

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The is a very dense and data rich paper that identifies a Akt-3/IWS singly pathway in lung cancer that results in generation of U2AF65 splice isoform which in turn activates alternative splicing of CDC5A gene leading to generation of Sororin-dependent regulation of pErk. Although the bulk of the data presented support the proposed model they remain significant gaps in our understanding the causal role of this pathway in NSCLC. If the authors can address the following concerns I would support publication of this interesting concept.

1. The observation that activation of Akt-3 is critical for phosphorylation cascade indicates that Akt-3 activation may be a driver oncogene. Do the authors have data indicating that an Activated form of Akt-3 (Akt-3DD) can drive transformation of immortalized lung epithelium or for that matter is ectopic expression of Sororin capable of driving oncogenic conversion of these cells.
2. In that regard do TCGA data indicate that mutation or rearrangement of Akt-3, splice mutations of either U2AF2, U2AF65 or CDC5A occur in human NSCLC. The inclusion of any data supporting this concept would go a long way in supporting the relevance of this pathway in human lung cancers.
3. Although data supporting Akt-3 involvement is presented does down regulation of Akt-1 or 2 impact on the splicing pathway proposed
4. The selective dependence on mutant EGFR NSCLC vs mutant Was on Sororin expression is interesting. How does Sororin regulate Erk activity independent of EGFR? This is a significant gap in the model.

Reviewer #2 (Remarks to the Author):

This manuscript presents a very solid experimental evidence for a new pathway that promotes proliferation and survival of EGFR-mutated lung adenocarcinoma cells. This pathway starts with the AKT-mediated phosphorylation of IWS1, which then associates with pol II CTD to trigger H3K36 trimethylation, thereby recruiting the reader LEDGF and splicing factor SRSF1 to U2AF2 gene and RNA to induce inclusion of exon 2. In turn, the protein encoded by U2AF2 mRNA with exon 2 regulates splicing of sororin which establishes a pattern of phosphorylated ERK for the maintenance of transformed conditions. To support these claims, authors use effective knockdowns, RT-PCR, RNA sequencing, chromatin pulldowns and related methods, immunoprecipitations, cell cycle assays, plus mice xenografts and expression patterns from actual lung adenocarcinomas, for a very solid and data-rich support of their findings. I find the data of high clarity and quality and their conclusions worth publishing in Nature Communications, if the authors can satisfactorily address the following minor yet important comments:

1. The phospho-IWS1 U2AF2 splicing pathway in lung cancer adds to a previous pathway in which phospho-IWS1 regulates FGFR2 alternative splicing via PTB to influence tumor progression and metastasis. In the current case, phospho-IWS1 regulates U2AF2 splicing to affect transformation. My question here is, are most or all the effects of phospho-IWS1 on transformation mediated by U2AF2, or there are other events important for this cellular process. This could be tested by 'rescuing' sh-IWS1 and Mutant-rescue (MT-R) lung cancer cell lines with exon 2 containing U2AF2 cDNA, to see if the cellular and xenograft phenotypes are rescued by this isoform. In addition, are all U2AF2 effects dependent on sororin? Again, this can be tested by rescuing sh-IWS1 and MT-R cells with additional sororin. Either answer would be ok, or else strongly justify why these experiments were not done to start with.
2. Similarly, while authors use the phosphorylation defective MT-R to rescue shIWS1, why not also use a phosphomimetic mutant? This would support the contribution of the charge in IWS1, which would make this mutant independent of AKT phosphorylation. Since authors use phosphomimetic mutant for sororin (DM-E), why not for IWS1?
3. On page 6, first section of results, when authors describe the common differentially expressed genes, it should be emphasized that these genes change their expression in the same direction for the analogous comparisons (all increase or all decrease together).

4. Authors claim that the shorter U2AF65 derived from skipping of exon 2 by shIWS1 exhibits reduced interaction to PRP19 complex. While the immunoprecipitation data look solid, a missing counter-test is to see the association of shorter U2AF2 (plus U2AF35 which is already blotted) with its more immediate partner which is U2 snRNP. Can authors blot the U2AF2 immunoprecipitates for a U2-specific protein? One would be enough. Also the N-terminal arginine/serine rich domain is most commonly named RS domain (RS domain and SR proteins, not vice versa).
5. Legend of Figure 1C, authors refer to changes in splicing as "inclusion level >20%", while this should read "increased inclusion level >20%". I would strongly recommend them to adopt the PSI (percentage spliced in) nomenclature for measuring splicing inclusion levels.
6. In Figure 3E, what is the significance of the abundant H3K36me3 peaks all along the U2AF2 gene?
7. In Figure 4A, authors rule out a role of PTB in U2AF2 splicing, by the lack of changes upon PTB knockdown. However, in other cells, knockdown of PTB upregulates its paralog PTBP2 which partially rescues its functions. Could this be the case for U2AF2?
8. Last, some of the font in Figure 8 is too small and should be enlarged.

Reviewer #3 (Remarks to the Author):

This manuscript describes a series of experiments that follow up the recent discovery that IWS1 regulates alternative splicing of the non-small lung cancer gene FGFR2. The authors initiate their studies by IWS1 knockdown RNA-seq approaches in lung adenocarcinoma cell lines and identify a new alternative splicing event in U2AF, an essential splicing factor. The skipping of U2AF exon 2 leads to the loss of its RS domain, which is required to coordinate spliceosomal assembly. Thus, U2AF exon 2 skipping will have profound effects of RNA splicing. The authors describe a series of logical experiments to show that the phosphorylated form of IWS1 modulates H3K36 methylation to increase the recruitment of LEDGF and the splicing factor SRSF1 to the nascent RNA, thereby increasing U2AF exon 2 inclusion. Interestingly, alternative splicing of U2AF is cell cycle dependent supporting its regulatory role. To investigate the biological significance of these findings the authors evaluate the correlation between IWS1 expression/U2AF alternative splicing and tumor growth and between IWS1 expression/U2AF alternative splicing and patient survival. While the mouse xenograft data are supportive of IWS1 expression/U2AF alternative splicing's importance in tumor growth, the patient survival data are less impressive, certainly not providing much improved predictions over the previously reported FGFR2 alternative splicing. Overall, the data presented are clean and they make a good case for the proposed mechanistic connections between IWS1 phosphorylation, U2AF alternative splicing and tumor growth.

1) The authors concentrate their discussion of alternative U2AF splicing on exon 2, providing evidence for IWS1, SETD2, SRSF1 association using Chip assays (Figs 1, 3, 4). Interestingly, the data also demonstrate that the same pattern of factor recruitment is seen for exon 3. Thus, it appears that the inclusion of U2AF exon 3 should also be under the control of AKT3 axis. In their analysis the authors do not discuss this possibility and their evaluation will not bring this potential added control to light because the primer pairs used to evaluate U2AF splicing are biased to detect exon 2 inclusion only.

2) U2AF is a general splicing factor involved in the initial assembly of the spliceosome. Losing its RS domain through exon 2 exclusion is expected to affect a large number of intron removal events. It is not clear from the data and from the discussion which differential splicing events are the consequence of direct or indirect effects. Presumably, exons that harbor SRSF1 binding sites could be direct targets, but essentially all other introns should be affected by the temporary loss of U2AF. In other words, what is unique about the exons affected by U2AF alternative splicing (CDCA5), or why does GAPDH splice without apparent defects?

3) It is unclear why the authors do not show how the manipulations carried out affect their previously identified FGFR2 alternative splicing. This data should be added, especially in light of the fact that the FGFR2 splicing parameter is a better survival predictor in the TCGA data analysis (Fig 8).

4) Figs 6, 6S and 7 – several panels are labeled % change, but the scale is fractions.

Response to the referees' comments.

IWS1 phosphorylation promotes cell proliferation and predicts poor prognosis in *EGFR* mutant lung adenocarcinoma patients, through the cell cycle-regulated *U2AF2* RNA splicing.

Laliotis et al.

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The is a very dense and data rich paper that identifies a AKT3/IWS1 singly pathway in lung cancer that results in generation of U2AF65 splice isoform which in turn activates alternative splicing of CDC5A gene leading to generation of Sororin-dependent regulation of p-ERK. Although the bulk of the data presented support the proposed model, they remain significant gaps in our understanding of the causal role of this pathway in NSCLC. If the authors can address the following concerns, I would support publication of this interesting concept.

Re : We thank the reviewer for her/his overall positive comments in our work.

1. The observation that activation of AKT3 is critical for phosphorylation cascade indicates that AKT3 activation may be a driver oncogene. Do the authors have data indicating that an Activated form of AKT3 (AKT3-DD) can drive transformation of immortalized lung epithelium or for that matter is an ectopic expression of Sororin capable of driving oncogenic conversion of these cells.

Re : We gratefully thank the reviewer for his/her time to read our manuscript and propose highly important experiments. Given that IWS1 phosphorylation by AKT3 controls the Sororin/ERK feedback loop through *U2AF2* alternative RNA splicing, we overexpressed myristoylated AKT3 (myr-AKT3), the phosphomimetic AKT3 T305/S472DD (AKT3-DD), phosphomimetic IWS1 S720D/T721E (IWS1 DE), the two spliced U2AF65 isoforms

(U2AF65 α , U2AF65 β) and several forms of Sororin (Sororin wt, Sororin DM-E, Sororin DM-A) in immortalized HBEC hTERT and performed cell transformation assays. The results are consistent with our overall findings, showing effective transformation of HBEC-hTERT cells induced by the active AKT3 (myr-AKT3, AKT3-DD) and IWS1 (IWS1 DE). Moreover, the downstream pathway of U2AF65 α spliced isoform and active Sororin (Sororin wt, Sororin DM-E) was sufficient to lead to cellular transformation, but the exon 2 deficient U2AF65 β and inactive Sororin mutant (Sororin DM-A), did not. The new data supporting these claims can be found in the revised manuscript in Figure S8 and in the text on page 21, line 442.

2. In that regard, do TCGA data indicate that mutation or rearrangement of Akt-3, splice mutations of either U2AF2, U2AF65 or CDC5A occur in human NSCLC. The inclusion of any data supporting this concept would go a long way in supporting the relevance of this pathway in human lung cancers.

Re : We gratefully thank the reviewer for her/his important point. Mutational analysis of six LUAD dataset via cBioportal for *AKT1*, *AKT3*, *IWS1*, *U2AF2* and *CDCA5* revealed the existence of amplifications (mainly for *AKT3*), mutations (gain of function *AKT1 E17K* and *D323Y*, *AKT3*, *U2AF2*, *IWS1* and *CDCA5*) and fusions (*U2AF2-DYNLT3*). Specifically, this analysis identified the known gain of function mutations of *AKT1 E17K* and *D323Y* (*Nature*, 448(7152), pp.439-444., *Nature biotechnology*, 34(2), pp.155-163.,) along with several missense, splice and truncating mutations on *AKT3* gene (*AKT3 G129E*, *AKT3 L233=*, *AKT3 R249S*), with unknown role in the function of AKT3 kinase. Interestingly, the same analysis revealed missense, frameshift and splice mutation of *U2AF2* RS domain (*Q10H*, *L11F*, *X77=*, *G98Rfs*3*), with not-known current significance. Furthermore, the same analysis showed mutations in *U2AF2 G176E/V/W* located in the RNA-Recognition Motif (RRM), which have been reported to alter RNA splicing (*Biochemistry*, 56(36), pp.4757-4761, *Journal of Biological Chemistry*, pp.jbc-RA120). Consistent with our overall findings, in the TCGA LUAD database,

AKT3 and CDCA5 expression is associated with poor prognosis. The new data supporting these claims can be found in the revised manuscript in Figure S12 and in the text on page 30, line 607.

3. Although data supporting Akt3 involvement is presented, does down regulation of Akt1 or Akt2 impact on the splicing pathway proposed.

Re : We gratefully thank the reviewer for her/his important point. To further investigate the role of all the AKT isoforms in the regulation of *U2AF2* alternative RNA splicing, we transduced NCI-H522 and NCI-H1299 cells with shAKT1, shAKT2 and shAKT3 along with shControl. Furthermore, the results showed that inhibition of AKT1 and AKT3, but not AKT2, inhibit the inclusion of *U2AF2* exon 2. Notably, the effect of AKT1 inhibition was less pronounced, as expected, given that IWS1 is primarily phosphorylated by the AKT3 kinase, as we have previously reported (*Molecular cell*, 53(4), pp.577-590). The new data supporting these claims can be found in the revised manuscript in Figure 2C and Figure S2C and in the text on page 10, line 245.

4. The selective dependence on mutant EGFR NSCLC vs mutant RAS on Sororin expression is interesting. How does Sororin regulate ERK activity independent of EGFR? This is a significant gap in the model.

Re : We gratefully thank the reviewer for her/his crucial point. These results were very interesting for our group as well. Recently, we have provided insight on the molecular events that regulate ERK phosphorylation levels. Based on previous observations, Sororin as part of the cohesin complex, interacts in its C-terminal domain with another member of the cohesin complex, STAG2 (*Cell Cycle*, 14(6), pp.820-826.). Based on our recent results, the downregulation of Sororin upon the loss of the described AKT/p-IWS1 pathway leads to downregulation of STAG2, due to protein degradation. It has been previously reported that loss of STAG2 enhances CCCTC-binding factor (CTCF)-mediated expression of dual

specificity phosphatase 6 (DUSP6), leading to downregulation of ERK signaling (*International Journal of Molecular Sciences* 20, no. 11 (2019): 2668.). Based on these observations, we confirmed that the knockdown of IWS1, downregulates Sororin, leading to loss of STAG2 expression. The latter leads to upregulation of DUSP6 and downregulation of ERK phosphorylation. These data are currently under preparation for publication.

Reviewer #2 (Remarks to the Author) :

This manuscript presents a very solid experimental evidence for a new pathway that promotes proliferation and survival of *EGFR* mutated lung adenocarcinoma cells. This pathway starts with the AKT-mediated phosphorylation of IWS1, which then associates with Pol II CTD to trigger H3K36 trimethylation, thereby recruiting the reader LEDGF and splicing factor SRSF1 to *U2AF2* gene and RNA to induce inclusion of exon 2. In turn, the protein encoded by *U2AF2* mRNA with exon 2 regulates splicing of Sororin which establishes a pattern of phosphorylated ERK for the maintenance of transformed conditions. To support these claims, authors use effective knockdowns, RT-PCR, RNA sequencing, chromatin pulldowns and related methods, immunoprecipitations, cell cycle assays, plus mice xenografts and expression patterns from actual lung adenocarcinomas, for a very solid and data-rich support of their findings. I find the data of high clarity and quality and their conclusions worth publishing in Nature Communications, if the authors can satisfactorily address the following minor yet important comments:

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1. *The phospho-IWS1 U2AF2 splicing pathway in lung cancer adds to a previous pathway in which phospho-IWS1 regulates FGFR2 alternative splicing via PTB to influence tumor progression and metastasis. In the current case, phospho-IWS1 regulates U2AF2 splicing to affect transformation. My question here is, are most or all the effects of phospho-IWS1 on*

transformation mediated by U2AF2, or there are other events important for this cellular process. This could be tested by 'rescuing' sh-IWS1 and Mutant-rescue (MT-R) lung cancer cell lines with exon 2 containing U2AF2 cDNA, to see if the cellular and xenograft phenotypes are rescued by this isoform. In addition, are all U2AF2 effects dependent on Sororin? Again, this can be tested by rescuing sh-IWS1 and MT-R cells with additional Sororin. Either answer would be ok, or else strongly justify why these experiments were not done to start with.

Re : We gratefully thank the reviewer for his/her time to read our manuscript and propose highly important experiments. We also gratefully thank the reviewer for her/his crucial point. We sincerely apologize for not being able to provide these data in the first place. The unprecedented pandemic and lockdown influenced our ability to perform these experiments in the original version of the manuscript. Nevertheless, we provide them in the revised manuscript. To further address these questions, we rescued NCI-H522 and NCI-H1299 shIWS1 cells with Sororin wt, Sororin DM-E and Sororin DM-A and we monitored their proliferation in culture. The results showed that phosphorylated Sororin, a downstream target of U2AF65 α , is also a critical regulator of the IWS1-dependent cell proliferation in lung adenocarcinoma cell lines. The new data supporting these claims can be found in the revised manuscript in Figure 6A and in the text on page 22, line 532.

To also address the role of U2AF2 alternative RNA splicing in cell proliferation and cell cycle progression, we rescued NCI-H522, NCI-H1299, A549 and NCI-H1975 shIWS1 cells with U2AF65 α and U2AF65 β isoforms. Cell proliferation and FACS analysis showed that the shIWS1 phenotype on reduced cell proliferation and G2/M arrest was rescued by U2AF65 α , but not U2AF65 β , suggesting that these IWS1-dependent effects are mediated through U2AF2 alternative RNA splicing. The new data supporting these claims can be found in the revised manuscript in Figure 6B, 6D, 6E and Figure S9B and in the text on page 23, line 542.

To further demonstrate the role of *U2AF2* alternative RNA splicing in tumor growth, we repeated the mouse xenografts models by injecting NCI-H1299 cells transduced with shIWS1 and rescued with the two U2AF65 isoforms, U2AF65 α and U2AF65 β , along with shControl cells. The results showed that the reduced tumor volume in shIWS1-derived tumors was rescued in U2AF65 α , but not U2AF65 β -derived tumors, confirming the role of the novel *U2AF2* RNA splicing pattern on tumor growth. The new data supporting these claims can be found in the revised manuscript in Figure 7B and Figure S10A and in the text on page 26, line 623.

Finally, in parallel with the proposed experiments, we also performed tumor xenograft models in the *EGFR* mutated NCI-H1975 cells, by injecting shControl and shIWS1 cells in the flanks of NSG mice. Furthermore, we analysed the lysates from these tumors for the expression of p-ERK and PCNA (by Western blot) and Ki-67 (by IHC). Consistent with our *in vitro* data on the effect of the loss of IWS1 on p-ERK levels (**Fig. 5H**), cell proliferation and cycle progression (**Fig. 6**) and the effect of IWS1 on the clinical outcome of patients with *KRAS* and *EGFR* mutations (**Fig. 8**), the *EGFR* mutated lung adenocarcinoma cells were more sensitive to the loss of IWS1 compared to the *KRAS* mutated. The new data supporting these claims can be found in the revised manuscript in Figure 7A., 7C., 7E., 7F. and Figure S10B and in the text on page 26, line 620-656.

2. Similarly, while authors use the phosphorylation defective MT-R to rescue shIWS1, why not also use a phosphomimetic mutant? This would support the contribution of the charge in IWS1, which would make this mutant independent of AKT phosphorylation. Since authors use phosphomimetic mutant for Sororin (DM-E), why not for IWS1?

Re : We gratefully thank the reviewer for her/his crucial point. Based on this important comment, we engineered shIWS1/IWS1 phosphomimetic mutant S720D/T721E rescue (shIWS1/IWS1 DE MT-R) NCI-H522 and NCI-H1299 cells, which has been shown to be constitutive active, in our previous observations (*Molecular cell*, 53(4), pp.577-590).

Monitoring the results of this transduction by RT-PCR and qRT-PCR, we revealed that this mutant rescues the shIWS1-phenotype, leading to inclusion of *U2AF2* exon 2 in the mature transcript. To further support these claims, we also revealed that the same mutant rescues the shIWS1-phenotype on *FGFR2* alternative RNA splicing, leading to exclusion of exon IIIb, as we have previously described (*Molecular cell*, 53(4), pp.577-590). The new data supporting these claims can be found in the revised manuscript in Figure 1A, 1F and Figure S1E and in the text on page 8, line 208. The expression of the DE mutant in both the NCI-H522 and NCI-H1299 cells is shown in the revised figure 1A.

3. On page 6, first section of results, when authors describe the common differentially expressed genes, it should be emphasized that these genes change their expression in the same direction for the analogous comparisons (all increase or all decrease together).

Re : We gratefully thank the reviewer for her/his crucial point. This very important proposed change was incorporated in the revised manuscript.

4. Authors claim that the shorter U2AF65 derived from skipping of exon 2 by shIWS1 exhibits reduced interaction to the PRP19 complex. While the immunoprecipitation data look solid, a missing counter-test is to see the association of shorter U2AF2 (plus U2AF35 which is already blotted) with its more immediate partner which is U2 snRNP. Can authors blot the U2AF2 immunoprecipitation for a U2-specific protein? One would be enough. Also, the N-terminal arginine/serine rich domain is most commonly named RS domain (RS domain and SR proteins, not vice versa).

Re : We gratefully thank the reviewer for her/his crucial point. The same U2AF65 immunoprecipitates from the described NCI-H522 and NCI-H1299 cells, were probed with antibody against SF3B1, an U2 snRNP protein. The results showed interaction of U2AF65 and SF3B1, independent of the splicing pattern of U2AF65 and IWS1 phosphorylation. The new data supporting these claims can be found in the revised manuscript in Figure 5C and

Figure S7A and in the text on page 17, line 435. We apologize for this error in the nomenclature. The corrected nomenclature in both figures and text, can be found in the revised manuscript.

5. Legend of Figure 1C, authors refer to changes in splicing as “inclusion level >20%”, while this should read “increased inclusion level >20%”. I would strongly recommend them to adopt the PSI (percentage spliced in) nomenclature for measuring splicing inclusion levels.

Re : We gratefully thank the reviewer for her/his crucial point. We apologize for the inconvenience. We have now adopted the PSI nomenclature in Figure 1 and throughout the paper.

6. In Figure 3E, what is the significance of the abundant H3K36me3 peaks all along the U2AF2 gene?

Re : We gratefully thank the reviewer for her/his crucial point. Phosphorylation of IWS1 promotes the recruitment of SETD2 to the SPT6/IWS1/ALY-REF complex in the CTD of RNA Pol II. During transcription SETD2 recruited in this complex catalyzes the trimethylation of histone H3 at K36. Therefore, the detection of SETD2 and H3K36me3 marks in the body of a given gene, indicates that this gene is a target of this pathway. Based on these considerations, the detection of SETD2 and histone H3K36me3 peaks along the *U2AF2* gene, provides additional evidence that *U2AF2* is indeed one of the target genes of this pathway. The distribution of histone H3K36me3 peaks all along the *U2AF2* gene, is likely to reflect the involvement of these histone marks in the regulation of other RNA processing activities, in addition to RNA splicing. Our preliminary data (not part of this paper) suggest the involvement of these marks in 3' end cleavage and polyadenylation and RNA transport.

7. In Figure 4A, authors rule out a role of PTB in U2AF2 splicing, by the lack of changes upon PTB knockdown. However, in other cells, knockdown of PTB upregulates its paralog PTBP2 which partially rescues its functions. Could this be the case for U2AF2?

Re : We gratefully thank the reviewer for her/his crucial point. To address this question, we first examined the expression of PTBP2 in the NCI-H522 and NCI-H1299 cells, with no significant increase in these cells (Revised manuscript Fig. 4A). To further investigate the possible involvement of PTBP2 in the regulation of *U2AF2* alternative RNA splicing, we knocked down PTBP2 using lentiviral shRNA construct. By using quantitative RT-PCR, the results showed that the regulation of *U2AF2* RNA splicing is independent of PTBP2, with an expected increase of *FGFR2* IIIb/IIIc ratio (*Sci Rep* 6, 20665-2016). The new data supporting these claims can be found in the revised manuscript in Figure S5B and in the text on page 13, line 335.

8. Last, some of the font in Figure 8 is too small and should be enlarged.

Re : We gratefully thank the reviewer for her/his crucial point. We apologize for the inconvenience. We tried to fix this issue as much as it was possible. The corrected Figure 8 can be found in the revised manuscript.

Reviewer #3 (Remarks to the Author) :

This manuscript describes a series of experiments that follow up the recent discovery that IWS1 regulates alternative splicing of the non-small lung cancer gene *FGFR2*. The authors initiate their studies by IWS1 knockdown RNA-seq approaches in lung adenocarcinoma cell lines and identify a new alternative splicing event in *U2AF2*, an essential splicing factor. The skipping of *U2AF2* exon 2 leads to the loss of its RS domain, which is required to coordinate spliceosomal assembly. Thus, *U2AF2* exon 2 skipping will have profound effects of RNA splicing. The authors describe a series of logical experiments to show that the phosphorylated form of IWS1 modulates H3K36 methylation to increase the recruitment of LEDGF and the splicing factor SRSF1 to the nascent RNA, thereby increasing *U2AF* exon 2 inclusion.

Interestingly, alternative splicing of U2AF is cell cycle dependent supporting its regulatory role. To investigate the biological significance of these findings the authors evaluate the correlation between IWS1 expression/U2AF alternative splicing and tumor growth and between IWS1 expression/*U2AF2* alternative splicing and patient survival. While the mouse xenograft data are supportive of IWS1 expression/*U2AF2* alternative splicing's importance in tumor growth, the patient survival data are less impressive, certainly not providing much improved predictions over the previously reported *FGFR2* alternative splicing. Overall, the data presented are clean and they make a good case for the proposed mechanistic connections between IWS1 phosphorylation, *U2AF2* alternative splicing and tumor growth.

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1) The authors concentrate their discussion of alternative U2AF2 splicing on exon 2, providing evidence for IWS1, SETD2, SRSF1 association using Chip assays (Figs 1, 3, 4). Interestingly, the data also demonstrate that the same pattern of factor recruitment is seen for exon 3. Thus, it appears that the inclusion of U2AF2 exon 3 should also be under the control of the AKT3 axis. In their analysis the authors do not discuss this possibility and their evaluation will not bring this potential added control to light because the primer pairs used to evaluate U2AF2 splicing are biased to detect exon 2 inclusion only.

Re : We gratefully thank the reviewer for his/her time to read our manuscript and propose highly important experiments. We also gratefully thank the reviewer for her/his crucial point. In order to address this question, we designed primers flanking *U2AF2* Exon 3 and Exon 5. By using this primer design, if the exon 3 is alternatively spliced by the AKT/IWS1 axis and excluded upon inhibition of the axis, the forward primer (for exon 3) will not align the designed sequence in shIWS1 and shIWS1/MT-R cells. RT-PCR experiments in NCI-H522 and NCI-H1299 showed that independent of IWS1 phosphorylation, exon 3 is constitutively spliced in the *U2AF2* mRNA. Notably, the constitutively active IWS1 mutant S720D/T721E

(shIWS1/IWS1 DE MT-R), had no effect in the inclusion of *U2AF2* exon 3. Furthermore, we utilized quantitative RT-PCR to measure the ratio of exon 3-containing/total *U2AF2* RNA transcripts in shIWS1, WT-R and MT-R NCI H522 and NCI-H1299 cells, relative to shControl. The results revealed that the knockdown of IWS1 and its replacement by the phosphorylation site IWS1 mutant do not affect the abundance of exon 3-containing *U2AF2* RNA transcripts. We conclude that the inclusion of exon 3 in the *U2AF2* mRNA is not regulated by IWS1 expression and/or phosphorylation. The new data supporting these claims can be found in the revised manuscript in Figure 1F and in the text on page 9, line 219.

2) U2AF2 is a general splicing factor involved in the initial assembly of the spliceosome. Losing its RS domain through exon 2 exclusion is expected to affect a large number of intron removal events. It is not clear from the data and from the discussion which differential splicing events are the consequence of direct or indirect effects. Presumably, exons that harbor SRSF1 binding sites could be direct targets, but essentially all other introns should be affected by the temporary loss of U2AF2. In other words, what is unique about the exons affected by U2AF2 alternative splicing (CDCA5), or why does GAPDH splice without apparent defects?

Re : We gratefully thank the reviewer for her/his crucial point. The reviewer is raising a very important question, which is not yet fully addressed. One set of genes whose RNA splicing may be affected by the loss of phosphorylated IWS1 may be genes with SRSF1 binding sites. In Figure S1 of this paper, we present validation data for the alternative RNA splicing of 4 additional genes in which IWS1 phosphorylation promotes exon inclusion in their mature mRNAs (*SLC12A2*, *IFT88*, *STXBP1*, *C1qTNF6*). Data presented in figures S6F, S6G, S6H and S6I and discussed in page 16, line 408 of the revised manuscript, show that all these genes contain SRSF1 binding motifs in the alternatively spliced exon and/or exons flanking the alternatively spliced one.

Moreover, these gene-selective results were very interesting for our group as well. U2AF65 is indeed a general splicing factor affecting the branchpoint assembly. However, earlier reports had shown that, although the effects may be global they are not universal, affecting some but not all the genes. Specifically, earlier observations on the regulation of *CDCA5* pre-mRNA, revealed that components of Prp19 Complex (Prp19 and Cdc5L) along with U2AF65, affected the intron removal of *CDCA5* pre-mRNA, but not the removal of intronic regions in other genes, such as *GUSB*, *ACTB* (β -actin gene) and *COIL* (p80-coilin gene) (EMBO Rep. 2014 Sep;15(9):948-55.), demonstrating a selective effect of the U2AF65-Prp19 on the intronic regions of target genes. To further support these results, other reports have shown that during DNA damage U2AF65 and Prp19 regulate the proper splicing and expression of *p21^{CIP1}*, but not *PUMA* and *NOXA* (*Genes & development*, 25(7), pp.701-716.) Consistently, our RIP data demonstrate the binding of Prp19 in the intronic areas of *CDCA5*, but not *GUSB*, when U2AF65 α (exon 2 containing isoform) is expressed, further supporting the selectivity of the latter complex on the target genes. Collectively, these data suggest that the removal of RS domain of U2AF65, may demonstrate a gene-specific indirect effect on intron removal events, through the regulation and recruitment of Prp19 on the target introns. The genome-wide distribution of U2AF65 and Prp19 upon loss of phosphorylated IWS1 and RS domain exclusion, will be addressed in future studies. This very important point suggested by the reviewer about the indirect and direct effects of U2AF65 spliced isoforms, has been included in the discussion section in the revised manuscript in page 33, line 827.

3) *It is unclear why the authors do not show how the manipulations carried out affect their previously identified FGFR2 alternative splicing. This data should be added, especially in light of the fact that the FGFR2 splicing parameter is a better survival predictor in the TCGA data analysis (Fig 8).*

Re : We gratefully thank the reviewer for her/his crucial point. To this extent, we have included in parallel qRT-PCR and ChIP experiments throughout Figure 1, Figure 2 (IGF/AKT axis), Figure 3 (The role of H3K36me3 and SETD2), Figure 4 (LEDGF/SRSF1 axis) the effect of these genetic manipulations and treatments in the regulation of *FGFR2* alternative RNA splicing. The new data supporting these claims, can be found in the revised manuscript in Figure S1E, Figure S2, Figure S3, Figure S5A, S5B, S5E, Figure S6A.

4) Figs 6, 6S and 7 – several panels are labeled % change, but the scale is fractions.

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REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have adequately addressed my concerns.

Reviewer #2 (Remarks to the Author):

All ok

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The authors return an extensively revised manuscript that addresses all points raised in the initial review. Overall, the added data and discussions further strengthen the proposed model. Recommend acceptance of the manuscript.

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Reviewer #3 (Remarks to the Author) :

The authors return an extensively revised manuscript that addresses all points raised in the initial review. Overall, the added data and discussions further strengthen the proposed model. Recommend acceptance of the manuscript.

Re : We collectively thank the reviewers for their overall evaluation, time and help through the peer-review process.