nature portfolio

Peer Review File

Defective N-Glycosylation of IL6 Induces Metastasis and Tyrosine Kinase Inhibitor Resistance in Lung Cancer

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript reports that the IL6-GP130-STAT pathway that promotes cancer progression and TKI drug resistance is altered by the N-glycosylation status of IL6. IL6 lacking the N-glycan at N73 (N73Q) stimulates SRC-YAP-SOX2 signaling instead of JAK/STAT3 signaling in lung cancer cells and thereby induces EMT, increased cell migration, invasion and metastasis. The authors provide evidence that osimertinib-resistant NSCLC cells produce under-N-glycosylated IL6 (loss of the H form by western)which correlates with reduced expression of N-glycan synthetic genes, and the switch to SRC-YAP-SOX2 signaling. They propose under-N-glycosylated IL6 as a prognostic factor for progression of TKI-resistant lung cancer cells.

The above conclusions rely on evidence that the form of IL6 identified as heavy (H) in western blots carries one or more N-glycan(s). This form is greatly reduced by PNGaseF treatment and by treatment of producing cells with the OST inhibitor NGI-1. However, it is not clear why the MW of the "light bands" is greater after PNGaseF treatment in Fig. 1g. In addition, the "H band" is distorted by a bubble. Finally, the histograms under each gel appear to represent quantitation of only the gel shown. Appropriate quantitation should compare several independent samples and gels, and histograms should include error bars.

Fig. 1D western needs quantitative and reproducibility data as in Fig. 2D. Should include H1299 line in the western analysis.

What occurs to the different western blot species when IL6 N73Q is treated with PNGase F or purified from cells treated with NGI-1?. One would expect reduction to a band of the same low MW in each case if treatments are complete. How do those data compare with treatments of WT IL6?

N73Q IL6 is secreted similarly to WT IL6 (see cytokine array) suggesting there is one or more remaining N-glycans, as also evidenced by the substantial lectin binding properties of N73Q IL-6 in Fig. 2C. IL6 with a different mutation to eliminate N73 (eg N73V, N73A) should be investigated to provide better evidence that the N-glycan and not the amino acid at position 73 is the cause of the change in IL6 properties (see Glycobiology 2018 May 1;28(5):276-283).

Lectin arrays should compare IL6 and IL6 after NGI-1 or PNGase F treatment. N73Q lectin binding data in Fig. 2C show lots of binding to lectins that recognize N-glycans. Also, DeNG-IL6 in Fig. 2B seems to include 2 bands. Better resolution and lower sample loading would help with interpretation.
All westerns need to have accompanying quantitation of reproduced gels with error bars

Evidence should be provided that the anti-IL6 antibody used recognizes all forms of N-glycosylated and DeNG-IL6.

IL6 has many isoforms. The authors should address this in relation to the isoforms produced by osmeritinib-resistant NSCLC cells and the isoforms detected by their anti-IL6 Ab. Are there Abs against recombinant IL-6 from bacteria which has no N-glycans? They should be tested.

The methods for immunoprecipitation are not adequately described.

Explain dotted circles in Fig. 3j.

The evidence that PC9-OR and HCC827-OR cells secrete under-N-glycosylated IL6 relies solely on western blots in which barely any IL6 was secreted from parental cells for comparison. Evidence that IL6 from OR cells has reduced lectin binding properties compared to IL6 from parental cells should be shown. Also, the OR-IL6 should be treated with PNGaseF and characterized. In addition, the IL6 from OR cells should be tested for functional properties - stimulation of cell migration and wound-healing, nuclear/cytoplasmic transfer etc. Finally, the authors should include evidence that DeNG-IL6 is produced in lung cancer cells or effusions from patients resistant to TKI inhibitor(s).

The N-glycan/IL6 shown in the summary diagram is not accurate.

Reviewer #2 (Remarks to the Author):

Authors explored the role of N-glycosylated (NG-IL6) and N-glycosylation-defective IL-6 (DeNG-IL6) on tumor cell function including the metastasis and drug resistance. They reported that both NG-IL-6 and DeNG-IL-6 could be produced by non-small cell lung cancer (NSCLC) cell lines. Using the expression system of DeNG-IL6 by replacing N73 with Q (N73Q), they demonstrated that DeNG-IL6 promotes EMT and metastasis mediating GP130/SRC/YAP axis activation. They also reported that IL-6 production was elevated in osimertinib resistant NSCLC cells and inhibition of YAP sensitized the cells to osimertinib.
This paper reported novel function of DeNG-IL6 in tumor progression and these findings seem to be very impressive. However, several issues listed below need to be addressed to increase the priority of this excellent paper.

Major comments,
1. In page 14, lines 324-339. Authors stated that osimertinib resistant cells (PC9-OR and HCC827-OR) secreted elevated levels of IL-6 compared with the parental cells. However, it was not stated whether the production of DeNG-IL6 was increased in the osimertinib resistant cells. Authors should state this point clearly in the text.

2. It is unclear whether biologically meaningful level of IL-6 was produced in the osimertinib resistant tumor cells. How much IL-6 was produced by these tumor cells (PC9-P, PC9-OR, HCC827-P and HCC827-OR)? Measurement of IL-6 by ELISA using culture supernatant would be helpful.

3. It is unclear whether IL-6 produced by PC9-OR and HCC827-OR really induced osimertinib resistance, while activation of SRC and YAP was well demonstrated in these cells. Authors should show the effect of IL-6 inhibition by anti-IL-6 antibody and IL-6 knockdown or knockout on osimertinib sensitivity in PC9-OR and HCC827-OR cells.

4. Authors mainly used PC14PE6/AS cells. Since the PC14PE6 cells are listed as a problematic cell line, which can be contaminated with PC-9 cells (Cellosaurus cell line PC14PE6 (CVCL_9W70) (expasy.org), authors should state the origin of PC14PE6 cells and how AS cells were generated from PC14PE6 cells in the Methods section.

Reviewer #3 (Remarks to the Author):

The cytokine IL6 has been implicated in many biological processes, including mediating resistance to EGFR tyrosine kinase inhibitors (10.1016/j.ccr.2014.05.019). Thus, elucidation of whether and how IL6 preferentially activates different signaling pathways downstream of the IL6 receptor in a context-dependent manner is of interest, particularly to those in the cancer and immunology fields.

In this manuscript, Hung and colleagues convincingly demonstrate that glycosylated IL6 is secreted by MPECs from patients with lung cancer and by NSCLC cells lines and that the Src-YAP-SOX2 axis promotes EMT and migration and is activated in osimertinib-resistant lung cancer cell lines. These findings are in line with those of previous studies demonstrating that IL6 is glycosylated on N73 (doi: 10.1084/jem.20031205) and that Src signaling promotes migration (numerous publications). The novel claim in this study is that deglycosylated IL6 (DeNG-IL6; the authors' term for IL6 that is not glycosylated at N73) preferentially activates Src rather than JAK-STAT3 signaling. However, due to technical difficulties isolating sufficient amounts of glycosylated or DeNG IL6 (mentioned in line 184), much of the work relies on conditioned media from cells overexpressing either WT or DeNG IL6. Though an understandable workaround, using this method raises the question of whether the differences observed were caused by differences in IL6 glycosylation or by differences in other components of the conditioned media (described in detail below). Moreover, some of the claims in the manuscript are unsupported by the data presented.

Overall, the manuscript presents an intriguing mechanism of IL6 regulation that adds to the body of literature on this important molecule and could serve as a jumping-off point for future studies on post-translational modifications of IL6 and their impact on signaling and function. However, a number of issues, particularly those affecting the central claim, preclude the publication of the manuscript as submitted.

Major points

1. As mentioned above, there are some issues with relying on conditioned media from cells overexpressing either WT or DeNG IL6 to determine the impact of glycosylated vs. deglycosylated IL6.
a. Each of these IL6-expressing cell lines were derived from individual AS2 clones. Because there is typically heterogeneity between cells even within the same cell line, differences observed in the cells treated with the CM from WT or DeNG IL6–expressing cells could be due to differences in secretion of other cytokines, independent of IL6 glycosylation. For example, in Suppl. Figure 5, it appears that levels of OPN (which has been reported to promote EMT; doi: 10.3390/jcm5040039) and OPG are increased in the N73Q-IL6-CM. (However, since the IL6 levels were adjusted to 1 ng/mL, it is difficult to draw any conclusions.) It would be helpful to include a cytokine array of undiluted CM from AS2-Vec-CM, WT-IL6-CM, and N73Q-IL6-CM so that cytokine levels could be compared across the lines.
b. The authors control for IL6 levels in the WT and DeNG CM by performing ELISAs, then diluting the CM so that the IL6 levels are 1 ng/mL in the CM. However, the results of the ELISAs are not included, making it impossible to compare the levels of IL6 secretion between the WT and DeNG IL6–expressing cells or determine whether one sample may have been diluted substantially more than the other, thereby diluting all of the other cytokines as well. Please include the results of the ELISAs performed, perhaps as supplementary information. In addition, please add whether the CM were diluted with RPMI 1640 (unsupplemented) or RPMI 1640 supplemented with FBS and anti-anti.
c. Experiments intended to demonstrate that the differences in cells treated with WT and DeNG IL6 CM were due to IL6, an α-gp130 neutralizing antibody is used. However, gp130 is a signal transducing component of not only the IL6 receptor, but also receptors for several other cytokines (doi: 10.3389/fimmu.2020.01424). Thus, a more appropriate reagent would be an IL6- or IL6Rα-neutralizing antibody.
2. The figure legend for Suppl. Figure 11 states that the IL6 quantity was measured and the IL6 levels in the CM were adjusted to 1 ng/mL before the cytokine array was performed, but according to the cytokine array, the secreted IL6 levels are quite different in the parental and osimertinib-resistant cells. Please address this.
3. Although the results presented in Figure 6 suggest that decreased N-glycosylation is associated with osimertinib resistance (Figure 6i) and that glycosylation of IL6 may be reduced in NSCLC cell lines that have acquired osimertinib resistance (Figure 6c), the claim of "a bypass mechanism that drives osimertinib resistance by reducing the N-glycosylation of IL6" (lines 393–394) is a bit overstated. Critically, the authors do not demonstrate that deglycosylated IL6, specifically, drives osimertinib resistance; rather, IL6 levels are increased and reduced IL6 glycosylation is correlated with osimertinib resistance in cell lines, and the identification of decreases in N-glycosylation in the RNAseq data from osimertinib-resistant tumors could be due to decreased N-glycosylation of any number of targets.
4. In all western blots shown, it appears that deglycosylated IL6 (L-IL6) is the predominant species, including in the WT-IL6 samples. It is unclear how a switch from ~70% deglycosylated to ~100% deglycosylated IL6 would enable preferential activation of Src, especially since the experiments in Figure 2 suggest that deglycosylated IL6 is able to promote STAT3 phosphorylation at early time points. Please address this, if not experimentally, at least with a discussion in the text.

Minor points

5. Please add more information about the IL6 receptor to the introduction, including the role of IL-

6Rα (gp80) in the receptor complex. Notably, the binding between IL6 and gp130 has been reported to be indirect (doi: 10.1002/cpt.782).
6. Figure 2d: Are all statistically significant differences indicated? If not, please indicate all statistically significant differences. (For example, it appears that perhaps the differences between WT- and N73Q-IL6 are different in the NL-20 cells at 3 and 6 h and in the Beas-2B cells at 6 h.)
7. Figure 2f: The legend mentions Suppl. Figure 4 – might this be Suppl. Figure 7 instead? Please check and edit if necessary.
8. Figure 4a: AS2-Vec is included in the legend, but not in the figure. Please correct this, ideally by adding AS2-Vec to the figure.
9. Figure 4b: This figure suggests that CSF2 expression is substantially increased in the N73Q-IL6 CM–treated cells, but based on Suppl. Fig. 5, levels of GM-CSF (which is encoded by CSF2) do not change. This is a minor point, but what might explain this?
10. Figure 4c: It is a bit difficult to tell on the PDF, but it appears that the N73Q-IL6 STAT3 image does not match the green pattern on the merged image. It is possible that the image was shifted slightly to the left or right or the brightness was changed. Please check this.
11. Figure 4e: The technique used for the wound healing assay is very nice – the images are consistent and clear. However, only the results for AS2-IL6-N73Q cells are shown. In addition, no IL6 inhibitor is used. Do the AS2-Vec and AS2-IL6-WT cells also exhibit migratory ability? If so, is this inhibited by JAK-STAT3 inhibitors and/or SRC inhibitors? Without this information, it is unclear whether N73Q-IL6 specifically promotes SRC-mediated migration (which is key for the claims of the paper) or if WT-IL6 can also do this.
12. Figure 6c: Is there a better WB available for the PC9 cells? It appears that there is a dot on the right-hand side of the band indicated to be Heavy-IL6, suggesting that there may indeed be Heavy-IL6 in the OR sample that was not visualized, perhaps due to a transfer issue or issues during the washing steps. In addition, please add more information to the figure legend so that it is clear what the blots under "IL6-WT" are – this is explained in the text, but not in the figure legend.
13. Suppl. Figure 8b: Please add which proteins were identified in spots 3 and 4.
14. Lines 188–191: The claim that IL6 is the predominant cytokine in the CM is not supported by the data. The antibodies in cytokine arrays typically have different detection ranges for different cytokines, and notably, IL-8 and OPN levels appear to be present, perhaps at high levels, as well.
15. Lines 575–576: Please add which ERK(s) the antibodies recognize.
16. Line 620: Hollow fiber culture is mentioned in the Materials and Methods, but not in the main text. What was hollow fiber culture used for?
17. Line 752: Did the authors use a Mann–Whitney test, Student's t-test, or both? Please clarify.
18. Please add which cytokine array was used, including the manufacturer and catalog number.
19. Please add which secondary antibodies were used for western blots and immunofluorescence, including the manufacturers and catalog numbers.

We thank all three reviewers for the constructive and professional comments for our manuscript. We believe that we have addressed all the comments raised by the reviewers, as summarized below.

Because we have presented new results in revised manuscript, demonstrating both increase in DeNG-IL6 and signaling shift in clinical specimens from NSCLC patients who received EGFR TKI treatments including gefitinib, afatinib, erlotinib, or osimertinib, we changed our title into **"N-Glycosylation-Defective IL6 Alternatively Activates the SRC-YAP-SOX2 Axis to Drive** (instead of "Potentiate") **Metastasis and EGFR TKI** (instead of "Osimertinib") **Resistance."**

Totally, Fig. 1/2/3/4/5/6/9 and Supplementary Fig. 5 have been modified or updated; Fig. 7/8 and Supplementary Fig. 7/8/12/14/15/17/19/20/23/24 have been added. Modifications in the manuscript are labeled with colored highlight. We believe the additional analyses have helped us to substantially improve our manuscript. We request you to consider this article for publication in *Nature Communications*.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript reports that the IL6-GP130-STAT pathway that promotes cancer progression and TKI drug resistance is altered by the N-glycosylation status of IL6. IL6 lacking the N-glycan at N73 (N73Q) stimulates SRC-YAP-SOX2 signaling instead of JAK/STAT3 signaling in lung cancer cells and thereby induces EMT, increased cell migration, invasion and metastasis. The authors provide evidence that osimertinib-resistant NSCLC cells produce under-N-glycosylated IL6 (loss of the H form by western) which correlates with reduced expression of N-glycan synthetic genes, and the switch to SRC-YAP-SOX2 signaling. They propose under-N-glycosylated IL6 as a prognostic factor for progression of TKI-resistant lung cancer cells.

The above conclusions rely on evidence that the form of IL6 identified as heavy (H) in western blots carries one or more N-glycan(s). This form is greatly reduced by PNGaseF treatment and by treatment of producing cells with the OST inhibitor NGI-1. However, it is not clear why the MW of the "light bands" is greater after PNGaseF treatment in Fig. 1g. In addition, the "H band" is distorted by a bubble. Finally, the histograms under each gel appear to represent quantitation of only the gel shown. Appropriate quantitation should compare several independent samples and gels, and histograms should include error bars.
Fig. 1D western needs quantitative and reproducibility data as in Fig. 2D. Should include H1299 line in the western analysis.

Response:

Thank you for the comments and suggestion. We have added quantitative results of the replicated WB in Fig. 1d, demonstrating the proportions of NG-IL6 and DeNG-IL6 secreted by each cell line. In addition, IP of IL6 in CM from H1299 was added in the experiment; this molecule was barely detected in WB, consistent with the low IL6 quantity measured by ELISAs as in Fig. 1c.

What occurs to the different western blot species when IL6 N73Q is treated with PNGase F or purified from cells treated with NGI-1? One would expect reduction to a band of the same low MW in each case if treatments are complete. How do those data compare with treatments of WT IL6?

Response:

Thank you for the comments and suggestion. As suggested, we have compared the molecular weight pattern of untreated N73Q-IL6 to that purified from AS2-IL6-N73Q cells pretreated with NGI-1 (Fig. 2c) or treated with PNGase F (Fig. 2d). The western blot patterns of IL6 from AS2-IL6-WT cells pretreated with NGI-1 or treated with PNGase F *in vitro* are similar to that of AS2-IL6-N73Q cells, while the western blot pattern of IL6 from AS2-IL6-N73Q cells was not changed by NGI-1 or PNGase F treatment.

N73Q IL6 is secreted similarly to WT IL6 (see cytokine array) suggesting there is one or more remaining N-glycans, as also evidenced by the substantial lectin binding properties of N73Q IL-6 in Fig. 2C. IL6 with a different mutation to eliminate N73 (eg N73V, N73A) should be investigated to provide better evidence that the N-glycan and not the amino acid at position 73 is the cause of the change in IL6 properties (see Glycobiology 2018 May 1;28(5):276-283).

Response:

Thank you for the comments and suggestion. To confirm that it was the N-glycan andt not the amino acid at position 73 that changes the properties of IL6, we constructed plasmids that express IL6 with the mutation N73A or N73V and then established stable cell lines, AS2-IL6-N73A and AS2-IL6-N73V cells, accordingly (Supplementary Fig. 15a-15c). N73A-IL6 and N73V-IL6 showed a similar pattern as N73Q-IL6 (Supplementary Fig. 15c). Both N73A-IL6-CM and N73V-IL6-CM induced similar patterns of STAT3 activation (Supplementary Fig. 15d) and nuclear localization (Supplementary Fig. 15e) as N73Q-IL6-CM did in AS2-Vec cells, respectively. The STAT3 activation by these three CMs was not prolonged until 24 h after treatment compared to that of WT-IL6-CM. Furthermore, both AS2-IL6-N73A and AS2-IL6-N73V cells showed strong *in vitro* migratory (Supplementary Fig. 15f) and *in vivo* metastatic capacity (Supplementary Fig. 15g and 15h) similar to AS2-IL6-N73Q cells. Those data provide evidence that the change of IL6 properties was driven by the alteration of N-glycan and not the amino acid at position 73.

Lectin arrays should compare IL6 and IL6 after NGI-1 or PNGase F treatment.

Response:

Thank you for the comments and suggestion. We compared the expression of glycans on IL6 secreted by AS2-IL6-WT cells before and after NGI-1 treatment using a lectin array. We added the data in Fig. 1m. The results demonstrated that NGI-1 effectively inhibited the N-glycosylation of WT-IL6 in the AS2-IL6-WT cells by 80%.

N73Q lectin binding data in Fig. 2C show lots of binding to lectins that recognize N-glycans. Also, DeNG-IL6 in Fig. 2B seems to include 2 bands. Better resolution and lower sample loading would help with interpretation.

Response:

Thank you for the comments and suggestion. We have replaced Fig. 2b with another image obtained from WB with lower sample loading and shorter exposure time, which clearly demonstrated a loss of the NG-IL6 band in N73Q-IL6.

All westerns need to have accompanying quantitation of reproduced gels with error bars

Response:

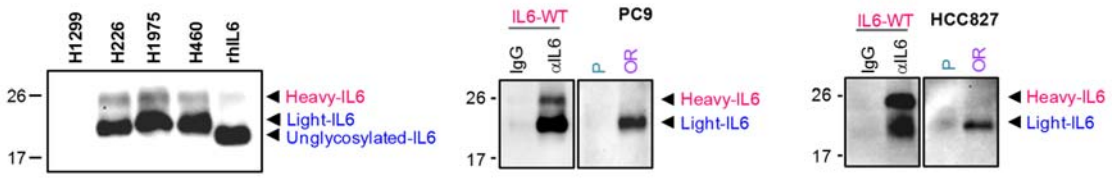
Thank you for the suggestion. We added accompanying quantitative results with error bars of repeated gels in all WB. Because cells from MPE (MPECs) of patients could not be maintained for a long period of time, the immunoprecipitation experiments of IL6 from CM of MPECs were performed once only. Therefore, there is no error bar attached for the experiment.

Evidence should be provided that the anti-IL6 antibody used recognizes all forms of N-glycosylated and DeNG-IL6.IL6 has many isoforms. The authors should address this in relation to the isoforms produced by osmeritinib-resistant NSCLC cells and the isoforms detected by their anti-IL6 Ab. Are there Abs against recombinant IL-6 from bacteria which has no N-glycans? They should be tested.

Response:

Thank you for the suggestion. We've added IL6 from H1299 into the figure (Fig. 1d), as well as the nonglycosylated recombinant IL6 produced by *E. coli*. (as depicted below, left). Consistent with the IL6 quantity determined by ELISAs in Fig. 1c, IL6 from H1299 cells was barely detectable in WB. In the same WB, we detect all forms of IL6, including N-glycosylated, under-N-glycosylated, and unglycosylated forms. The rhIL6 shows the lowest molecular weight on the blot.

Secreted IL6 from OR cells (Fig. 6g, as depicted below, middle and right) were detected on WB at the position of L-IL6, as detected in the NSCLC cell lines (Fig. 1d), or DeNG-IL6, as detected in Fig. 1k, indicating that the main component of OR-IL6 was DeNG-IL6.



The methods for immunoprecipitation are not adequately described.

Response:

Thank you for the comments. Since we added a description of the collection of CM in the revised Methods section, we modified our description of immunoprecipitation experiments as follows:

Immunoprecipitation (IP)

For WB, ten milliliters of adjusted CM supernatant was used for each IP reaction. The concentration of IL6 in the CM supernatant was measured by ELISAs and adjusted to 1 ng/mL with culture media containing supplements. The adjusted CM was then precleaned with nProtein A Sepharose beads (GE Healthcare, GE17-5280-01) to remove nonspecific binding. IL6 antibody (2 µg, GeneTex, GTX110527; RRID:AB_10721789) was used in each IP reaction, rotated at low speed overnight at 4 °C. The Ag-Ab complex was pulled down with 50 µL of nProtein A at 4 °C for 2 h and released in 26 µL of RIPA lysis buffer plus 6x sample buffer by boiling. The IL6 products were then subjected to WB for pattern analysis. *For the lectin microarray*, CM supernatant from a hollow fiber bioreactor in which the IL6 concentration was adjusted to 1 µg/mL was applied. A total amount of 100 µg IL6 was pulled down following the above procedures.

Explain dotted circles in Fig. 3j.

Response:

Fig. 3j and 3i show independent experiments. Because experiments for Fig. 3j were conducted earlier, the cells used in Fig. 3j did not carry pLuc; thus, we terminated each replicate experiment when the first tested animal was dead. The dotted circles indicated the first dead animal in which the lungs were necrotic; thus, they were not applied for further experiments.

The evidence that PC9-OR and HCC827-OR cells secrete under-N-glycosylated IL6 relies solely on western blots in which barely any IL6 was secreted from parental cells for comparison. Evidence that IL6 from OR cells has reduced lectin binding properties compared to IL6 from parental cells should be shown. Also, the OR-IL6 should be treated with PNGaseF and characterized. In addition, the IL6 from OR cells should be tested for functional properties - stimulation of cell migration and wound-healing, nuclear/cytoplasmic transfer etc. Finally, the authors should include evidence that DeNG-IL6 is produced in lung cancer cells or effusions from patients resistant to TKI inhibitor(s).

Response:

Thank you for the suggestion. To decipher the role of OR-IL6 in EGFR-TKI resistance, we performed the following experiments and reorganized these data. Together with the data in original Fig. 6, we presented these findings in revised Fig. 6, Fig. 7, and Fig. 8.

To confirm that the PC9-OR and HCC827-OR cells secreted underglycosylated IL6, we examined the lectin binding properties of OR-IL6 by WGA-lectin blotting (Fig. 6j), which detects N-glycosylation on the loaded proteins. The WGA-lectin signal was reduced by approximately 70% in both OR-IL6s (not specified) compared to their corresponding WT-IL6. Furthermore, we analyzed the N-glycan on WT-IL6 and OR-IL6 with a lectin microarray. Because PC9-OR cells secreted much lower levels of IL6 than HCC827-OR cells (Fig. 6b), we only analyzed the N-glycan on WT-IL6 and OR-IL6 from HCC827 cell series (Fig. 6k). In addition, we treated the OR-IL6 from PC9-OR and HCC827-OR cells with PNGase F. As a control experiment, WT-IL6 from PC9-P and HCC827-P cells showed a decrease in NG-IL6 after treatment with PNGase F. Compared to the WT-IL6, there is no difference in WB patterns of OR-IL6 from cells with or without treatment with PNGase F (Fig. 6l), indicating that OR-IL6 contains few N-glycans.

We next examined the biological properties of OR-IL6 from both PC9-OR and HCC827-OR cells. Compared to those of the WT-IL6 from PC9-IL6-WT and HCC827-IL6-WT cells, the OR-IL6-stimulated STAT3 activation (Fig. 7a) and nuclear transfer (Fig. 7b) were not prolonged to 24 h, as N73Q-IL6 did (Fig. 2f and 2g). In addition, the OR-IL6 promoted prominent migration (Fig. 7g) and faster wound-healing (Supplementary Fig. 19) in the corresponding parental cells than the WT-IL6 cells.

To decipher the role of OR-IL6 in EGFR-TKI resistance, we inhibited OR-IL6 by IL6 neutralizing Ab or shRNA against IL6 in the PC9-OR and HCC827-OR cells. In resistant cells, which were maintained in medium containing osimertinib at the tolerant concentration 5 μM, the addition of IL6 neutralizing Ab restored the cytotoxicity of osimertinib by approximately 15% (Supplementary Fig. 17). When shRNA was used to knock down IL6 (Fig. 6c and 6d), the cytotoxicity was effectively restored by approximately 50% in PC9-OR cells and 80% in HCC827-OR cells (Fig. 6e and 6f). These data support that the OR-IL6 contributes to the resistance to osimertinib in NSCLC cells.

To explore the clinical relevance, we collected lung cancer cells from malignant pleural effusions (MPECs) of EGFR-mutant-positive lung cancer patients who were naïve or resistant to EGFR TKI treatment (Supplementary Fig. 20). After short-term culture, the IL6 from the CM was analyzed. A higher expression of DeNG-IL6 in CM from MPECs of EGFR TKI-resistant NSCLC patients (Fig. 8a) was observed, compared to EGFR TKI-naïve patients. Lectin blotting showed a decrease of N-glycosylation in TKI-resistant MPECs-derived IL6 (Fig. 8b). Furthermore, we collected paired tissues

from TKI-naïve and TKI-resistant patients for IF staining (Supplementary Fig. 23, Supplementary Fig. 24, and Fig. 8e).

In the EGFR-TKI-naïve tissues, we observed strong nuclear STAT3 (Fig. 8e, upper panel) but weak membranous SRC expression. In contrast, in the EGFR-TKI-resistant group, the expression of membranous SRC was prominent and the nuclear STAT3 staining was barely observed (Fig. 8e, lower panel). The difference was highlighted by TissueQuest quantification (Fig. 8f).

In summary, those data provide evidence that TKI-resistant NSCLC cells produce elevated DeNG-IL6 and subsequently induced a signal shift from JAK-STAT3 to SRC-YAP. The changes facilitated cellular migration and drug resistance.

The N-glycan/IL6 shown in the summary diagram is not accurate.

Response:

Thank you for the comment. We substituted the N-glycan structure with an IUPAC standard graphical demonstration of N-glycan with a specific shape and color of each monosaccharide (Fig. 9).

Reviewer #2 (Remarks to the Author):

Authors explored the role of N-glycosylated (NG-IL6) and N-glycosylation-defective IL-6 (DeNG-IL6) on tumor cell function including the metastasis and drug resistance. They reported that both NG-IL-6 and DeNG-IL-6 could be produced by non-small cell lung cancer (NSCLC) cell lines. Using the expression system of DeNG-IL6 by replacing N73 with Q (N73Q), they demonstrated that DeNG-IL6 promotes EMT and metastasis mediating GP130/SRC/YAP axis activation. They also reported that IL-6 production was elevated in osimertinib resistant NSCLC cells and inhibition of YAP sensitized the cells to osimertinib.
This paper reported novel function of DeNG-IL6 in tumor progression and these findings seem to be very impressive. However, several issues listed below need to be addressed to increase the priority of this excellent paper.

Major comments,
1. In page 14, lines 324-339. Authors stated that osimertinib resistant cells (PC9-OR and HCC827-OR) secreted elevated levels of IL-6 compared with the parental cells. However, it was not stated whether the production of DeNG-IL6 was increased in the osimertinib resistant cells. Authors should state this point clearly in the text.

Response:

Thank you for the comment and suggestion. The OR cells secreted elevated levels of IL-6 compared with the parental cells. However, in our original version, we did not show convincing evidence that the main component of OR-IL6 was DeNG-IL6 (only based on the shift of molecular weight). Thanks to the suggestion from reviewer-1, we have performed more experiments examining the lectin affinity (Fig. 6j and 6k) and the pattern properties of OR-IL6 after PNGase F digestion (Fig. 6l). The OR-IL6 showed weak affinity to WGA, which recognized N-glycosylation of proteins. In addition, the molecular weight of OR-IL6 did not change after PNGase F digestion. Those data indicate that the elevated IL6 from OR cells was DeNG-IL6. Therefore, we have added the following description into the text:

To examine whether the main component of OR-IL6 was DeNG-IL6, we analyzed the IL6 from PC9-IL6-WT, PC9-OR, HCC827-IL6-WT, HCC827-OR cells by lectin blotting probed with wheat-germ-agglutinin (WGA), which recognizes N-acetylglutamate on the target protein (Fig. 6j). The WT-IL6 from PC9 and HCC827 cells showed positive bands, suggesting they harbor N-glycosylation. In comparison, the band density suggested that NG-IL6 was reduced by approximately 80% in both OR cells. Furthermore, we analyzed the N-glycan on WT-IL6 and OR-IL6 with a lectin microarray. Because PC9-OR cells secreted a much lower quantity of IL6 than HCC827-OR cells (Fig. 6b), we only analyzed the N-glycan on WT-IL6 and OR-IL6 from the HCC827 cell series (Fig. 6k). After treatment with PNGase F (Fig. 6l), the heavy forms of WT-IL6 were reduced in both the PC9 and HCC827 cell

series, whereas little influence was observed on that of the OR-IL6. These data provide evidence that the OR-IL6 is under-N-glycosylated.

2. It is unclear whether biologically meaningful level of IL-6 was produced in the osimertinib resistant tumor cells. How much IL-6 was produced by these tumor cells (PC9-P, PC9-OR, HCC827-P and HCC827-OR)? Measurement of IL-6 by ELISA using culture supernatant would be helpful.

Response:

Thank you for the comment and suggestion. In the original submitted manuscript (Fig. 6b), we used the relative fold intensity to show IL6 expression in the CM of PC9-P, PC9-OR, HCC827-P and HCC827-OR cells. In the revised edition, we show the data using the absolute concentration of IL6, measured by ELISAs (Fig. 6b). The IL6 concentration in the medium of both OR cells is more than 1000 pg/mL. Usually, an IL6 concentration at 1-10 pg/mL is capable of inducing signal transduction *in vitro*. Notably, in our previous study, the IL6 quantity in plasma of lung cancer patients is at the pg/mL level (doi: 10.1002/ijc.27892). According to the plasma IL6 concentration we divided patients into low, intermediate and high groups. Patients with high IL6 (>25.16 pg/mL) had poor survival outcomes.

In summary, the IL6 levels produced by the osimertinib-resistant tumor cells were biologically meaningful and sufficient to induce signaling transduction and regulate cellular behaviors.

3. It is unclear whether IL-6 produced by PC9-OR and HCC827-OR really induced osimertinib resistance, while activation of SRC and YAP was well demonstrated in these cells. Authors should show the effect of IL-6 inhibition by anti-IL-6 antibody and IL-6 knockdown or knockout on osimertinib sensitivity in PC9-OR and HCC827-OR cells.

Response:

Thank you for the comment and suggestion. To decipher the role of OR-IL6 in EGFR-TKI resistance, we inhibited the OR-IL6 either by IL6 neutralizing Ab or shRNA against IL6 in the PC9-OR and HCC827-OR cells. In resistant cells, after treatment with maintenance medium containing osimertinib at the tolerant concentration 5 μM, the addition of IL6 neutralizing Ab restored the sensitivity to cytotoxicity of osimertinib by approximately 15% (Supplementary Fig. 17). When shRNA was used to knock down IL6 (Fig. 6c and 6d), the cytotoxicity was effectively restored the sensitivity by approximately 50% in PC9-OR cells and 80% in HCC827-OR cells (Fig. 6e and 6f). These data support that OR-IL6 contributes to the resistance to osimertinib in NSCLC cells.

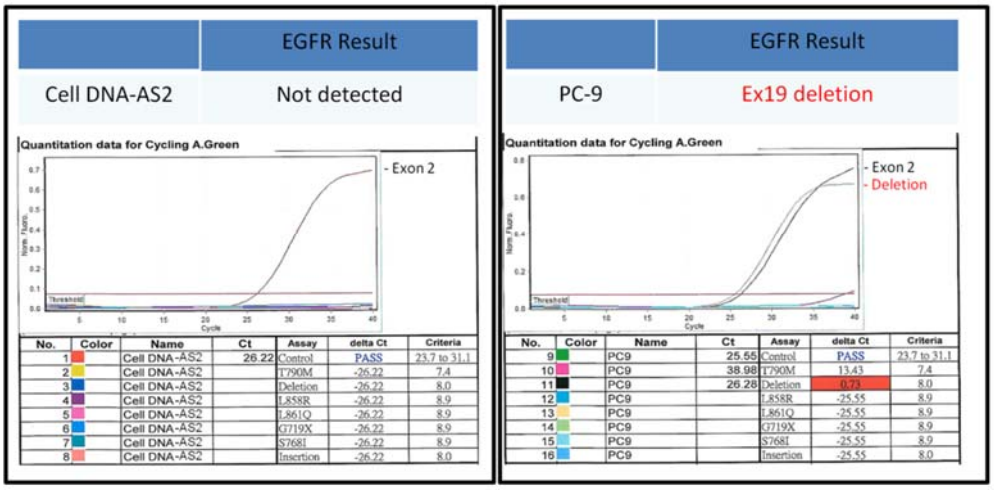
4. Authors mainly used PC14PE6/AS cells. Since the PC14PE6 cells are listed as a problematic cell line, which can be contaminated with PC-9 cells (Cellosaurus cell line PC14PE6 (CVCL_9W70) (expasy.org), authors should state the origin of PC14PE6 cells and how AS cells were generated from PC14PE6 cells in the Methods section.

Response:

Thank you for the comment and suggestion. The human lung adenocarcinoma cell line PC14PE6 was provided by Dr. Fidler (MD Anderson Cancer Center, Houston, TX). The PC14PE6/AS2 (AS2) line (Yeh et al., 2006, doi:10.1038/sj.onc.1209464) was established from ascites in SCID mice generated by inoculation of PC14PE6 cells into peritoneal cavity. We have stated this information in the revised Methods section. We originally obtained the cells directly from Dr. Fidler. The PC-9 cell line is known to harbor the EGFR del19 mutation. Notably, we did not detect EGFR mutations in our AS2 cells in comparison to PC9 cells, as demonstrated in the qPCR data shown below. In addition, the AS2 cells were authenticated by STR-PCR, and results shared a similarity of only 22% to the STR profile of PC14 cells in Expasy, which is listed with EGFR del19. This evidence indicates that the PC14PE6/AS2 cell line we used was not contaminated by PC-9 cells.

At the present time, we found that the information of PC14PE6/AS2 (RRID:CVCL_9W72) was listed on ExPasy, which was updated on 20-May-2021. However, our profile of AS2 was not able to be compared with this cell line because the STR profile of CVCL_9W72 was not provided on the website.

<Characterization of EGFR mutation status>



<Results of cell line authentication>

	Sample	TH01	D5S818	D13S317	D7S820	D16S539	CSF1PO	AMEL	vWA	TPOX	Match %
Sample Result	AS2	9	13,14	8	9	12	13	X	16,18,20	8	-
ExPASy Database Result	PC-14	7	11	8	10	9	11	X	17	11	22
ATCC STR Database Result	There are no results.										

<Interpretation>

After searching the genotypes of the sample in the ATCC STR database, no matching result is found. The customer requested to compare genotyping data with PC-14 (ExPASy database, https://web.expasy.org/cellosaurus/CVCL_1640) and the result shows a 22% match.

Reviewer #3 (Remarks to the Author):

The cytokine IL6 has been implicated in many biological processes, including mediating resistance to EGFR tyrosine kinase inhibitors (10.1016/j.ccr.2014.05.019). Thus, elucidation of whether and how IL6 preferentially activates different signaling pathways downstream of the IL6 receptor in a context-dependent manner is of interest, particularly to those in the cancer and immunology fields.

In this manuscript, Hung and colleagues convincingly demonstrate that glycosylated IL6 is secreted by MPECs from patients with lung cancer and by NSCLC cells lines and that the Src-YAP-SOX2 axis promotes EMT and migration and is activated in osimertinib-resistant lung cancer cell lines. These findings are in line with those of previous studies demonstrating that IL6 is glycosylated on N73 (doi: 10.1084/jem.20031205) and that Src signaling promotes migration (numerous publications). The novel claim in this study is that deglycosylated IL6 (DeNG-IL6; the authors' term for IL6 that is not glycosylated at N73) preferentially activates Src rather than JAK-STAT3 signaling. However, due to technical difficulties isolating sufficient amounts of glycosylated or DeNG IL6 (mentioned in line 184), much of the work relies on conditioned media from cells overexpressing either WT or DeNG IL6. Though an understandable workaround, using this method raises the question of whether the differences observed were caused by differences in IL6 glycosylation or by differences in other components of the conditioned media (described in detail below). Moreover, some of the claims in the manuscript are unsupported by the data presented.

Overall, the manuscript presents an intriguing mechanism of IL6 regulation that adds to the body of literature on this important molecule and could serve as a jumping-off point for future studies on post-translational modifications of IL6 and their impact on signaling and function. However, a number of issues, particularly those affecting the central claim, preclude the publication of the manuscript as submitted.

Major points

1. As mentioned above, there are some issues with relying on conditioned media from cells overexpressing either WT or DeNG IL6 to determine the impact of glycosylated vs. deglycosylated IL6.
a. Each of these IL6-expressing cell lines were derived from individual AS2 clones. Because there is typically heterogeneity between cells even within the same cell line, differences observed in the cells treated with the CM from WT or DeNG IL6–expressing cells could be due to differences in secretion of other cytokines, independent of IL6 glycosylation. For

example, in Suppl. Figure 5, it appears that levels of OPN (which has been reported to promote EMT; doi: 10.3390/jcm5040039) and OPG are increased in the N73Q-IL6-CM. (However, since the IL6 levels were adjusted to 1 ng/mL, it is difficult to draw any conclusions.) It would be helpful to include a cytokine array of undiluted CM from AS2-Vec-CM, WT-IL6-CM, and N73Q-IL6-CM so that cytokine levels could be compared across the lines.

Response:

Thank you for the comment and suggestion. We have included a cytokine array result of undiluted AS2-Vec-CM, WT-IL6-CM, and N73Q-IL6-CM. As shown in revised Supplementary Fig. 5c, the constituents and expression levels of cytokines in undiluted WT-IL6-CM were similar to that of N73Q-IL6-CM. In comparison, the AS2-Vec-CM expressed high RANTES and low OPN.

The results of the adjusted CM (Supplementary Fig. 5d) were similar to that of undiluted CM (Supplementary Fig. 5c). Because inhibition of IL6 or its receptors by antibodies reduced the activation of STAT3 (Supplementary Figs. 6, 7, 8) and cellular EMT (Fig. 3f and 3g, and Supplementary Fig. 11 and Supplementary Fig. 12), we suspected that the IL6 in CM plays a major role in the observed biological behavior. Of course, we cannot rule out the possibility that other cytokines, independent of IL6 glycosylation, may have contributions. Extended future works are required to answer these questions.

b. The authors control for IL6 levels in the WT and DeNG CM by performing ELISAs, then diluting the CM so that the IL6 levels are 1 ng/mL in the CM. However, the results of the ELISAs are not included, making it impossible to compare the levels of IL6 secretion between the WT and DeNG IL6–expressing cells or determine whether one sample may have been diluted substantially more than the other, thereby diluting all of the other cytokines as well. Please include the results of the ELISAs performed, perhaps as supplementary information. In addition, please add whether the CM were diluted with RPMI 1640 (unsupplemented) or RPMI 1640 supplemented with FBS and anti-anti.

Response:

Thank you for the comments and suggestion. Prior to treatment, the CM was diluted with MEMα medium with 10% FBS, 1% antibiotics, and 0.5% MEM with vitamins. We measured the IL6 concentration by ELISAs, and the results were added in Supplementary Fig. 5d, showing similar levels of IL6 in the diluted WT-IL6-CM and N73Q-IL6-CM.

c. Experiments intended to demonstrate that the differences in cells treated with WT and DeNG IL6 CM were due to IL6, an α-gp130 neutralizing antibody is used. However, gp130 is a signal transducing component of not only the IL6 receptor, but also receptors for several

other cytokines (doi: 10.3389/fimmu.2020.01424). Thus, a more appropriate reagent would be an IL6- or IL6Rα-neutralizing antibody.

Response:

Thank you for the comment and suggestion. In addition to GP130 neutralizing Ab (Supplementary Fig. 6), we also examined the inhibitory effect of neutralizing Abs against GP80 (IL6 Rα) (revised Supplementary Fig. 7) and IL6 (revised Supplementary Fig. 8) on the WT-IL6-CM- and N73Q-IL6-CM-induced activation of STAT3. Both neutralizing Abs compromised the levels of pSTAT3 in three different cell lines in a dose-dependent manner, indicating that the pSTAT3 was induced by the IL6 in the CM via classic pathway.

2. The figure legend for Suppl. Figure 11 states that the IL6 quantity was measured and the IL6 levels in the CM were adjusted to 1 ng/mL before the cytokine array was performed, but according to the cytokine array, the secreted IL6 levels are quite different in the parental and osimertinib-resistant cells. Please address this.

Response:

Thank you for the comment and suggestion. We only adjusted CM to 1 ng/mL before the cytokine array in the experiments using AS2-IL6-WT and AS2-IL6-N73Q cells (Supplementary Fig. 5) to demonstrate that the depletion of N-glycosylation on IL6 did not affect the production of other cytokines. In the experiments using the parental and osimertinib-resistant cells, we used undiluted OR-CM for the cytokine array. For clarification, we have added a description in the Methods section.

3. Although the results presented in Figure 6 suggest that decreased N-glycosylation is associated with osimertinib resistance (Figure 6i) and that glycosylation of IL6 may be reduced in NSCLC cell lines that have acquired osimertinib resistance (Figure 6c), the claim of "a bypass mechanism that drives osimertinib resistance by reducing the N-glycosylation of IL6" (lines 393–394) is a bit overstated. Critically, the authors do not demonstrate that deglycosylated IL6, specifically, drives osimertinib resistance; rather, IL6 levels are increased and reduced IL6 glycosylation is correlated with osimertinib resistance in cell lines, and the identification of decreases in N-glycosylation in the RNAseq data from osimertinib-resistant tumors could be due to decreased N-glycosylation of any number of targets. Provide evidence that OR-IL6 in OR cells was under-Nglycosylated, and contributes to the EGFR-TKI resistance

Response:

Thank you for the comments and suggestions. To decipher the role of OR-IL6 in EGFR-TKI resistance, we performed the following experiments and reorganized these data. Together with the data in original Fig. 6, we presented these findings in revised Fig. 6, Fig. 7, and Fig. 8.

To decipher the role of OR-IL6 in EGFR-TKI resistance, we inhibited OR-IL6 by IL6 neutralizing Ab or shRNA against IL6 in the PC9-OR and HCC827-OR cells. In resistant cells, which were maintained in medium containing osimertinib at the tolerant concentration 5 μM, the addition of IL6 neutralizing Ab restored the cytotoxicity of osimertinib by approximately 15% (Supplementary Fig. 17). When shRNA was used to knock down IL6 (Fig. 6c and 6d), the cytotoxicity was effectively restored by approximately 50% in PC9-OR cells and 80% in HCC827-OR cells (Fig. 6e and 6f). These data indicate that OR-IL6 contributes to the resistance to osimertinib in NSCLC cells.

To prove that the PC9-OR and HCC827-OR cells secreted underglycosylated IL6, we examined the lectin binding properties of OR-IL6 by WGA-lectin blotting (Fig. 6j), which detects N-glycosylation on the loaded proteins. The WGA-lectin signal was reduced by approximately 70% in both OR-IL6s (not specified) in comparison to their corresponding WT-IL6. Furthermore, we analyzed the N-glycan on WT-IL6 and OR-IL6 with a lectin microarray. Because PC9-OR cells secreted much lower levels of IL6 than HCC827-OR cells (Fig. 6b), we only analyzed the N-glycan on WT-IL6 and OR-IL6 from the HCC827 cell series (Fig. 6k). In addition, we treated the OR-IL6 from PC9-OR and HCC827-OR cells with PNGase F. As a control experiment, WT-IL6 from PC9-P and HCC827-P cells showed a decrease in NG-IL6 after treatment with PNGase F. Compared to the WT-IL6, there is no difference in WB patterns of OR-IL6 from cells with or without treatment with PNGase F (Fig. 6l), indicating that OR-IL6 contains few N-glycans.

We next examined the biological properties of OR-IL6 from both PC9-OR and HCC827-OR cells. Compared to those of the WT-IL6 from PC9-IL6-WT and HCC827-IL6-WT cells, the OR-IL6-stimulated STAT3 activation (Fig. 7a) and nuclear transfer (Fig. 7b) failed to last until 24 h, as that of N73Q-IL6 did (Fig. 2f and 2g). In addition, OR-IL6 promoted prominent migration (Fig. 7g) and faster wound-healing (Supplementary Fig. 19) in the corresponding parental cells compared to the WT-IL6 cells.

To explore the clinical relevance, we collected lung cancer cells from malignant pleural effusions (MPECs) of EGFR-mutant-positive lung cancer patients who were naïve or resistant to EGFR TKI treatment (Supplementary Fig. 20). After short-term culture, the IL6 from the CM was analyzed. A higher expression of DeNG-IL6 in CM from MPECs of EGFR TKI-resistant NSCLC patients (Fig. 8a) was observed compared to that of EGFR TKI-naïve patients. Lectin blotting showed a decrease of N-glycosylation in TKI-resistant MPECs-derived IL6 (Fig. 8b). Furthermore, we collected paired tissues from TKI-naïve and TKI-resistant patients for IF staining (Supplementary Fig. 23, Supplementary Fig. 24, and Fig. 8e).

In the EGFR-TKI-naïve tissues, we observed strong nuclear STAT3 (Fig. 8e, upper panel) but weak membranous SRC expression. In contrast, in the EGFR-TKI-resistant group, the expression of

membranous SRC was prominent and the nuclear STAT3 staining was barely observed (Fig. 8e, lower panel). The difference was highlighted by TissueQuest quantification (Fig. 8f).

In summary, those data provide evidence that TKI-resistant NSCLC cells produce elevated DeNG-IL6 and subsequently induced a signal shift from JAK-STAT3 to SRC-YAP. The changes facilitated cellular migration and drug resistance.

4. In all western blots shown, it appears that deglycosylated IL6 (L-IL6) is the predominant species, including in the WT-IL6 samples. It is unclear how a switch from ~70% deglycosylated to ~100% deglycosylated IL6 would enable preferential activation of Src, especially since the experiments in Figure 2 suggest that deglycosylated IL6 is able to promote STAT3 phosphorylation at early time points. Please address this, if not experimentally, at least with a discussion in the text.

Response:

Thank you for the comments and question. As shown in Fig. 4c, both IL6 glycoforms stimulated recruitment of STAT3 and SRC to GP130 but with a different preference.

Yes, in the CM of established lung cancer cell lines, it appears that deglycosylated IL6 (L-IL6) is the predominant species. However, in the CM of cancer cells from patient MPE, most cases (10/12) showed equal expression of the two species (Fig. 1b). The data indicate that the glycosylation patterns in cells might be modified by the environment. In revised Supplementary Fig. 6 and Supplementary Fig. 7, we showed that blockade of the IL6 receptors GP130 and GP80 reduced the STAT3 activity induced by both WT-IL6 and N73Q-IL6 in various cell lines. Notably, the inhibition was more effective in the N73Q-IL6-treated cells than in the WT-IL6-treated cells, suggesting that WT-IL6 (N-glycosylated) may have stronger binding affinity to its receptors and greater resistance to antibody blockade. We assume that NG-IL6, with stronger receptor binding ability, even with only 30% occupancy in CM, may outperform DeNG-IL6 in signal transduction. However, when the occupancy of NG-IL6 in CM is reduced to below 10%, DeNG-IL6 becomes dominant in signaling.

Minor points

5. Please add more information about the IL6 receptor to the introduction, including the role of IL-6Rα (gp80) in the receptor complex. Notably, the binding between IL6 and gp130 has been reported to be indirect (doi: 10.1002/cpt.782).

Response:

Thank you for the suggestion. We have added the following text to the introduction section: the first paragraph of the original introduction was replaced by the following paragraph.

Interleukin-6 (IL6) is produced by both cancerous and noncancerous human cells and binds to IL6-receptor alpha (IL6Rα, also known as GP80). The IL6-IL6Rα complex then associates with coreceptor GP130, primarily triggering the GP130-JAK-STAT3 signaling[1, 2, 3] axis to regulate immune responses and cancer pathogenesis[4, 5, 6, 7]. In non-small cell lung cancer (NSCLC), an increase in circulating IL6 is associated with poor survival of patients, and autocrine IL6-mediated GP130-JAK-STAT3 signaling is positively correlated with tumor progression, apoptotic resistance, and drug resistance[8, 9, 10]. In addition to JAK-STAT3, IL6 activates the ERK, AKT, and SRC-YAP pathways via GP130 to regulate diverse biological processes[1, 11, 12].

6. Figure 2d: Are all statistically significant differences indicated? If not, please indicate all statistically significant differences. (For example, it appears that perhaps the differences between WT- and N73Q-IL6 are different in the NL-20 cells at 3 and 6 h and in the Beas-2B cells at 6 h.)

Response:

Thank you for the suggestion. Indeed, the STAT3 phosphorylation was changing at different time points, in different cells. We focused on the 24 h point because it represented the persistence of STAT3 activation, and the phenomenon was common in different cell lines. We have added all statistically significant data in Fig. 2d and highlighted the significance at 24 h.

7. Figure 2f: The legend mentions Suppl. Figure 4 – might this be Suppl. Figure 7 instead? Please check and edit if necessary.

Response:

Thank you for the kind reminder. The original Fig. 2f was moved to Fig. 2h, and the original Supplementary Fig. 7 was moved to Supplementary Fig. 9. We have changed the description of the legend in Fig. 2h to that of Supplementary Fig. 9.

8. Figure 4a: AS2-Vec is included in the legend, but not in the figure. Please correct this, ideally by adding AS2-Vec to the figure.

Response:

Thank you for the suggestion. Originally, we performed the WB with the sample order (from left to right) as AS2-Vec, AS2-IL6-WT, and AS2-IL6-N73Q. For a clearer demonstration and comparison, we cropped the original WB images and used only data from AS2-IL6-WT and AS2-IL6-N73Q. We

replaced Fig. 4a with a recropped WB results demonstrated protein expression in AS2-Vec, AS2-IL6-WT, and AS2-IL6-N73Q cells.

9. Figure 4b: This figure suggests that CSF2 expression is substantially increased in the N73Q-IL6 CM–treated cells, but based on Suppl. Fig. 5, levels of GM-CSF (which is encoded by CSF2) do not change. This is a minor point, but what might explain this?

Response:

Thank you for the question. To our knowledge, the expression of protein levels and its corresponding mRNA levels may not always be consistent (doi: 10.1016/j.cell.2016.03.014). In our case, there might be unidentified mechanisms that promote the degradation or inhibit the expression of GM-CSF proteins. In the current study, we focused on the biological properties of IL6. Further detailed experiments are needed to elucidate this discrepancy.

10. Figure 4c: It is a bit difficult to tell on the PDF, but it appears that the N73Q-IL6 STAT3 image does not match the green pattern on the merged image. It is possible that the image was shifted slightly to the left or right or the brightness was changed. Please check this.

Response:

Thank you for the careful review. We found that there was a shift of the photo possibly due to the file transformation into PDF file. We have replaced Fig. 4c with the re-exported images.

11. Figure 4e: The technique used for the wound healing assay is very nice – the images are consistent and clear. However, only the results for AS2-IL6-N73Q cells are shown. In addition, no IL6 inhibitor is used. Do the AS2-Vec and AS2-IL6-WT cells also exhibit migratory ability? If so, is this inhibited by JAK-STAT3 inhibitors and/or SRC inhibitors? Without this information, it is unclear whether N73Q-IL6 specifically promotes SRC-mediated migration (which is key for the claims of the paper) or if WT-IL6 can also do this.

Response:

Thank you for the comments and suggestions. Indeed, the AS2-Vec and AS2-IL6-WT cells also exhibit migration, as shown in Fig. 3h. We examined the effect of inhibitors against STAT3 or SRC signaling on the migratory capacity of AS2-Vec and AS2-IL6-WT cells. The results of AS2-Vec cells were added in revised Supplementary Fig. 14, and results of AS2-IL6-WT cells were inserted in revised Fig. 4e. In addition, the effect of IL6 neutralizing Ab was evaluated in these cells.

In summary, IL6 neutralizing Ab effectively inhibited migration of all these cells. Compared to the mock control, the SRC inhibitors efficiently inhibited the migration of the AS2-IL6-N73Q cells, while the STAT3 inhibitor S3I-201 and the JAK2 inhibitor ruxolitinib showed modest inhibitory effects. In contrast, S3I-201 and ruxolitinib inhibited the migration of AS2-vec and AS2-IL6-WT cells more effectively than

SRC inhibitors did (Supplementary Fig. 14), suggesting that STAT3 has a weaker contribution to migration than SRC does in AS2-IL6-N73Q cells.

12. Figure 6c: Is there a better WB available for the PC9 cells? It appears that there is a dot on the right-hand side of the band indicated to be Heavy-IL6, suggesting that there may indeed be Heavy-IL6 in the OR sample that was not visualized, perhaps due to a transfer issue or issues during the washing steps.
In addition, please add more information to the figure legend so that it is clear what the blots under "IL6-WT" are – this is explained in the text, but not in the figure legend.

Response:

Thank you for the comments and suggestions. The figure was moved to revised Fig. 6g. We replaced the original WB results of PC9-OR-IL6 in Fig. 6g with data from another replicate. For the figure legend, we also added the following description in the legends:

Because the PC9-P and HCC827-P cells secreted little IL6, which prevented analysis of its glycosylation pattern, we established PC9-IL6-WT and HCC827-IL6-WT cells as control cell lines (Fig. 6g and 1k).

13. Suppl. Figure 8b: Please add which proteins were identified in spots 3 and 4.

Response:

Thank you for the suggestion. Because we added additional results to the Supplementary data as Supplementary Fig. 7 and Supplementary Fig. 8, the original Supplementary Fig. 8 was changed to revised Supplementary Fig. 10. We added proteins identified by MS in spots 3 and 4 in an extended table in the revised Supplementary Fig. 10b.

14. Lines 188–191: The claim that IL6 is the predominant cytokine in the CM is not supported by the data. The antibodies in cytokine arrays typically have different detection ranges for different cytokines, and notably, IL-8 and OPN levels appear to be present, perhaps at high levels, as well.

Response:

Thank you for the comments and kind reminder. We have replaced the original description in lines 188-191 with the following text:

In addition, the cytokine array results demonstrated that the constituents and quantity of cytokines contained in both undiluted and diluted WT-IL6-CM and N73Q-IL6-CM were nearly the same and the differences were negligible (Supplementary Fig. 5c and 5d); thus, the observed biological changes are likely induced primarily by different N-glycosylations of IL6.

15. Lines 575–576: Please add which ERK(s) the antibodies recognize.

Response:

Thank you for the kind reminder. We have examined the total ERK (ERK1/2) by antibody from Santa Cruz Biotechnology, Inc. (sc-514302). We have added this description in the manuscript text and changed the label in Fig. 4a.

16. Line 620: Hollow fiber culture is mentioned in the Materials and Methods, but not in the main text. What was hollow fiber culture used for?

Response:

Thank you for the careful review and question. The IL6-containing CM produced from cells cultured in 10 cm dish was used for most of the experiments in this study. However, detection of IL6 glycans by lectin array requires a large amount of protein. Therefore, we seeded appropriate cells into a hollow fiber bioreactor (HFB) to increase the IL6 production. IL6 generated from AS2-IL6-WT cells treated with and without NGI-1 (10 μM) and from HCC827-IL6-WT as well as HCC827-OR cells was used.

17. Line 752: Did the authors use a Mann–Whitney test, Student's t-test, or both? Please clarify.

Response:

Thank you for the kind reminder. We used Student's *t*-test for statistical analysis. We have clarified this in the revised Methods section.

18. Please add which cytokine array was used, including the manufacturer and catalog number.

Response:

Thank you for the kind reminder. We used Ray-Bio C-Series Human Cytokine Antibody Array (Cat# AAH-CYT-5-8 <8 Samples Kit>) in Supplementary Fig. 5 and Supplementary Fig.13. This information has been included in the revised Methods and Supplementary Table 3.

19. Please add which secondary antibodies were used for western blots and immunofluorescence, including the manufacturers and catalog numbers.

Response:

Thank you for the kind reminder. This information has been stated in the revised Supplementary Table 3.

Additional changes in the Methods section were labeled with colored highlight.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have responded very well to the suggestions and requirements of the reviewers and the manuscript is significantly improved. The combined data support the conclusion that the N-glycosylation of N73 in IL6 results in preferential stimulation of YAP-JAK-STAT signaling by NG-IL6 whereas mutation of N73 to A, Q or V gives DeNG-IL6 which preferentially stimulates SRC signaling. However, the existence of DeNG-IL6 in secretions of TKI-resistant tumors is not proved. In addition, the description of the experiments and their interpretation with respect to the glycosylation status of IL-6 remain confused and inaccurate. The diagram in Fig. 9 shows an incorrect structure for the N-glycan precursor. If the authors mean to depict the immature N-glycan transferred in the ER to IL6, it should initially be Glc3Man9GlcNAc2 which is then processed to ultimately give the complex N-glycans depicted on secreted NG-IL6. The microarray lectin data provide evidence that the mature N-glycan structures shown in Fig. 9 are a reasonable guess.
In Fig. 9, two complex N-glycans are shown on IL6. However, throughout the text, authors go to great lengths to conclude that the potential N-glycan site at N172 as not glycosylated. But the lectin array data for DeNG-IL6 missing only the N-glycan at N73 (Fig. 2e) belie this interpretation since the mutant N73A-IL6 binds well to many lectins that recognize complex N-glycans (L-PHA, LCA, AAL to name a few), whereas IL6 produced in the presence of NGI-1 has essentially no binding to these lectins (Fig. 1m). The lectin array data provide good evidence that N172 in IL6-N73A carries a complex N-glycan - as depicted in Fig. 9 by the authors!!! Thus, the N172 N-glycan should not be dismissed but described as likely, although it can still be concluded that its significance for IL6-induced signaling activity is minimal given the data for IL6 versus N73A/Q/V-IL6. Acknowledging that N172 carries an N-glycan based on the lectin array evidence for N73A-IL6 means that all IL-6 western data should be re-interpreted. It may help to re-organize the text so that the fact of N172 N-glycosylation is presented early. Then it follows that N73A-IL6 is not devoid of an N-glycan and it would be much more accurate to call this molecule uNG-IL6 for under-N-glycosylated-IL6. Fig. 9 actually presents this scenario appropriately, and the text needs to conform to that summary.
The O-glycans of IL6 should also be mentioned since their presence is apparent in Fig. 1g in which PNGase F treatment does not reduce IL6 WT to the lowest MW form. To avoid a lot of confusion, the authors need to re-interpret all western data concerning IL6 and mutant forms. For example, in several panels in Fig.1 there appear to be 3 bands of IL6 – a faint "heavy" band, and two "light" dark bands, often of equal intensity. The faint "heavy" band may be IL6 with both N-glycans, the next band may be IL6 with one N-glycan (N73 or N172), and the lowest band may be IL6 lacking N-glycosylation. To properly dissect the N-glycosylation status of IL6 isoforms, the manuscript should compare in the same gel IPed IL6 from E. coli (no glycans), with IL6-WT +/- PNGase F with IL6-N73A+/-PNGaseF, with IL6-N172A+/-PNGase F (at different exposures so that clear evidence for each isoform is presented). The same IL6 constructs should be compared after inhibition by NGI-1 +/- PNGase F to show that NGI-1 inhibition of N-glycan addition is equivalent to PNGase F removal of N-glycans. In the current manuscript there is no evidence that NGI-1 or PNGaseF treatments were titrated to completion. A few MPEC lines should also be analyzed in the same gel with these controls so the IL6 isoforms in their CM are clear.
Finally, evidence that uNG-N73A-IL6 is found in secretions of TKI-resistant tumors preferentially signaling via SRC would be highly desirable. MS analysis of IL6 peptides treated with PNGase F would give Asp in place of Asn at aa 73 if N73 carried an N-glycan that was removed by PNGase F, and it would give Asn at N73 if N73 was never N-glycosylated. The authors could use ion monitoring MS to focus on peptides that include N73 in IL6 from control versus TKI-resistant tumors. This type of definitive data would greatly improve the biological relevance of the manuscript and more strongly support the authors' conclusions.

Additional points
Histograms with one point per sample of unreplicated data should be removed.

The dashed circle in images of tumors should be explained

Reviewer #2 (Remarks to the Author):

Authors responded appropriately.

Reviewer #3 (Remarks to the Author):

In their initial submission, Hung and colleagues presented intriguing (though in some cases incompletely supported) findings suggesting that IL6 that lacks N-glycosylation at N73 preferentially activates the SRC-YAP-SOX2 axis rather than the JAK-STAT3 axis downstream of GP130, thereby driving migration, metastasis, and resistance to the EGFR TKI osimertinib.

In the revised manuscript, the authors substantially bolstered the validity and significance of their findings, as the experiments that were added to the manuscript strengthened the support for their claims. Thus, the revised manuscript provides important insight into the functional and potential clinical implications of IL6 N-glycosylation that would be of great interest to the IL6 and EGFR TKI resistance fields.

Major comment:

Fig. 7f shows a decrease in survival of PC9-OR and HCC827-OR cells treated with osimertinib plus increasing doses of the YAP inhibitor VP. The results are interpreted to indicate that treatment with VP overcomes resistance to osimertinib. However, because no data on treatment with VP alone (without osimertinib) are provided, it is unclear whether treatment with VP sensitizes the OR cells to osimertinib or these cells are simply sensitive to VP. Please provide survival data for PC9-OR and HCC827-OR cells treated with increasing doses of VP alone and, based on the results, clarify whether treatment with VP sensitizes the cells to osimertinib.

Minor comments:

Lines 164–165 state that IL6 from AS2-IL6-WT “exhibited complete N-glycosylation”; however, Fig. 1j indicates that most of the IL6 in AS2-IL6-WT cells is Light-IL6. Please update the text.

This may be a PDF transfer issue, but there are horizontal lines across the images in Fig. 7c.

There is a typo in the label for the y-axis in Fig. 8c.

We thank all three reviewers for the constructive and professional comments for our manuscript. We believe that we have addressed all the comments raised by the reviewers, as summarized below.

Modifications in the manuscript for revision-1 are labeled with grey highlight. In the current revision-2, Fig. 1/2/6/8/9 have been modified or updated; Supplementary Fig. 4/14/21 have been added. Modifications in the manuscript for revision-2 are labeled with yellow highlight, and the changes of figure numbers for revision-2 are labeled with cyanine highlight. We believe the additional analyses have helped us to substantially improve our manuscript. We request you to consider this article for publication in *Nature Communications*.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have responded very well to the suggestions and requirements of the reviewers and the manuscript is significantly improved. The combined data support the conclusion that the N-glycosylation of N73 in IL6 results in preferential stimulation of YAP-JAK-STAT signaling by NG-IL6 whereas mutation of N73 to A, Q or V gives DeNG-IL6 which preferentially stimulates SRC signaling. However, the existence of DeNG-IL6 in secretions of TKI-resistant tumors is not proved.

Response:

Thank you for the insightful comments and suggestion. Fig. 6b shows an ~5-fold increase in the secretion of IL6 in TKI-resistant OR cells. The majority of OR-IL6 was characterized as DeNG-IL6 by lectin blotting (Fig. 6j). The results from both the lectin array analysis (Fig. 6k) and mass spectrometry (Fig. 6l and Supplementary Fig. 21) showed a decrease in N-glycans on OR-IL6 by ~30%. PNGase F digestion reduced the intensity of the NG-IL6 band associated with WT-IL6 but had a minimal effect on the pattern of OR-IL6 (Fig. 6m). In addition, IL6 isolated from patient-derived TKI-resistant MPE cells was defective in N-glycosylation (Fig. 8a and 8b, right panel) compared to that isolated from TKI-naïve MPE cells. Thus, the above evidence indicates that DeNG-IL6 accounts for the majority of IL6 secreted from both TKI-resistant cell lines and patient-derived TKI-resistant tumor cells. The explanation of the MS results is detailed later in the response letter.

In addition, the description of the experiments and their interpretation with respect to the glycosylation status of IL-6 remain confused and inaccurate. The diagram in Fig. 9 shows an incorrect structure for the N-glycan precursor. If the authors mean to depict the immature N-glycan transferred in the ER to IL6, it should initially be Glc3Man9GlcNAc2 which is then processed to ultimately give the complex N-glycans depicted on secreted NG-IL6.

Response:

Thank you for the insightful comments and suggestion. We substituted the initial glycan structure of the core N-glycan precursor with Dol-PP using an IUPAC standard graphical representation with a specific shape and color for each monosaccharide in the updated Fig. 9.

The microarray lectin data provide evidence that the mature N-glycan structures shown in Fig. 9 are a reasonable guess. In Fig. 9, two complex N-glycans are shown on IL6. However, throughout the text, authors go to great lengths to conclude that the potential N-glycan site at N172 as not glycosylated. But the lectin array data for DeNG-IL6 missing only the N-glycan at N73 (Fig. 2e) belie this interpretation since the mutant N73A-IL6 binds well to many lectins that recognize complex N-glycans (L-PHA, LCA, AAL to name a few), whereas IL6 produced in the presence of NGI-1 has essentially no binding to these lectins (Fig. 1m). The lectin array data provide good evidence that N172 in IL6-N73A carries a complex N-glycan - as depicted in Fig. 9 by the authors!!! Thus, the N172 N-glycan should not be dismissed but described as likely, although it can still be concluded that its significance for IL6-induced signaling activity is minimal given the data for IL6 versus N73A/Q/V-IL6. Acknowledging that N172 carries an N-glycan based on the lectin array evidence for N73A-IL6 means that all IL-6 western data should be re-interpreted. It may help to re-organize the text so that the fact of N172 N-glycosylation is presented early. Then it follows that N73A-IL6 is not devoid of an N-glycan and it would be much more accurate to call this molecule uNG-IL6 for under-N-glycosylated-IL6. Fig. 9 actually presents this scenario appropriately, and the text needs to conform to that summary.

The O-glycans of IL6 should also be mentioned since their presence is apparent in Fig. 1g in which PNGase F treatment does not reduce IL6 WT to the lowest MW form. To avoid a lot of confusion, the authors need to re-interpret all western data concerning IL6 and mutant forms. For example, in several panels in Fig.1 there appear to be 3 bands of IL6 – a faint "heavy" band, and two "light" dark bands, often of equal intensity. The faint "heavy" band may be IL6 with both N-glycans, the next band may be IL6 with one N-glycan (N73 or N172), and the lowest band may be IL6 lacking N-glycosylation.

Response:

We are very grateful that the reviewer acknowledged that N172 carries an N-glycan based on evidence from the lectin array analysis and gave us a constructive suggestion regarding naming N73Q-IL6 uNG-IL6 for reinterpreting our results. However, after conducting new experiments and obtaining additional evidence, we provide the following interpretation:

To answer the question raised by the reviewer, in addition to the original AS2-IL6-WT and AS2-IL6-N73Q cell lines, we constructed IL6-N172Q and IL6-N73Q+N172Q plasmids and established AS2-IL6-N172Q and AS2-IL6-N73Q+N172Q cell lines in the current revision (Supplementary Fig. 4a). Secreted WT-IL6, N73Q-IL6, N172Q-IL6, and N73Q+N172Q-IL6 were quantified (Supplementary Fig. 4b and 4c) and immunoprecipitated. The pattern was analyzed on the same Western blot with nonglycosylated rhIL6 expressed in *E. coli* (Supplementary Fig. 4d). The WT-IL6 pattern basically contained three bands: the heavy-IL6 (H-IL6) band, the light-IL6 (L-IL6) band and, occasionally, a third faint band at the lowest MW among the three bands at longer exposure times. The N73Q-IL6 pattern typically showed two bands: the L-IL6 band and, occasionally, the lowest-MW faint band at longer exposure times. Intriguingly, the N172Q-IL6 pattern showed four bands: the H-IL6, L-IL6, and lowest-MW faint bands and an additional faint band between H-IL6 and L-IL6 bands (as indicated with a gray asterisk). Finally, the pattern of N73Q+N172Q-IL6 was similar to that of N73Q-IL6,

containing the L-IL6 band and the lowest-MW faint band, whose intensity was stronger than that in the N172Q-IL6 pattern.

When applying MS to identify the glycosylation status of IL6, because of the limited recognition sites of proteases, the N73-containing IL6 peptide could be generated only via digestion with Glu-C, and the N172-containing IL6 peptide was generated only via digestion with trypsin. Thus, the precise percentages of IL6 with single or double N-glycosylation could not be characterized simultaneously with the peptide products from a single digestion.

The MS results (Fig. 6l and Supplementary Fig. 21) showed that more than 99% of secreted IL6 molecules carried a glycan on the T170 residue, indicating the universal existence of O-glycosylation in IL6. Regarding N-glycosylation, in HCC827-IL6-WT cells, 28% of the secreted WT-IL6 molecules were glycosylated on the N73 residue, and 4% were glycosylated on the N172 residue. In HCC827-OR cells, 20% of the secreted OR-IL6 molecules were glycosylated on the N73 residue, and 3% were glycosylated on the N172 residue. These data indicate that N73 is the dominant N-glycosite in IL6 and that the amount of attached N-glycan was reduced by approximately one-third (from 28% to 20%) when the cells acquired resistance to osimertinib.

The H-IL6 band in the Western blot represented IL6 with N-glycosylation at N73 (Fig. 2b and Supplementary Fig. 4d), and mutation of N73 to Q led to the disappearance of the H-IL6 band. These results indicate that the majority of N-glycosylation on IL6 occurs at N73 and that the N73Q mutation reduces the N-glycosylation of IL6 by ~90%. Because the abundance of IL6 with N172 glycosylation was low (3-4%) (Fig. 6l and Supplementary Fig. 21) and there was no obvious band detected at a higher MW than the H-IL6 band (Fig. 2b and Supplementary Fig. 4d), it is reasonable to conclude that the quantity of IL6 with double N-glycosylation (on both N73 and N172) is below the detection limit.

Furthermore, we examined the autonomous migration capacity of AS2-IL6-WT, AS2-IL6-N73Q, AS2-IL6-N172Q, and AS2-IL6-N73Q+N172Q cells (Supplementary Fig. 14). Consistent with the results demonstrated previously, AS2-IL6-N73Q cells showed the strongest migratory capacity (Fig. 3h). AS2-IL6-N172Q cells showed no significant difference in cell migration compared to AS2-IL6-WT cells. The migratory capacity of AS2-IL6-N73Q+N172Q cells was comparable to that of AS2-IL6-N73Q cells. These data therefore indicate that the DeNG-IL6-mediated promotion of cell migration was attributed mainly to the loss of N-glycosylation on N73.

Accordingly, we have updated the nomenclature for the IL6 glycoforms in the WB results from top to bottom: ***NG-IL6*** (N-glycosylated IL6, carrying both O-glycans and N-glycans, equivalent to H-IL6)***, DeNG-IL6*** (N-glycosylation-defective IL6, carrying only O-glycans, equivalent to L-IL6), and ***NoG-IL6*** (IL6 that is not glycosylated, carrying neither N- nor O-glycans). We are very grateful for the reviewer's constructive comments, and we have reorganized the text as highlighted in yellow and revised the labeling in all WB results in the revised manuscript.

To properly dissect the N-glycosylation status of IL6 isoforms, the manuscript should compare in the same gel IPed IL6 from E. coli (no glycans), with IL6-WT +/- PNGase F with IL6-N73A+/-PNGaseF, with IL6-N172A+/-PNGase F (at different exposures so that clear evidence for each isoform is presented).

Response:

Thank you for the insightful comments and suggestion. We compared the pattern of IL6 expressed in *E. coli* (nonglycosylated) with those of IL6-WT, IL6-N73Q, IL6-N172Q, and IL6-N73Q+N172Q from the corresponding source cells w/wo pretreatment with NGI-1 (Supplementary Fig. 4e). Treatment with NGI-1 showed no influence on the quantity of NoG-IL6. Unexpectedly, both the N172Q and N73Q+N172Q mutations led to an increase in the proportion of NoG-IL6, suggesting that the amino acid change in residue 172 from N to Q may interfere with the O-glycosylation of IL6. According to the prediction of NetOGlyc and the MS data (shown later in the response letter), abundant O-glycosylation was detected at T170 in the IL6 peptide, confirming the reviewer's deduction.

The same IL6 constructs should be compared after inhibition by NGI-1 +/- PNGase F to show that NGI-1 inhibition of N-glycan addition is equivalent to PNGase F removal of N-glycans.

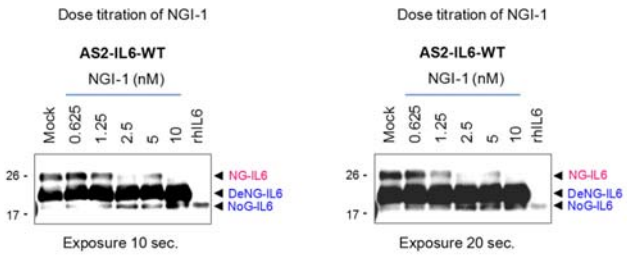
Response:

Thank you for the suggestion. We compared the patterns of WT-IL6 and N73Q-IL6 after inhibition by NGI and digestion with PNGase F on the same Western blot (Fig. 2c) and have replaced the original figure with the new results.

In the current manuscript there is no evidence that NGI-1 or PNGase F treatments were titrated to completion.

Response:

Thank you for the suggestion. We examined the inhibitory effect of NGI-1 on NG-IL6 at titrated concentrations from 10 to 0.625 nM. As shown below, NGI-1 completely inhibited NG-IL6 at 10 nM. Therefore, we chose 10 nM as the treatment concentration in our study.



A few MPEC lines should also be analyzed in the same gel with these controls so the IL6 isoforms in their CM are clear.

Response:

Thank you for the suggestion. In the original study, we conducted the experiments sequentially according to the time of sample collection; thus, we performed IP and WB first for IL6 isolated from TKI-naïve MPECs and then for IL6 isolated from TKI-resistant MPECs (in a first collected, first analyzed manner). Because the amount of secreted IL6 in the conditioned medium (CM) was limited, we had to use all of the CM for the IP assay. Therefore, no CM was left. To perform the experiments suggested by the reviewer, we have to initiate a new clinical study and obtain approval from the IRB. It is not possible to accomplish this in a short time period. Please allow us to do this experiment in a future research program. Thank you so much.

Finally, evidence that uNG-N73A-IL6 is found in secretions of TKI-resistant tumors preferentially signaling via SRC would be highly desirable. MS analysis of IL6 peptides treated with PNGase F would give Asp in place of Asn at aa 73 if N73 carried an N-glycan that was removed by PNGase F, and it would give Asn at N73 if N73 was never N-glycosylated. The authors could use ion monitoring MS to focus on peptides that include N73 in IL6 from control versus TKI-resistant tumors. This type of definitive data would greatly improve the biological relevance of the manuscript and more strongly support the authors' conclusions.

Response:

Thank you for the insightful comments and suggestion. The glycoforms of the two IL6 glycoproteins, i.e., IL6 isolated from HCC827-IL6-WT cells (namely, WT-IL6) and IL6 isolated from HCC827-OR cells (namely, OR-IL6), were examined. Purified WT-IL6 and OR-IL6 were subjected to digestion with the protease Glu-C or trypsin to generate IL6 peptides containing Asn73 or Asn172, respectively. N-glycans were subsequently removed by treatment with PNGase F. Digested IL6 samples were analyzed by LC–ESI-MS on an Orbitrap Fusion mass spectrometer (see the **Methods** section). Both WT-IL6 and OR-IL6 were found to contain three glycosylation sites: Asn73 (N73), Asn172 (N172) and Thr170 (T170). However, these three sites are not homogeneously glycosylated. As shown in Fig. 6l, 28% and 20% of WT-IL6 and OR-IL6 proteins were glycosylated at N73, respectively, i.e., OR-IL6 displayed a 29% reduction in N73 glycosylation compared to WT-IL6. In contrast, there were no significant differences in the percentages of proteins glycosylated at either N172 or T170. The glycosylation percentage was minimal at N172 (4% and 3% of WT-IL6 and OR-IL6, respectively), whereas glycosylation was prevalent at T170 (>99% and 96% of WT-IL6 and OR-IL6, respectively).

Therefore, there are presumably four different glycoforms of WT-IL6. Twenty-eight percent of WT-IL6 molecules have two glycosylation sites at N73 and T170, 68% of WT-IL6 molecules are monoglycosylated at T170, and the remaining 4% of WT-IL6 molecules may contain two (at N172 and T170) or three (at N73, N172, and T170) glycan chains. Accordingly, the percentage of heavily glycosylated IL6, which contains three glycan chains, is expected to be 4% at most. However, owing to the limited recognition sites of proteases mentioned before, the precise percentages of IL6 with single or double N-glycosylation could not be determined simultaneously with the peptide products from a single digestion. The conclusion appears to be consistent with the results of the Western blots shown in Fig. 6k. In the blots, there are two major bands corresponding to WT-IL6: one dense L-IL6 band corresponding to DeNG-IL6 and the H-IL6 band near the marker for 26 kDa (NG-IL6). Because there was no obvious band detected at a higher MW than the H-IL6 band (Fig. 2b and Supplementary Fig. 4d), the quantity of heavily glycosylated IL6 (at N73, N172 and T170) must be below the detection limit and was too low to be detected.

Additional points

Histograms with one point per sample of unreplicated data should be removed.

Response:

Thank you for the suggestion. We removed the single points from the histograms of unreplicated data.

The dashed circle in images of tumors should be explained

Response:

Thank you for the suggestion. Fig. 3j and 3i show the results of independent experiments. Because the experiments in Fig. 3j were conducted earlier, the cells used in Fig. 3j did not carry pLuc; thus, we terminated each replicate experiment upon the death of the first animal. The dotted circles indicate the necrotic lungs in the first mouse to succumb; thus, these lungs were not applied for further experiments. We have added the description in the results and the figure legends in the manuscript.

Reviewer #2 (Remarks to the Author):

Authors responded appropriately.

Response:

Thank you so much.

Reviewer #3 (Remarks to the Author):

In their initial submission, Hung and colleagues presented intriguing (though in some cases incompletely supported) findings suggesting that IL6 that lacks N-glycosylation at N73 preferentially activates the SRC-YAP-SOX2 axis rather than the JAK-STAT3 axis downstream of GP130, thereby driving migration, metastasis, and resistance to the EGFR TKI osimertinib.

In the revised manuscript, the authors substantially bolstered the validity and significance of their findings, as the experiments that were added to the manuscript strengthened the support for their claims. Thus, the revised manuscript provides important insight into the functional and potential clinical implications of IL6 N-glycosylation that would be of great interest to the IL6 and EGFR TKI resistance fields.

Major comment:

Fig. 7f shows a decrease in survival of PC9-OR and HCC827-OR cells treated with osimertinib plus increasing doses of the YAP inhibitor VP. The results are interpreted to indicate that treatment with VP overcomes resistance to osimertinib. However, because no data on treatment with VP alone (without osimertinib) are provided, it is unclear whether treatment with VP sensitizes the OR cells to osimertinib or these cells are simply sensitive to VP. Please provide survival data for PC9-OR and HCC827-OR cells treated with increasing doses of VP alone and, based on the results, clarify whether treatment with VP sensitizes the cells to osimertinib.

Response:

Thank you for the comments and suggestion. We tested the sensitivity of OR cells to VP alone or in combination with osimertinib at various concentrations. To further confirm the synergistic effect, we tested the

sensitivity of OR cells to the combination of VP and osimertinib at different concentration combinations. Furthermore, as demonstrated in the updated Fig. 7f, we calculated the combinatorial effect of the drugs with Synergy Finder 3.0 (https://synergyfinder.fimm.fi/synergy/synfin_docs/#inpdata) using the ZIP calculation model {reference: https://doi.org/10.1093/nar/gkac382}. The resulting heatmap demonstrates that osimertinib and VP exhibit significant synergism in both PC9-OR (p= 3.65e-07) and HCC827-OR (p=2.69e-14) cells (Fig. 7f).

Minor comments:

Lines 164–165 state that IL6 from AS2-IL6-WT "exhibited complete N-glycosylation"; however, Fig. 1j indicates that most of the IL6 in AS2-IL6-WT cells is Light-IL6. Please update the text.

Response:

Thank you for the comments and suggestion. We have updated the text as follows:

"Moreover, we transfected AS2 and HCC827 cells (which produce relatively low amounts of IL6) with a plasmid harboring human wild-type IL6 (WT-IL6), establishing AS2-IL6-WT and HCC827-IL6-WT cells. In these cells, both the mRNA (Fig. 1h) and protein (Fig. 1i) levels of IL6 were increased, and both H-IL6 and L-IL6 were detected (Fig. 1j), as then verified by MS (Supplementary Fig. 3)."

This may be a PDF transfer issue, but there are horizontal lines across the images in Fig. 7c.

Response:

Thank you for the comments and suggestion. We checked the original uploaded file, and there were no horizontal lines across the images in Fig. 7c. Therefore, we suspected that it may be a PDF conversion issue.

There is a typo in the label for the y-axis in Fig. 8c.

Response:

Thank you for the helpful reminder. We have fixed the typo in the label.

Additional changes in the **Abstract** and **Methods** section were labeled with colored highlight.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have responded appropriately to most concerns raised in review. However, the nomenclature for the different forms of IL6 remains very confusing. The extensive data and novel conclusions of this paper will have low impact unless readers can easily grasp the different forms of IL6 being discussed. Figure 2 in the text should include the information on N- and O-glycans now in supp Fig. S4 and clearly describe the predicted composition of each band on gels of IL6. Fig. 2a should show the in-silico predictions for N- and O-glycans on wild type IL6. The data in supp Fig. S4 show nicely that both N73 and N172 get an N-glycan since the lowest MW band above recombinant IL6 from E. coli is markedly enhanced in the double Q versus single Q mutants. The authors should put a stick diagram against each form in supp Fig. S4d (moved to Fig. 2) to show the N- and O-glycans predicted to be present. Ideally, the authors should show that the double Q mutant runs at the same MW as NGI-1-treated and PNGase F-treated IL6 in the same gel. This lowest MW band with O-glycans (often obscured by the form with a single N+four O-glycans) should be called deNG-IL6 for deN-glycosylated IL6. The single Q mutants should be called uNG-IL6 for under-N-glycosylated IL6. It should be clear to the reader that the 4 O-glycans remain on all forms of IL6, except the recombinant form from E. coli. Thus, H-IL6 in Fig. 1 will be redefined in Fig. 2 as the fully glycosylated, wild type form termed NG-IL6 for N-glycosylated IL6; and L-IL6 in Fig. 1 would be redefined in Fig. 2 as uNG-IL6 for under N-glycosylated IL6. The band generated by the loss of two N-glycans in the double Q mutant would be called deNG-IL6 for deN-glycosylated IL-6. The recombinant form lacking all glycans would be called deG-IL6 for deglycosylated IL-6. The stick diagram opposite each band will show the N- and O-glycans predicted to be on that form of IL6.
The fact that N73Q-IL6 includes no H-IL6 form whereas N172Q-IL6 retains the H-IL6 form presumably reflects the MW and conformational effects of the N-glycan at the respective sites. It is clear from the microarray data that N73Q-IL6 is well occupied with an N-glycan at N172 since binding is reduced by only ~50% for N-glycan-binding lectins. The N73Q form that carries an N-glycan at N172 runs within the broad L-IL6/uNG-IL6 band. The authors should describe their western data accurately.
While the data show that N73Q-IL6 stimulates the SRC-YAP-SOX2 signaling axis, the authors do not appear to have tested N172Q-IL6 for signaling activities. If they have tested N172Q for the signaling switch, those data should be included in the manuscript. If not, the authors should be clear that their data do not exclude this possibility, although the loss of the H-IL6 band is characteristic of N73Q-IL6 as well as TKI-resistant cells and N172Q-IL6 does not enhance wound closing in contrast to N73Q-IL6.
Also relevant to accurate interpretation is Fig. 9 which needs further revision. NG-IL6 should be diagrammed with 2 N-glycans and 4 O-glycans. Since no glycan structures are actually known, use of N- and O- or generic symbols for N- and O-glycans is fine. The Glc3Man9GlcNAc2 glycan now included should be removed. That glycan is added in the ER and would not be found on secreted IL6. Based on the lectin array data, mature IL6 seems to carry two biantennary N-glycan(s). The N73Q form should be shown with one N-glycan at N172 and 4 O-glycans.

Additional points
1) Intro – "We demonstrated ---" should be "Here we demonstrate ----

2) Fig. 1L—lectin microarray NGI-1 relevant lectins are PSA, LCA, ECA, ConA – not AAL or SNA

3) Fig. 2D – lectin microarray shows that half the N-glycans are gone by mutating N73 in terms of binding to PSA, LCA, ECA and ConA so N172 does have an N-glycan which should be accurately acknowledged.

4) Line 211 "These results suggested that rather than N172, N73 is the dominant N-glycosite on IL6." This statement is not correct. The result shows that the glycosite at N73 is primarily responsible for the H-IL6 form.

5) Line 220: The nomenclature should be redefined as "NG-IL6 (N-glycosylated IL6, carrying two N-glycans and four O-glycans, equivalent to H-IL6); uNG-IL6 (under N-glycosylated IL-6 carrying

one N-glycan and four O-glycans, equivalent to L-IL6; deNG-IL6 (deN-glycosylated IL6, carrying only O-glycans, equivalent to the band below L-IL6 in the double N/Q mutant), and deG-IL6 (IL6 that is not glycosylated, carrying neither N- nor O-glycans)."
The nomenclature must include N172Q-IL6 which migrates within the broad band of L-IL6. The amount of the N172Q form is clear from the intensity of the lowest band on the N73/N172 mutant lane (supp Fig. S4e). The N73Q mutation increases the intensity of this lowest band slightly, the N172Q mutant increases it much more and the double mutant increases it most. From the gel in Fig. S4e it seems that both glycosites are similarly occupied as indicated by the lectin microarray data in Fig. 2D.

The data in Fig. S4e for the double mutant do not agree with the data in Fig. 1g which shows that removal of all N-glycans leads to an increase in MW for the form with only O-glycans. This is probably because the cell lines and thus glycoforms are different. To avoid confusion, it would be better to remove the H460 data and include the gel in Fig. S4e in the main text.

Reviewer #3 (Remarks to the Author):

Authors responded appropriately.

We thank all three reviewers for the constructive and professional comments for our manuscript. We believe that we have addressed all the comments raised by the reviewers, as summarized below.

In the current revision-3, modifications in the manuscript for revision-3 are labeled with yellow highlight. Fig. 1/2/9 have been modified or updated; Supplementary Fig. 2/4/15 have been added. We believe the additional analyses have helped us to substantially improve our manuscript. We request you to consider this article for publication in *Nature Communications*.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have responded appropriately to most concerns raised in review. However, the nomenclature for the different forms of IL6 remains very confusing. The extensive data and novel conclusions of this paper will have low impact unless readers can easily grasp the different forms of IL6 being discussed.

Figure 2 in the text should include the information on N- and O-glycans now in supp Fig. S4 and clearly describe the predicted composition of each band on gels of IL6. Fig. 2a should show the in-silico predictions for N- and O-glycans on wild type IL6. The data in supp Fig. S4 show nicely that both N73 and N172 get an N-glycan since the lowest MW band above recombinant IL6 from E. coli is markedly enhanced in the double Q versus single Q mutants. The authors should put a stick diagram against each form in supp Fig. S4d (moved to Fig. 2) to show the N- and O-glycans predicted to be present.

Ideally, the authors should show that the double Q mutant runs at the same MW as NGI-1-treated and PNGase F-treated IL6 in the same gel. This lowest MW band with O-glycans (often obscured by the form with a single N+four O-glycans) should be called deNG-IL6 for deN-glycosylated IL6. The single Q mutants should be called uNG-IL6 for under-N-glycosylated IL6. It should be clear to the reader that the 4 O-glycans remain on all forms of IL6, except the recombinant form from E. coli. Thus, H-IL6 in Fig. 1 will be redefined in Fig. 2 as the fully glycosylated, wild type form termed NG-IL6 for N-glycosylated IL6; and L-IL6 in Fig. 1 would be redefined in Fig. 2 as uNG-IL6 for under N-glycosylated IL6. The band generated by the loss of two N-glycans in the double Q mutant would be called deNG-IL6 for deN-glycosylated IL-6. The recombinant form lacking all glycans would be called deG-IL6 for deglycosylated IL-6. The stick diagram opposite each band will show the N- and O-glycans predicted to be on that form of IL6.

The fact that N73Q-IL6 includes no H-IL6 form whereas N172Q-IL6 retains the H-IL6 form presumably reflects the MW and conformational effects of the N-glycan at the respective sites. It is clear from the microarray data that N73Q-IL6 is well occupied with an N-glycan at N172 since binding is reduced by only ~50% for N-glycan-binding lectins. The N73Q form that carries an N-glycan at N172 runs within the broad L-IL6/uNG-IL6 band. The authors should describe their western data accurately.

Response:

Thank you for the insightful comments and constructive suggestions. *In silico* analysis predicts four O-glycosites (T166, T170, T171, S174) and two N-glycosites (N73 and N172) in the full-length IL6 peptide (Supplementary Fig. 1, Supplementary Fig. 2a, and Fig. 2a). Although four O-glycosites were predicted, the MS data of WT-IL6 (Fig. 6l and

Supplementary Fig. 22) provided compelling evidence that over 99% of the IL6 molecules possess O-glycosylation, and the location of O-glycan was detected only on T170. Therefore, we demonstrated O-glycan solely on T170, thereby contradicting the initial assumption of O-glycosylation at all four predicted sites. Additionally, the MS data revealed that 28% of total IL6 molecules carries glycan on N73, whereas only 4% carries glycan on N172.

Based on the predicted combination of N- and O-glycans, theoretically, there would be five IL6 bands consisting of the following glycoforms on Western blot from top to bottom: (1) IL6 carries glycan at N73+N172+T170, (2) IL6 carries glycan at N73+T170 or N172+T170, (3) IL6 carries glycan at N73 or N172, (4) IL6 carries glycan solely at T170, and (5) IL6 without any glycans. However, there were only four IL6 bands detected on the blot (Fig. 2b). Site-directed mutagenesis (Fig. 2b) and NGI-1 treatment (Fig. 2c) approaches revealed that the four detected IL6 bands on the Western blot from top to bottom were actually **(1)** IL6 carries glycan at N73+T170 or N172+T170, **(2)** IL6 carries glycan at N73 or N172, **(3)** IL6 carries glycan at T170, and **(4)** IL6 without any glycans. Because the H-IL6 band was completely lost in the N73Q-IL6 form, we consider the H-IL6 band represents IL6 carries glycan at N73+T170. The presence of the IL6 glycoform carrying glycans at N172+T170 cannot be ruled out, but its abundance appears to be below the detection threshold of Western blot analysis. No discernible bands higher than the H-IL6 band were observed, indicating that the quantity of the tri-glycosylated IL6 (N73+N172+T170), if present, is once again below the limit of detection for Western blot analysis. The MS data (Fig. 6l and Supplementary Fig. 22) indicated that the predominant (>85%) fraction of the H-IL6 band consists of IL6 molecules bearing N-glycosylation at N73 and O-glycosylation at T170. Conversely, IL6 variants with N-glycosylation on N172 and O-glycosylation on T170 make minimal contributions to this band, thereby corroborating this interpretation.

The reviewer suggests that IL6 carries glycan at N172+T170 runs within the broad L-IL6 band of the N73Q-IL6, but we would like to raise other possibility. While the L-IL6 band of N73Q-IL6 appears thicker than that of N172Q-IL6 (Fig. 2b), the MS data (Fig. 6l and Supplementary Fig. 22) revealed a very low abundance of IL6 with glycans on N172+T170. Thus, the disparity in L-IL6 band intensity between these two cell lines (N73Q-IL6 cells and N172Q-IL6 cells) cannot be attributed solely to the absence of IL6 with N172+T170 in the N172Q-IL6 cell line. Furthermore, the intensity of the L-IL6 band in the N73Q+N172Q-IL6 is equivalent to that of the N73Q-IL6 (Fig. 2b), providing additional evidence that IL6 glycosylated at N172+T170 does not significantly contribute to the intensity of the L-IL6 band. We suppose that the decrease of L-IL6 band intensity in N172Q-IL6 cells is caused by a reduction of O-glycosylation at T170 in these cells and a subsequent appearance of IL6 form which carries glycan at N73 exclusively, labeled with a gray asterisk in Fig. 2b. Certainly, further investigation is needed to validate the hypothesis that the loss of N172 glycosylation might diminish O-glycosylation at T170 of IL6, potentially leading to an increase in IL6 variants glycosylated solely at N73.

Accordingly, we propose the update nomenclature as follows: Because the fully glycosylated IL6 (N73+N172+T170) cannot be detected in Western blot, we prefer to name the H-IL6 band as N-glycosylated IL6 (**NG-IL6**) band to represent IL6 carries glycan at T170 along with either N73 or N173. Alternatively, as suggested by the reviewer, it could be designated as under-N-glycosylated IL6 (uNG-IL6), as it does not encompass fully glycosylated IL6 (N73+N172+T170). According to the mass spectrometry (MS) data, IL6 molecules carrying glycans at N73+T170 constituted the majority of N-glycosylated IL6 (28/28+4, >85%). Hence, we opt to designate it as NG-IL6 rather than uNG-IL6 on Western blot analyses. We kindly request permission from the reviewer to proceed with this decision. We agree with reviewer to name the L-IL6 band as N-glycosylation-defective IL6 (**deNG-IL6**) band. The mutation of N172 to Q generated a new band, labeled with asterisk, between the NG-IL6 and the deNG-IL6 bands. The distance between

the asterisk-labeled band and the deG-IL6 band equals the distance between the NG-IL6 and deNG-IL6 band (Fig. 2b). Moreover, treatment with NGI-1 decreased the intensity of the faint asterisk-labeled band (Fig. 2c); therefore, we deduce the band may represent IL6 carries only N-glycan at N73 and without O-glycan at T170, and the appearance of the asterisk-labeled band and the increase in deG-IL6 suggested that the change in the amino acid at residue 172 may interfere with glycosylation at T170 (described in the discussion part). Finally, the lowest band beneath the deNG-IL6 band, of which the molecular weight equals that of the recombinant IL6 band containing no glycan, is designated as glycosylation-deficient IL6, **deG-IL6**.

We have updated the description in the manuscript as follows:

" Henceforth, we propose a nomenclature for each IL6 band based on its molecular weight position as depicted on the Western blot (from top to bottom), as follows: The H-IL6 band in Fig. 1, redefined in Fig. 2, possesses the highest molecular weight and is designated as **NG-IL6** for N-glycosylated IL6. The majority of NG-IL6 glycoform carries glycans on N73+T170, while a minority carries glycans on N172+T170, albeit rarely detected. The L-IL6 band in Fig. 1, redefined in Fig. 2, is termed **deNG-IL6** for N-glycosylation-defective IL6, as the glycan chain resided solely on T170. Additionally, the IL6 band with the smallest molecular weight (Fig. 2b and 2c) is named **deG-IL6** (glycosylation-deficient IL6), indicating the absence of both N- and O-glycans. This form was observed in various glycosylation-defective IL6 variants (N73Q-IL6, N172Q-IL6, N73Q+N172Q-IL6) and rhIL6 from *E. coli*."

Additionally, in combination with the reviewer's suggestion, we have updated the arrangements of the figures. Supplementary Fig. 2a is currently moved to Fig. 2a, which shows the *in silico* predictions for N- and O-glycans on full-length IL6 peptide. We have also moved Supplementary Fig. 4d to Fig. 2b accompanied with stick diagram demonstrating the actual locations of glycan chains on N-glycosites and O-glycosites (Fig. 2b, Fig. 6l and Supplementary Fig. 22) as both H-IL6 and L-IL6 also contained an O-glycan at T170, in accordance with the MS analysis (Fig. 6l). Furthermore, we have moved Supplementary Fig. 4e to Fig. 2c, showing the double Q mutant (N73Q+N172Q-IL6) runs at the same molecular weights NGI-1-treated IL6 in the same gel.

We believe this clarification enhances the comprehensibility of our findings for both the reviewer and readers alike. We are grateful for the reviewer's thorough explanation and generous suggestions.

While the data show that N73Q-IL6 stimulates the SRC-YAP-SOX2 signaling axis, the authors do not appear to have tested N172Q-IL6 for signaling activities. If they have tested N172Q for the signaling switch, those data should be included in the manuscript. If not, the authors should be clear that their data do not exclude this possibility, although the loss of the H-IL6 band is characteristic of N73Q-IL6 as well as TKI-resistant cells and N172Q-IL6 does not enhance wound closing in contrast to N73Q-IL6.

Response:

Thank you for the insightful suggestion. We have examined the influence of N172Q-IL6 on the signaling by WB (Supplementary Fig. 17). For whole cell lysates, as compared to AS2-IL6-WT cells, AS2-IL6-N73Q cells showed a switch of intracellular signaling preference from STAT3 activation to SRC-YAP-SOX2 axis. This signaling switch was not detected in AS2-IL6-N172Q cells (Supplementary Fig. 17a). The analysis of cytosolic (Supplementary Fig. 17b) and nuclear (Supplementary Fig. 17c) protein fractions showed a similar trend. In combination with the results of migration assay (Supplementary Fig. 16), we defined that N-glycan on N73 is responsible for the signaling switch to SRC-YAP-SOX2 axis and subsequent cell plasticity.

We have included the results in the manuscript as follows:

“To determine whether the glycan attached to N73 or N172 is accountable for the signaling switch, we evaluated the signaling expression in whole-cell lysates, as well as nuclear and cytosolic fractions, extracted from AS2-Vec cells, AS2-IL6-WT cells, AS2-IL6-N73Q cells, and AS2-IL6-N172Q cells. Compared to AS2-IL6-WT cells, a signaling shift from the JAK-STAT3 pathway to the alternatively activated SRC-YAP-SOX axis was exclusively observed in AS2-IL6-N73Q cells (but not in AS2-IL6-N172Q cells) in both whole-cell lysates (Supplementary Fig. 17a) and nuclear (Supplementary Fig. 17b) and cytosolic (Supplementary Fig. 17c) fractions. These findings suggest that N-glycosylation on N73, rather than N172, is accountable for the switch in IL6 downstream signaling from the STAT3 axis to the SRC axis.”

Also relevant to accurate interpretation is Fig. 9 which needs further revision. NG-IL6 should be diagrammed with 2 N-glycans and 4 O-glycans. Since no glycan structures are actually known, use of N- and O- or generic symbols for N- and O-glycans is fine. The Glc3Man9GlcNAc2 glycan now included should be removed. That glycan is added in the ER and would not be found on secreted IL6. Based on the lectin array data, mature IL6 seems to carry two biantennary N-glycan(s). The N73Q form should be shown with one N-glycan at N172 and 4 O-glycans.

Response:

Thank you for the comments and suggestion. We have updated Fig.9, the corresponding text in the manuscript, and our response elsewhere in this letter according to the reviewer’s suggestion.

Additional points

1) Intro – “We demonstrated ---" should be “Here we demonstrate ----

Response:

Thank you so much. We have updated the description according to the reviewer’s suggestion.

2) Fig. 1L—lectin microarray NGI-1 relevant lectins are PSA, LCA, ECA, ConA – not AAL or SNA

Response:

Thank you so much. We have updated the description according to the reviewer’s suggestion.

3) Fig. 2D – lectin microarray shows that half the N-glycans are gone by mutating N73 in terms of binding to PSA, LCA, ECA and ConA so N172 does have an N-glycan which should be accurately acknowledged.

Response:

Thank you for the kind suggestion. As described elsewhere, although N73 is the most promising N-glycosite, the intensity of N-glycan signal of N73Q-IL6 was reduced by approximately 50% (Fig. 2d) in the lectin microarray. This incomplete prevention of N-glycosylation indicates that N73 may not the only N-glycosite in the IL6 protein. However, MS-mediated glycan analysis of WT-IL6 showed that both N73 and N172 were N-glycosylated, with percentages of 28% and 3%, respectively (Fig. 6l), indicating that N73, rather than N172, is the dominant N-glycosite in IL6. Therefore, it is reasonable that the remaining ~50% N-glycan signal of N73Q-IL6 could not be entirely attributed to this minor quantity of N-glycans on N172. The input IL6 samples for MS analysis, WB analysis, and lectin microarray analysis were IP products pulled-down using polyclonal anti-IL6 antibody. The IL6 on the blotted membrane was detected with monoclonal anti-IL6 antibody, which showed high specificity for IL6 molecules. In comparison, the glycan signals were

detected through the binding of lectins, which may also recognized glycans on any other non-specific proteins other than IL6 in the sample. Because the detection with lectin microarray was very sensitive, we deduce that the remaining ~50% of lectin signal on N73Q-IL6 (Fig. 2d) may be resulted from the antibody fragments for IP or non-specific proteins that were co-pulled down from the conditioned medium (CM).

Accordingly, we have included the following description in the discussion section:

"N73 emerges as the predominant N-glycosylation site, a conclusion reinforced by the MS-based glycan analysis of WT-IL6 (Fig. 6l). However, lectin microarray analysis revealed only an approximately 50% reduction in the signal intensity of N-glycan in N73Q-IL6 (Fig. 2d), presenting a somewhat contradictory outcome. This discrepancy is likely due to the utilization of a polyclonal anti-IL6 antibody for the IP-based isolation process. Consequently, the resultant mixture possibly encompassed not only the desired N73Q-IL6 but also other components, such as antibody fragments and/or nonspecific proteins co-precipitated from the conditioned medium."

4) Line 211 "These results suggested that rather than N172, N73 is the dominant N-glycosite on IL6." This statement is not correct. The result shows that the glycosite at N73 is primarily responsible for the H-IL6 form.

Response:

Thank you for your comments. We have updated the description according to the reviewer's suggestion.

5) Line 220: The nomenclature should be redefined as "NG-IL6 (N-glycosylated IL6, carrying two N-glycans and four O-glycans, equivalent to H-IL6); uNG-IL6 (under N-glycosylated IL-6 carrying one N-glycan and four O-glycans, equivalent to L-IL6; deNG-IL6 (deN-glycosylated IL6, carrying only O-glycans, equivalent to the band below L-IL6 in the double N/Q mutant), and deG-IL6 (IL6 that is not glycosylated, carrying neither N- nor O-glycans)."

The nomenclature must include N172Q-IL6 which migrates within the broad band of L-IL6. The amount of the N172Q form is clear from the intensity of the lowest band on the N73/N172 mutant lane (Supp Fig. S4e). The N73Q mutation increases the intensity of this lowest band slightly, the N172Q mutant increases it much more and the double mutant increases it most. From the gel in Fig. S4e it seems that both glycosites are similarly occupied as indicated by the lectin microarray data in Fig. 2D.

Response:

Thank you for the insightful comments and nice suggestion. As described elsewhere, we suggest the following nomenclature of the IL6 glycoforms based on their molecular weight position separated on Western blot rather than the mutation status as following:

Because the fully glycosylated IL6 (N73+N172+T170) cannot be detected in Western blot, we prefer to name the H-IL6 band as N-glycosylated IL6 (**NG-IL6**) band to represent IL6 carries glycan at T170 along with either N73 or N173. Alternatively, as suggested by the reviewer, it could be designated as under-N-glycosylated IL6 (uNG-IL6), as it does not encompass fully glycosylated IL6 (N73+N172+T170). According to the mass spectrometry (MS) data, IL6 molecules carrying glycans at N73+T170 constituted the majority of N-glycosylated IL6 (28/28+4, >85%). Hence, we opt to designate it as NG-IL6 rather than uNG-IL6 on Western blot analyses. We kindly request permission from the reviewer to proceed with this decision. We agree with reviewer to name the L-IL6 band as N-glycosylation-defective IL6 (**deNG-IL6**) band. The mutation of N172 to Q generated a new band, labeled with asterisk, between the NG-IL6 and

the deNG-IL6 bands. The distance between the asterisk-labeled band and the deG-IL6 band equals the distance between the NG-IL6 and deNG-IL6 band (Fig. 2b). Moreover, treatment with NGI-1 decreased the intensity of the faint asterisk-labeled band (Fig. 2c); therefore, we deduce the band may represent IL6 carries only N-glycan at N73 and without O-glycan at T170, and the appearance of the asterisk-labeled band and the increase in deG-IL6 suggested that the change in the amino acid at residue 172 may interfere with glycosylation at T170 (described in the discussion part). Finally, the lowest band beneath the deNG-IL6 band, of which the molecular weight equals that of the recombinant IL6 band containing no glycan, is designated as glycosylation-deficient IL6, **deG-IL6**.

We have updated the description in the manuscript as follows:

" Henceforth, we propose a nomenclature for each IL6 band based on its molecular weight position as depicted on the Western blot (from top to bottom), as follows: The H-IL6 band in Fig. 1, redefined in Fig. 2, possesses the highest molecular weight and is designated as **NG-IL6** for N-glycosylated IL6. The majority of NG-IL6 glycoform carries glycans on N73+T170, while a minority carries glycans on N172+T170, albeit rarely detected. The L-IL6 band in Fig. 1, redefined in Fig. 2, is termed **deNG-IL6** for N-glycosylation-defective IL6, as the glycan chain resided solely on T170. Additionally, the IL6 band with the smallest molecular weight (Fig. 2b and 2c) is named **deG-IL6** (glycosylation-deficient IL6), indicating the absence of both N- and O-glycans. This form was observed in various glycosylation-defective IL6 variants (N73Q-IL6, N172Q-IL6, N73Q+N172Q-IL6) and rhIL6 from *E. coli*."

The data in Fig. S4e for the double mutant do not agree with the data in Fig. 1g which shows that removal of all N-glycans leads to an increase in MW for the form with only O-glycans. This is probably because the cell lines and thus glycoforms are different. To avoid confusion, it would be better to remove the H460 data and include the gel in Fig. S4e in the main text.

Response:

Thank you for the insightful comments and suggestions. We have moved Supplementary Fig. 4e to Fig. 2c as the reviewer recommended. Additionally, the IL6 sample analyzed in Fig. 1g was derived from the conditioned media (CM) of short-term cultured lung cancer cells isolated from patient-derived MPEs. Treatment with PNGase F resulted in a reduction of the H-IL6 band intensity in both patient samples. We speculate that the unexpected shift observed in the L-IL6 band (representing forms with only O-glycans) may be attributed to the conjugation of different patient-specific O-glycans, thereby leading to distinct IL6 glycoforms. Consequently, we have opted to retain this data.

Reviewer #3 (Remarks to the Author):

Authors responded appropriately.

Response:

Thank you so much.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have extensively revised their manuscript which is now suitable for publication.

We thank all three reviewers for the constructive and professional comments for our manuscript. We believe that we have addressed all the comments raised by the reviewers.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have extensively revised their manuscript which is now suitable for publication.

Response:

We appreciate all your constructive suggestions and insights in previous reviews to improve our study. Thank you so much.