked on the receptor; TG is not defined in the legend. Figures 1d and 1e are very helpful, however.

Figure 1 a, b, c have been revised in terms of removing redundant labels, domain coloring, and more efficient representation as shown:



3. In Figure 3, which portion of the cytokine contacts the receptor? The hydrophobic region?

ALKAL1 and ALKAL2 use helices α B and α C to form the site 1 interface with ALK/LTK while helix α A is used to engage the second receptor molecule. In our structures of the ALK_{TG}—ALKAL2 and LTK_{TG}—ALKAL1 the central hydrophobic cavity remains exposed.

This engagement is described in the main text as follows:

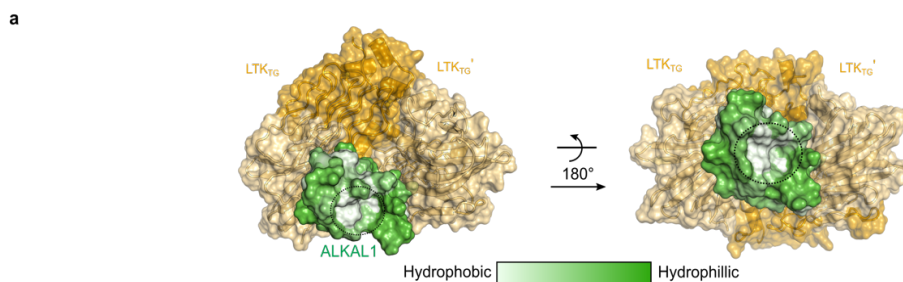
lines 140-142:

"The cytokine ligands are asymmetric three-helix bundles and use helices B and C to contact one copy of their receptors via site 1, and helix A to engage a second copy of the receptor via site 2 (Fig. 2c,f)."

lines 156-159:

"Despite their engulfment by the receptor dimers, both ALKAL1 and ALKAL2 display a solvent-exposed hydrophobic cavity defined by conserved residues: those lining the internal BC face (Leu117, Phe94, Tyr98) and the AB loop (Val84, Phe86) and those around the outside rim contributing hydroxyl groups (Fig. 3b, Extended Data Fig. 5a)."

Extended Data Figure 5



Reviewer Reports on the First Revision:

Referee #1 (Remarks to the Author):

The authors revision of their manuscript has addressed many of the issues raised. This reviewer appreciates that they are struggling with measuring ALK activity in potentially activating somatic ALK mutations, as well as their honest responses. The detailed investigation of activating mutations in the ligand binding regions of ALK will certainly require future work. This reviewer is satisfied with the revised version of the manuscript and supportive of publication.

Referee #2 (Remarks to the Author):

The authors responded extensively to the reviews, and strengthened the manuscript while making it more clear and enjoyable to read. I am enthusiastic about publishing this study in its current form.

One outstanding item that requires clarification is whether Fab has been resolved in the high resolution (1.9Å) structure of the LTK receptor with ALKAL1 (which is AUG-beta), which I understand is stabilized by a Fab (Nb3.1b). I could not find this information in any form in the text or figures. Could the bound Fab affects the way by which ALKAL1 interacts with LTK?

Referee #3 (Remarks to the Author):

The authors have satisfactorily addressed my concerns, and the addition of the site 3 mutagenesis experiments strengthen the paper's conclusions.