

O-GlcNAcylation of FOXK1 co-opts BAP1 to orchestrate the E2F pathway and promote oncogenesis

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In the manuscript titled "O-GlcNAcylation of FOXK1 orchestrates the E2F pathway and promotes oncogenesis," the authors reported the oncogenic role of FOXK, a subunit of the BAP1 complex, in facilitating cell transformation. Additionally, demonstrated that FOXK1 is highly expressed in patient samples and correlated with E2F targets. Importantly, they demonstrated that FOXK1 could be O-Glycosylated which promotes BAP1 recruitment to chromatin. The overall experiments were well-designed, and the results were convincing. However, I have a few concerns that should be addressed by the authors.

Specific comments

- 1) A primary concern is the potential presence of FOXK1 in other epigenetic complexes, such as the SIN3A complex. The authors should deplete BAP1 in FOXK1 over-expressing cells to see if the gene expression and other phenotypes could be rescued.
- 2) In Figure 3, the authors need to validate the specificity of the FOXK1/2 antibodies in ChIP-seq, particularly due to the notable overlap between FOXK1 and FOXK2 peaks.
- 3) It is established that FOXK1 and FOXK2 are mutually exclusive in the BAP1 complex. If the proposed model is true, overexpressing FOXK2 should diminish the BAP1-FOXK1 interaction, thereby suppressing cell transformation and tumor growth. Have the authors observed this phenomenon?
- 4) The oncogenic functions of BAP1 and its sub-complexes have been elucidated in various cancer types such as leukemia and lung cancer. The authors should reference these studies and delve deeper into the potential of targeting BAP1 in FOXK1-induced malignant transformation.

Reviewer #2

(Remarks to the Author)

In this manuscript, the authors investigate the role of FOXK1 in cell proliferation and its interplay with the E2F transcription factor. The role of FOXK1 in cancer and proliferation is well established. The authors show that FOXK1 is modified by O-GlcNAcylation, and while this is an interesting observation, the study does not convincingly show a functional role for this modification in cell cycle control or regulation of FOXK1 target genes. As such, the paper does not convincingly support the authors' major conclusions.

Major points

1. In Fig. 2, the authors state the number of E2F target genes regulated by FOXK1, including analysis of "the 200 E2F target genes." The number of target genes vastly exceeds this number, and it is not clear how the authors arrived at this group of genes. More importantly, these genes may be regulated as a consequence of cell cycle perturbations caused by over-expression of FOXK1 rather than by FOXK1 directly, and further functional studies are lacking. The existence of a binding

site by ChIP-seq does not guarantee a functional impact.

2. FOXK1 and FOXK2 bind to nearly identical regions (Fig. 3), yet the authors make the claim that the two factors function differently, namely, that the former activates E2F target genes, but the latter does not. The authors ascribe this to O-GlcNAcylation, which is regulated both by cell cycle and metabolic cues, which the authors have not distinguished in this study. Both cell cycle state and metabolism is altered by the perturbations used to synchronize cells.

3. Major questions remain about the FOXK1-11A mutant used in key studies in the paper. For example, it is not known whether the structure and activity of this protein is altered by the mutations. Does the mutant protein bind to the genome normally and recapitulate the chromatin modifications seen with the wild-type protein? According to Fig. 6 and extended Fig. 8, there are no obvious differences between the proteins. Does the mutant normally regulate target genes, and does it directly recruit BAP and OGT? In short, it is hard to determine the mechanistic impact of the FOXK1-11A mutation on gene regulation. Importantly, Fig. 6e, shows a quantitative reduction in binding of the mutant to the genome, and this could reflect the modest diminution of other histone marks in a subsequent figure panel (fig. 6g). Altogether, this series of key experiments falls short of presenting a convincing case for the mechanistic defects associated with loss of the O-GlcNAcylation modification on FOXK1.

4. Another major shortcoming of the study is that nearly all experiments rely on over-expression of FOXK1 and other proteins. Another drawback is that multiple, unrelated normal and cancer cell lines (IMR90, U2OS, K562, HLF, 3T3L1, PC3, HCT116) are used throughout the study, rather than using a single line to systematically perform all experiments.

Reviewer #3

(Remarks to the Author)

Mascello et al.

O-GlcNAcylation of FOXK1 orchestrates the E2F pathway and promotes oncogenesis

This manuscript explored the nuanced differences between FOXK1 and FOXK2 transcription factors in cancer. FOXK1 expression alone correlated with increased proliferation, delayed senescence, and tumor initiation in transformed fibroblast cells. FOXK, but not FOXK2, overexpression promotes increased proliferation of non-transformed fibroblasts and can increase E2F1 gene expression and tumor growth in vivo when combined with known oncogenes. Authors show that FOXK1, but not FOXK2, is O-GlcNAcylated and mutating eleven O-GlcNAc sites reduced FOXK1 O-GlcNAcylation and reduced effect on proliferation and tumor growth in vivo. Lastly, authors show that FOXK1 O-GlcNAcylation regulates BAP1 recruitment to E2F target gene promoters.

Noteworthy: This study identified a novel regulation of E2F1-regulated genes by FOXK1. It also identifies FOXK1 as an O-GlcNAcylated protein.

Not clear if work will be of significance: FOXK1 and FOXK2 are both known to have tumor promoting activity. Also, its relevance in cancer and oncogenesis is not well studied here as nearly all experiments were performed in fibroblast cells. In addition, many of the main conclusions are not well supported by the data.

Possible flaws: Also, major model to compare difference between FOXK1 vs FOXK2 is problematic since model used overexpressed FOXK1 nearly 5 fold over FOXK2 in fibroblasts.

Major Points

1. Figure 1: Need to show expression levels between FOXK1 vs FOXK2 via western blot and to also compare overexpression to normal levels (see issue below).
2. Figure 2F: IMR90 cells used here (also used in Fig. 1?) have very high amounts of FOXK1 levels compared to FOXK2 levels. This could mean the changes in phenotypes seen in Fig. 1 and gene expression in Fig. 2 could be result of nearly 4-5 fold higher levels of FOXK1. Authors should overexpress similar levels of FOXK1 and FOXK2 and examine effects on cell phenotypes & E2F1 genes to support conclusion of differential effects of these transcription factors.
3. Line 77 & 124 authors conclude FOXK1 “..constitutes a potent oncogene.”. Data presented shows FOXK1 is a potent tumor promotor in fibroblasts. Authors would need to add more data to support conclusion or alter conclusion.
4. Figure 4, 5 & 6 was nearly all done in fibroblasts and thus its relevance to cancer and oncogenesis is preliminary and needs to be verified in cancer cells.
5. The data linking FOXK1 O-GlcNAcylation to BAP1 recruitment was not very convincing. There is no functional data showing BAP1 play a role in FOXK1-regulation of E2F1 regulated genes and that FOXK1 O-GlcNAcylation play a role.

Minor Points

1. Fig 1D: Fox2 graph does not look like 59%, looks way lower than the control 60%
2. Fig 1I: Statistical significance? Explain how HDM2/FOXK1 seemed sufficient for growth, but this was not seen with the addition of the third oncogenic factor RASG12V.
3. Figure 1N image should be a representative of Figure 1M. These figures not well labeled thus not clear what is being shown.
4. Figure 2D: Vector1 seemingly has significantly different E2F target expression (authors should explain).
5. Figure 2G: Not clear what type of cancer is being evaluated. Just solid tumors? All cancers?
6. Line 162 & 164 figures are mislabeled in text.
7. Figure 3C,F : reads mTROC1. Is this mTORC1?
8. Figure 3D: the dotted lines do not directly align with columns – slightly confusing to interpret.

9. Figure 4A-D: Consider putting (-) signs in for clarity, and aligning the (+) signs
10. Figure 4C: Input FOXK1 expression decreased upon OSMI-4, interferes with IP interpretation.
11. Figure 4F: Fraction F and E are either switched or mislabeled on the graph
12. Figure 4H: Label C and N terminus
13. Figure 5B: difficult to determine the alignment of the statistical markers, esp on the "10 days" column
14. Figure 5D: Appears to be n=2 for FOXK111A, based on number of squares visible and lack of statistical markers
15. Figure 5I: consider looking at overall O-GlcNAcylation levels within the transformed cells.

Reviewer #4

(Remarks to the Author)

1. Overview and General Comments

The manuscript submitted by Affar and colleagues investigates the oncogenic properties observed in the FOXK1 transcription factor but not in FOXK2. The increased expression of FOXK1 is associated with the activation of E2F target genes. The study suggests a connection between FOXK1's oncogenic traits and its modification by O-GlcNAcylation. FOXK1 mutants lacking O-GlcNAcylation exhibit a reduced oncogenic capacity and decreased expression of E2F pathway genes. The glycosylation-impaired FOXK1 variants show decreased association with BAP-1 compared to their glycosylated counterparts. Furthermore, the glycosylation-impaired FOXK1 exhibits elevated histone H2AK119ub and reduced H3K4m1, leading to a shift towards a transcriptionally repressive state and a reduction in oncogenic properties.

While the manuscript is technically sound and presents novel findings, certain areas require clarification for a better understanding of the implications of these findings.

2. Key Observations and Findings

FOXK1's role as an oncogene has been previously reported, and this study provides additional experimental evidence showing that it promotes cell growth and tumorigenesis.

This study reports the molecular mechanisms underpinning FOXK1's function, illustrating its association with the E2F pathway and the critical role of O-GlcNAcylation in modulating FOXK1's oncogenic capacity.

O-GlcNAcylation is associated with BAP-1 recruitment to target genes. The tumor suppressor BAP-1 seems to stimulate the oncogenic properties of FOXK1.

3. Strengths

The manuscript is well-structured, offering extensive experimental data and its significance in the context of cancer biology. The link of FOXK1 to E2F pathways and the identification of O-GlcNAcylation as a key post-translational modification influencing FOXK1's activity add a valuable dimension to the current understanding of transcription factor regulation in oncogenesis.

4. Areas for Improvement and clarification

In Figure 1i, when comparing FOXK1/HDM2 and FOXK1/HDM2/RASG12V, FOXK1/HDM2/RASG12V shows no growth stimulation. There is no discussion on this variance.

In Figure 1n, the negative control should be E1A+RASG12V+Vector instead of Vector.

In Figure 2f, the protein expression level of FOXK2 is significantly lower than FOXK1, albeit its high RNA level is reported in Extended Figure 2b. This makes it difficult to determine if the observed elevation of E2F1, CDC6, and cyclin A levels with FOXK1 overexpression is specific to FOXK1 or can be attributed to variations in protein expression.

Figure 4b, align the gel figure + signs.

P9, line 204: catalytically dead form of OGT data are not shown?

P9, line 207: OGA is not defined previously.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors conducted sufficient experiments and have addressed most of my concerns.

Reviewer #2

(Remarks to the Author)

The authors have largely responded adequately to my concerns with new experiments.

However, the Palbociclib and HiC experiments do not substantively add to the conclusions. They are add-ons that should be removed from the manuscript.

Reviewer #3

(Remarks to the Author)

Authors have addressed all major & minor issues.

Manuscript is acceptable for publication.

Reviewer #4

(Remarks to the Author)

The revised manuscript submitted by Affar and colleagues addresses the reviewers' comments and incorporates additional data to strengthen the conclusions.

The study provides substantial evidence linking the well-established oncogenic properties of FOXK1 to E2F pathways. While this association is not unexpected given the oncogenic nature of FOXK1, the findings strengthen this connection with additional data. Moreover, the study identifies O-GlcNAcylation as a key post-translational modification that regulates FOXK1's activity and mediates its association with BAP-1. The newly added Figure 7A highlights the direct link between FOXK1 glycosylation and its association with BAP-1.

One of this reviewer's comments was on the difference in expression levels between FOXK1 and FOXK2. This issue has been addressed by incorporating half-titer expression vectors where applicable in the revised manuscript.

Regarding concerns about the cross-reactivity of FOXK1 and FOXK2 antibodies, Supplementary Figures 4a, b, and c demonstrate the specificity of the FOXK1 antibody to FOXK1 and the FOXK2 antibody to FOXK2. However, since the blot does not display both molecular weight ranges for the FOXK1 and FOXK2 for each probe, it remains difficult to fully assess potential cross-reactivity between endogenous FOXK1 and FOXK2. Including both molecular weight ranges in the blot would help resolve this issue and effectively rule out cross-reactivity concerns.

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REBUTTAL

We sincerely thank the reviewers and the editor for their valuable comments and suggestions. To further strengthen our data and conclusions, we have conducted additional studies as detailed in the rebuttal below. We have addressed the majority of the comments raised and provided a thorough response to each point. We believe that, as a result, our study has been significantly improved and now represents a substantial and novel contribution to our understanding of transcription regulation, the E2F pathway and oncogenesis.

Please note that, in formulating our responses, we have used the following format: Common reviewers comments outlined by the Editor (E) and Specific reviewers' comments for each of the four reviewers (R1, R2, R3, R4). Accordingly, responses are labeled as Response E-1, E-2, etc. (for the editor and all reviewers) and Responses R1-1, R1-2, R2-1, R2-2, etc. (for the specific comments of each reviewer).

(COMMON REVIEWERS COMMENTS)

1. Experimentally validate the functional role of FOXK1 O-GlcNAcylation in regulating E2F target genes via recruiting BAP1 (Reviewers #1,2,3):

Response E-1: We carefully considered the concerns regarding the functional link between FOXK1 O-GlcNAcylation and BAP1 in the regulation of E2F target genes, and have performed additional experiments to address this comment. We initially showed that expression of wild-type FOXK1, but not the O-GlcNAcylation defective mutant, FOXK1^{11A}, promotes the recruitment of BAP1 at enhancers and promoters. Specifically, we identified 2,130 regions with reduced BAP1 recruitment in cells expressing the O-GlcNAcylation-defective mutant compared to wild-type FOXK1 (**Figure 7f**). Gene ontology indicated that E2F target genes are the preeminent gene expression program that show differential recruitment of BAP1 depending on FOXK1 O-GlcNAcylation state (**Figure 7g**). To further consolidate our results, we performed in vitro interaction assays and now report that FOXK1 O-GlcNAcylation promotes its interaction with BAP1, but not with SIN3A, NCOR1, or HDAC3, known repressors and partners of FOXK1 (**new Figure 7e**). We also performed additional genome occupancy (Cut&Run) experiments for BAP1 in U2OS cells and found that the recruitment of BAP1 is decreased, at the same enhancers and promoters that regulate E2F target genes, following expression of the FOXK1 O-GlcNAc defective mutant (**new Figure S11a**). Thus, in both IMR90 and U2OS cells, BAP1 recruitment is decreased in FOXK1 O-GlcNAcylation-defective mutant versus the wild type form. Moreover, the recruitment of SIN3A in IMR90 cells remained similar between the wild-type and the O-GlcNAc-defective mutant form of FOXK1 (**new Figure S11b**). To further link FOXK1 O-GlcNAcylation to BAP1 function in regulating cell proliferation, we also expressed FOXK1 wild type and FOXK1 lacking the FHA domain (FOXK1^{ΔFHA}), which is responsible for its interaction with BAP1. We found that expression of FOXK1^{ΔFHA} does not enhance cell proliferation. Instead, this mutant appears to even reduce cell proliferation, suggesting a possible dominant negative effect (**new Figure 8b**). We also expressed a FOXK1 mutant that does not interact with DNA (FOXK1^{H355A}), and while this mutant is relatively stable comparatively to other forms of FOXK1, it did not increase cell proliferation. Instead, expression of this mutant also reduced cell proliferation possibly acting in a

dominant negative manner (**new Figure 8b**). Next, we expressed FOXK1 in U2OS cells with a stable knockdown of BAP1 to determine its impact on cell proliferation in the absence of BAP1. As expected, BAP1-depleted cells grow slowly comparatively to cells expressing a control shRNA (**new Figure 8c,d**). We found that FOXK1 overexpression does not enhance cell proliferation in BAP1-depleted cells comparatively to control cells. Of note, FOXK2 was also included as a control, and despite its expression levels, similar to those of FOXK1, it did not affect U2OS cell proliferation. We also re-examined our previously published microarray data in U2OS depleted in BAP1¹. BAP1 depletion results in the downregulation of a significant number of E2F target genes. Gene set enrichment analysis indicated a significant enrichment of FOXK1 on promoters of genes regulated by BAP1 compared to control cells (**new Figure 8e,f,g**). Among the 308 genes that were downregulated (at least two-fold) following BAP1 depletion, we found 43 genes belonging to the E2F target gene family. Moreover, FOXK1 was found enriched on 33 of these promoters as well as on their distal regions (**new Figure 8g**).

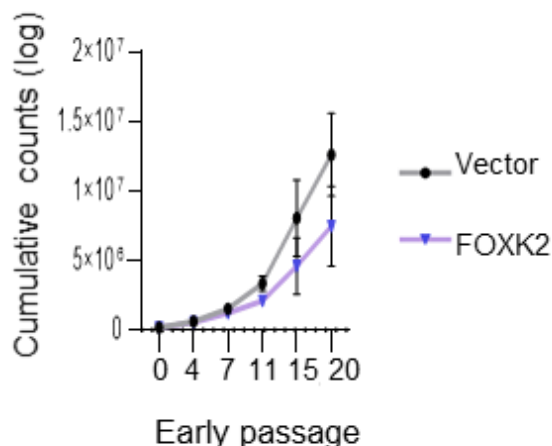
To provide additional evidence for the relevance of BAP1 and FOXK1 interaction in cancer, we analysed the impact of FOXK1 overexpression in a series of pleural mesothelioma and lung cancer cell lines that are BAP1-proficient or BAP1-deficient. BAP1 being highly mutated in mesothelioma cancers. We found that expression of FOXK1, but not its O-GlcNAcylation defective mutant, increases cell proliferation in the majority of BAP1 expressing cells, but not BAP1-deficient cells (**new Figure 8h and Figure S12**). These results are extremely interesting as the presence of the non-mutated form of BAP1 is associated with a poor prognosis in mesothelioma^{2,3}. Finally, we also explored the cancer cell genetic dependency map, which compiles genome-wide knockout data for genes essential for cancer cell proliferation, and found that cells depending on FOXK1 expression for survival show a dependence for BAP1 as well, whereas no dependence for FOXK2 expression was observed in these cell lines (**Figure 8a**).

Taken altogether, these results confirm the functional role of FOXK1 O-GlcNAcylation in regulating E2F target genes through BAP1 recruitment and strengthen the link of this pathway with cancer.

2. Address the concerns on proteins over-expression (Reviewers #2,3):

Response E-2: We acknowledge the concerns regarding potential issues with protein overexpression and have included additional data to justify that the effects of FOXK1 and FOXK2 could be compared, and that only FOXK1 promotes cell proliferation (**new Figure S2a,b**) and (**new Figure S3a**).

We have initially observed that in IMR90 cells, FOXK2 expression become occasionally lower comparatively to that of FOXK1, particularly under conditions of prolonged cell culture, as we showed in **Figure 2f**. However, we note that FOXK1 or FOXK2 overexpressing cells show generally about four-fold or a ten-fold increase, respectively, of their expression levels relative to



their corresponding endogenous proteins (**new Figure S2a**). Thus, FOXK2 is even more overexpressed relative to its endogenous form than FOXK1. Interestingly, we also observed that there is a slight negative effect of FOXK2 expression on IMR90 cell proliferation, likely resulting in the protracted selection overtime of cells with reduced FOXK2 expression. In fact, when we express FOXK1 or FOXK2 using early passage IMR90 cells, we indeed noted that FOXK2 reduces slightly cell proliferation (**Figure at the left, not included in the manuscript**). This is also consistent with our

results showing that FOXK2, but not FOXK1, increases senescence-associated PML bodies (**Figure 1g**). Moreover, expression of FOXK2, but not FOXK1, induces the expression of the cell cycle inhibitor p21 (**Figure 2e,f**). Of note, we also repeated our viral transduction experiments by titrating down the FOXK1 viral suspension. We noted that even with similar protein expression levels observed at the end of the experiment, expression of FOXK1, but not FOXK2, promotes cell proliferation (**new Figure S2b**).

On the other hand, we harvested the cells for the RNA-seq experiments immediately after viral transduction and antibiotic selection, ensuring that FOXK1 and FOXK2 are expressed at similar protein levels (**new Figure S3a**). The protein expression levels are also corroborated with the RNA-seq data (**new Figure S3a,b**). We also note that the levels of FOXK1 and FOXK2 in our overexpression system are comparable to those found in cancer (**Figure S3c**). Under these conditions, our RNA-seq experiments indicate that, while FOXK1 promotes cell cycle genes including E2F targets, FOXK2 induces other gene expression programs that are associated with differentiation, and even, genes that negatively regulate cell cycle, including the p53 pathway (**Figure S3f**).

In U2OS cells, the second cell type extensively used in our studies, expression of FOXK1, but not FOXK2 promotes cell proliferation, and western blot analysis confirmed that the expression levels of overexpressed FOXK1 and FOXK2 are very comparable (**new Figure 8c,d**).

Finally, we improved some panels by reloading samples (Flag and tubulin blot for **Figure 2f**, FOXK1 IP and FOXK1 O-GlcNAc levels in IP, tubulin blot **Figure 5j** and **Figure 6i**). We have also updated the visual representation of **Figure 7f** and **Figure S10d,e**.

3. Improve the consistency in the use of cell lines (Reviewer #2) and strengthen the relevance to cancer (Reviewer #3):

Response E-3: We acknowledge that the use of multiple cell lines might seem somewhat ambiguous. However, our approach is intentionally designed to validate, as much as possible, our findings across diverse cell types for broader reproducibility and reliability. In addition, we always strive to include male and female cells. We would like to emphasize that FOXK1 is known to have an ubiquitous expression and FOXK1 is amplified or overexpressed in several cancers of various tissue origins. Thus, we initially sought to demonstrate the relevance of FOXK1 overexpression in the context of cancer cell models of diverse tissue and sex origin. However, as we progressed on the mechanistic characterization of O-GlcNAcylation, we also expanded our studies to include additional cell types in order to address specific questions. For instance, we used IMR90, a widely used cell model to study senescence and oncogenic transformation. IMR90 and K562 are widely used for genomic studies and existing data have been initially exploited to interpret our studies. IMR90 and HLF are two models of normal human fibroblasts that can be easily synchronized by contact inhibitions. 3T3L1 can robustly exit cell cycle and differentiate providing a robust model to analyse FOXK1 O-GlcNAcylation in cycling versus differentiated cells. It also allowed us to conclude that FOXK1 O-GlcNAcylation also occurs in mouse cells. During the revisions, while we consolidated our data mostly on IMR90 and U2OS cell lines, we also included mesothelioma cell lines, as this cancer type is characterized by a high frequency of BAP1 mutations.

Overall, our experiments showed that FOXK1 overexpression leads to increased cell proliferation and tumor progression through its O-GlcNAcylation, confirming its oncogenic role across different cancer types. We emphasize that the main figures and data throughout the paper are primarily derived from IMR90 and U2OS cell lines. Additional information from other cell lines has been mostly included in the supplemental data.

To further emphasize the relevance to cancer, we expanded our study on the interaction between FOXK1 and BAP1 both in vitro and in cells (**new Figure 7e and new Figure 8**, Please see also **Response E-1** above).

4. Clarify the differential roles of FOXK1 and FOXK2 (All Reviewers):

Response E-4: We performed additional experiments and analysis to further clarify this aspect. We have showed in the initial version that FOXK1, but not FOXK2, promotes cell proliferation and delays cell senescence (**Figure 1**). FOXK1, but not FOXK2 is O-GlcNAcylated by OGT, and FOXK1 O-GlcNAc-defective mutant have reduced capacities in promoting cell proliferation and tumor progression (**Figure 5, 6**). Global gene expression analysis showed that only FOXK1, but not FOXK2, significantly upregulates E2F target genes (**Figure 2 and Figure S3**). FOXK2 is associated with genes involved in the epithelial mesenchymal transition stress responses, and negative cell cycle regulation (**Figure S3**). As indicated above, we ensured that the differential effect of FOXK1 and FOXK2 is not due to differences in protein expression (**Response E2**). Thus, at the functional level, expression of these two factors result in different outcomes. Genome occupancy studies indicated that FOXK1 and FOXK2 occupy similar gene regulatory regions, suggesting that their functional impact might be dictated by the chromatin context and protein interactions (**Figure 3**). To further investigate this, we performed ChIP followed by ReChIP of FOXK1 and FOXK2 and found that these factors do not simultaneously occupy the same DNA regulatory regions (**new Figure 3g, h**). Taking into account that similar genome binding patterns were observed on unsynchronized cells, we asked whether a differential recruitment of FOXK1 versus FOXK2, not seen on asynchronous cells, could be observed as a function of cell cycle. We synchronized cells in G0 and G1/S, and performed Cut&Run genome occupancy and found that FOXK1 recruitment is strongly induced relatively to FOXK2 at the onset of G1/S transition. However, FOXK2 binding was stronger than that of FOXK1 in G0 (**new Figure 3i, j**). We note that FOXK1-O-GlcNAcylation is also maximal at the G1/S cell cycle transition (**Figure 5e, Figure S6c, d**). On the other hand, we selected the set of differentially expressed genes between FOXK1 and FOXK2 and transposed their promoters and enhancers binding sites to previous HiC 3D chromatin data obtained in IMR90. Remarkably, we can observe a very significant promoter-enhancer interaction for differentially expressed genes (FOXK1-associated) that we do not observe for non-differentially expressed genes (**new Figure 4d, e**). These differences altogether demonstrate the distinctive regulatory and oncogenic roles of FOXK1 compared to FOXK2. Our results are in agreement with others also showing distinct functional roles of FOXK1 and FOXK2, and we have expanded the discussion to better cover this aspect.

~~On the other hand, while part of another manuscript in preparation, we are also providing additional genetic data to support the functional link between FOXK1 and BAP1 in the regulation of the E2F pathway. We generated a mouse model by mutating the single threonine 492 of BAP1 (T493 in human) to inhibit its phosphorylation thereby disrupting the interaction between BAP1 and FOXK1/2 and studying the resulting phenotypic and molecular defects in vivo (**Figure immediately below**). Interestingly, our results show that homozygous *Bap1*^{T492A/T492A} mice exhibit partial embryonic lethality (**panel b**). We found that most *Bap1*^{T492A/T492A} embryos exhibit a developmental retardation that can be clearly observed at E16.5 (**panel c**). Moreover, few *Bap1*^{T492A/T492A} mice escape the embryonic lethality barrier (representing only 4.7 % of the 25% expected ratio). These mice are remarkably small and show developmental abnormalities at adult age (**panels c and d**). Genome-wide recruitment of BAP1 in murine embryonic fibroblast (MEFs) (Cut&Run analysis) revealed that BAP1 recruitment to some genomic regions is abrogated (**panel f**). Gene ontology analysis indicated that promoters showing differential recruitment of BAP1 are~~

important for mouse viability (**panel g left**) and the E2F pathway (**panel g right**). Interestingly, *Bap1*^{T492A/T492A} phenotypes are strikingly similar to those of FOXK1 KO mice, which are also partially embryonic lethal, with only 6% surviving beyond embryogenesis. The viable FOXK1 KO animals are also smaller and show cell proliferation defects⁴. In contrast, FOXK2 KO mice do not display any reported phenotype (<https://www.mousephenotype.org/data/genes/MGI:1916087>). These observations strongly support our conclusions on the role of BAP1-FOXK1 interaction in the regulation of cell cycle progression and the E2F pathway.

[FIGURE REDACTED]

Figure for review only : BAP1-FOXK1/2 interaction mutant mouse model exhibits embryonic lethality and developmental defects reminiscent of FOXK1 KO.

a) Schematic representation of the CRISPR/Cas9 *Bap1*^{T492A} knocking mice model strategy. **b)** Percentage of distribution of offspring genotypes from heterozygous crosses. **c)** Representative comparative photographs between wild type and homozygous embryos at E16.5 as well as young and old adult mice. **d)** Comparison of body weight between wild type and *Bap1*^{T492A} mutant mice. The asterisks represent the statistical significance determined by a two-way ANOVA test. **e)** Immunoprecipitation of endogenous BAP1 from either wild type or *Bap1*^{T492A} liver of adult animal to validate disruption of interaction between BAP1 and FOXK1/2 factors. **f)** Cut&Run analysis of BAP1 performed on MEFs extracted from wild type and *Bap1*^{T492A} embryos at E13.5 to examine BAP1 recruitment to its target genes in the absence of its interaction with FOXK1/2. **g)** Gene ontology analysis performed on promoters associated with the loss of BAP1 recruitment to chromatin in *Bap1*^{T492A} MEFs

Sex and gender considerations in our study.

Due to limited funding, we were unable to systematically test sex as a variable across all experimental conditions. However, we have made every effort to incorporate sex considerations into our study. Specifically, we ensured the inclusion of cells derived from both males and females wherever possible, and mouse xenograft experiments were conducted using both male and female animals. These steps were taken to increase the applicability of our findings despite the resource constraints. In our analysis of human cancers, we included samples from both male and female patients. We analyzed patient survival data, as well as FOXK1 and FOXK2 expression and their correlation with E2Fs, separately by gender (**Figure 2 h-i** and **Figure S1d**).

RESPONSE TO SPECIFIC REVIEWER'S COMMENTS

Reviewer #1 (Remarks to the Author):

In the manuscript titled "O-GlcNAcylation of FOXK1 orchestrates the E2F pathway and promotes oncogenesis," the authors reported the oncogenic role of FOXK, a subunit of the BAP1 complex, in facilitating cell transformation. Additionally, demonstrated that FOXK1 is highly expressed in patient samples and correlated with E2F targets. Importantly, they demonstrated that FOXK1 could be O-Glycosylated which promotes BAP1 recruitment to chromatin. The overall experiments were well-designed, and the results were convincing. However, I have a few concerns that should be addressed by the authors.

Specific comments

1) A primary concern is the potential presence of FOXK1 in other epigenetic complexes, such as the SIN3A complex. The authors should deplete BAP1 in FOXK1 over-expressing cells to see if the gene expression and other phenotypes could be rescued.

Response R1-1: This is indeed an important consideration. FOXK1 interact with two major complexes, the BAP1 complex and the SIN3A complex, either of which could be impacted by FOXK1 O-GlcNAcylation.

To further support our data, we performed in vitro interaction assays and observed an increased binding of BAP1 with O-GlcNAcyated FOXK1, but not with SIN3A, NCOR1 or HDAC3 (**new Figure 7e**). This is consistent with our data, obtained *in cellulo*, showing a decreased association of BAP1 with the FOXK1-O-GlcNAcylation defective mutant on gene regulatory regions and notably E2F targets (**Figure 7f**).

We also used the FOXK1 delta FHA (FOXK1^{ΔFHA}) that cannot interact with BAP1 and performed overexpression in IMR90. We now found that FOXK1^{ΔFHA} does not increase proliferation, as does the wildtype form (**new Figure 8b**).

We also overexpressed FOXK1 in U2OS cells stably depleted of BAP1 using shBAP1. We found that FOXK1 does not increase proliferation in BAP1-depleted cells as it does in cells expressing a non-target control shRNA (**new Figure 8c, d**). Moreover, FOXK1, but not its O-GlcNAcylation defective mutant form, promote cell proliferation in BAP1 proficient, but not in BAP1-deficient cancer cells (**new Figure 8h**). Furthermore, as previous gene expression profiling indicated that BAP1 depletion also downregulates E2F target genes¹, we further exploited these data to demonstrate the functional link between BAP1 targets and FOXK1 target genes (**new Figure 8e,f,g**).

Finally, we performed Cut&Run experiments for SIN3A in the context of the wildtype and O-GlcNAcylation-defective mutant. Our genomic analysis of SIN3A did not show a differential recruitment of SIN3A on E2Fs target genes and their enhancers following the expression of FOXK1 or its O-GlcNAcylation defective mutant (**new Figure S11b**).

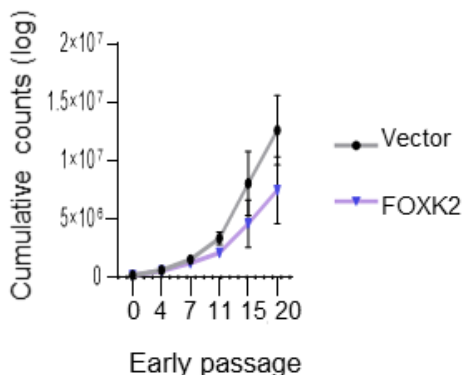
(Please also see **Response E-1**).

2) In Figure 3, the authors need to validate the specificity of the FOXK1/2 antibodies in ChIP-seq, particularly due to the notable overlap between FOXK1 and FOXK2 peaks.

Response R1-2: This was indeed an important consideration in our experimental design. We performed ChIP using anti-Flag following overexpression of individual proteins and the Flag signal for each protein corresponds to the signal of the corresponding endogenous protein (**Figure 3d, and Figure S4d**). In respect to the antibodies against endogenous proteins, these are highly specific, as we do not detect FOXK1 with anti-FOXK2 and vice versa (**Figure S2a**). Moreover, we performed siRNA or CRISPR-KO of either FOXK1 or FOXK2 and rigorously validated antibody specificity (**new Figure S4a-c**). Thus, both protein overexpression and protein knockdown show that there is no cross-reactivity. Furthermore, our experiments of ChIP followed by Re-ChIP did not show any enrichment of FOXK1 targets with FOXK2 and vice versa (**new Figure 3g,h**). We concluded that the FOXK1 and FOXK2 antibodies we used are indeed highly specific.

3) It is established that FOXK1 and FOXK2 are mutually exclusive in the BAP1 complex. If the proposed model is true, overexpressing FOXK2 should diminish the BAP1-FOXK1 interaction, thereby suppressing cell transformation and tumor growth. Have the authors observed this phenomenon?

Response R1-3: We appreciate the reviewer's insightful comments regarding this interesting aspect. Overall, our findings indicate that FOXK2 overexpression does not have a significant effect



on cell proliferation. However, when FOXK2 was overexpressed in low-passage IMR90 cells, we observed a slight decrease in cell proliferation compared to the control (**see figure at the left**). While this effect was marginal, we opted not to include these data in the current manuscript. Additionally, we found that FOXK2 overexpression, but not FOXK1, increases the formation of senescence-associated PML bodies (**Figure 1g, h**). Moreover, FOXK2 expression induces a gene expression program associated with stress responses and negative regulation of the cell cycle, including

the upregulation of p21 (**Figure 2e, f and Figure S3f**), whereas FOXK1 promote cell cycle and E2F gene expression.

Interestingly, while ChIP-seq experiments performed on asynchronous cell populations showed similar genomic binding patterns for FOXK1 and FOXK2, ReChIP analysis revealed that these transcription factors do not bind the same genomic regions simultaneously (**new Figure 3g, h**). This indicates that FOXK1 and FOXK2 may compete for binding at certain loci to differentially influence gene expression. These findings support the idea that FOXK1 and FOXK2 might have different (even opposing) roles in specific contexts.

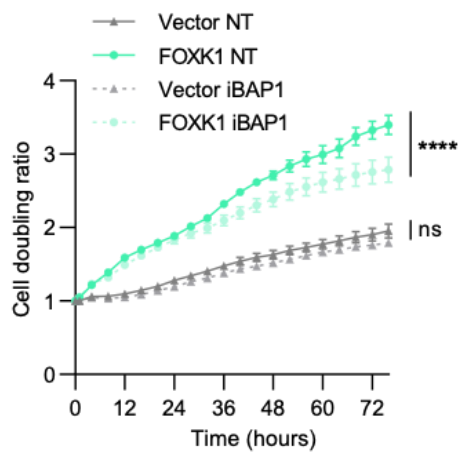
Another, not mutually exclusive, possibility is that FOXK1 and FOXK2 may interact differentially with BAP1 depending on the cell cycle phase, selectively influencing specific gene expression programs. Supporting this, our data now demonstrate that FOXK1 chromatin binding is strongly enhanced during the G1/S transition, time at which its O-GlcNAcylation is also increased (**new Figure 3i, j**). O-GlcNAcylation may further stabilize the FOXK1-BAP1 interaction, as shown by our pulldown assays and genomic occupancy studies (**new Figure 7e and Figure 7f**).

Thus, while our findings overall provide important insights, we believe that systematically characterizing the dynamic interactions between FOXK1, FOXK2, BAP1, and chromatin requires further investigation, which may extend beyond the scope of the current study.

4) The oncogenic functions of BAP1 and its sub-complexes have been elucidated in various cancer types such as leukemia and lung cancer. The authors should reference these studies and delve deeper into the potential of targeting BAP1 in FOXK1-induced malignant transformation.

Response R1-4: This is a highly interesting aspect of BAP1 biology. We believe that the reviewer is refereeing to the gain of function of BAP1 caused by mutations in ASXLs proteins^{5,6}. These mutations are indeed associated with a gain of function of BAP1 by enhancing its deubiquitinase activity in cancer cells. We included a concise section in the discussion with appropriate references. As suggested by the reviewer, we consolidated the link between FOXK1 and BAP1. In vitro interaction assays demonstrated that FOXK1 O-GlcNAcylation promotes its interaction with BAP1 but not with repressors such as SIN3A, NCOR1, or HDAC3 (**new Figure 7e**). Additional, genome occupancy (Cut&Run) experiments revealed reduced BAP1 recruitment to enhancers and promoters of E2F target genes in U2OS cells expressing the O-GlcNAcylation-defective FOXK1 mutant (**Figure S11a**). We also found that FOXK1 mutants lacking the FHA domain (FOXK1^{ΔFHA}) responsible for interaction with BAP1 failed to enhance cell proliferation (**new Figure 8b**). FOXK1 overexpression did not rescue proliferation in BAP1-depleted U2OS cells (**new Figure 8c,d**). Gene set enrichment analysis of microarray data from BAP1-depleted U2OS cells confirmed downregulation of E2F target genes, with FOXK1 enriched on 33 of 43 affected promoters (**new Figure 8e-g**). Analysis of the cancer cell genetic dependency map further showed that FOXK1-dependent cells for survival are also BAP1-dependent cells, while no dependency on FOXK2 was observed (**new Figure 8a**). These findings underscore the critical role of FOXK1 O-GlcNAcylation in BAP1 recruitment, E2F gene regulation, and cancer progression. To further strengthen our data on the role of BAP1-FOXK1 interaction in cancer, we performed FOXK1 overexpression across BAP1-proficient and BAP1-deficient cell lines (mostly cells derived from mesothelioma tumors). Our findings showed that FOXK1, but not its O-GlcNAcylation-deficient mutant, enhanced cell proliferation in cells expressing BAP1, while having no impact on BAP1-deficient cells (**new Figure 8h**). This observation is particularly interesting given that BAP1 expression is linked to a poor prognosis in mesothelioma^{2,3}.

On the other hand, we tested Palbociclib, a well-established and clinically relevant inhibitor of CDK4/CDK6 kinases, major drivers of the E2F pathway, and found that it can significantly reduce cell proliferation in FOXK1-expressing IMR90 cells probably due to the overactivated E2Fs pathway in these cells (**new Figure 2g**). These data further support our findings on the role of FOXK1 in promoting E2F gene expression and cell proliferation. In addition, we conducted preliminary experiments using a promising BAP1 inhibitor⁶. At high doses, the inhibitor induced



cell death in both FOXK1-expressing IMR90 cells and controls. Interestingly, at lower doses, we observed a more pronounced inhibition of proliferation in FOXK1-overexpressing cells compared to controls (**Figure in the left**). These results suggest that BAP1 inhibition may have selective therapeutic potential in the context of FOXK1 overexpression. Future studies will focus on a detailed evaluation of BAP1 inhibitors, including their therapeutic potential in human tumors with FOXK1 overexpression, as well as exploring the efficacy of combination treatments targeting complementary pathways.

(Please also see **Response E1**).

Reviewer #2 (Remarks to the Author):

In this manuscript, the authors investigate the role of FOXK1 in cell proliferation and its interplay with the E2F transcription factor. The role of FOXK1 in cancer and proliferation is well established. The authors show that FOXK1 is modified by O-GlcNAcylation, and while this is an interesting observation, the study does not convincingly show a functional role for this modification in cell cycle control or regulation of FOXK1 target genes. As such, the paper does not convincingly support the authors' major conclusions.

Response R2-1: We have now included additional results to further demonstrate the functional significance of FOXK1 O-GlcNAcylation in regulating E2F target genes and cell proliferation. The conclusions of these and our previous data are summarized as follows:

First, the use of O-GlcNAcylation-defective mutants of FOXK1 (FOXK1^{7A} and FOXK1^{11A}) shows that these mutants have a reduced ability to promote cell proliferation compared to wild-type FOXK1 (**Figure 6a-c**). This is evidenced by cell counts and a slower engagement in the S phase in cells expressing O-GlcNAc-defective FOXK1 mutants. We have previously shown that O-GlcNAc-defective FOXK1 mutants do not increase E2F1 expression as does the wildtype form. We now expanded gene expression analysis to include other important E2F target genes, in addition to E2F1 (**new Figure 6d**). Furthermore, we have provided in vivo evidence showing that cells expressing O-GlcNAcylation-defective mutants of FOXK1 have a delayed onset of tumor formation and reduced tumor growth in xenograft models compared to wild-type FOXK1-expressing cells (**Figure 6e-h**). This underscores the importance of O-GlcNAcylation in mediating the oncogenic properties of FOXK1.

FOXK1 O-GlcNAcylation is modulated during the cell cycle, with the highest levels occurring during E2F pathway activation at G1/S (**Figure 5e and Figure S6c,d**). We now provide additional data to show that this also coincides with increased recruitment of FOXK1 on gene regulatory regions (**new Figure 3i,j and new Figure S4g**). We also now show that O-GlcNAcylation promotes the interaction of FOXK1 with BAP1 using an in vitro system, but not of FOXK1 with components of the SIN3A complex (**new Figure 7e**).

O-GlcNAcylation-defective mutants of FOXK1 also exhibit impaired recruitment of BAP1 to gene regulatory regions, resulting in altered histone modifications, as shown in **Figure 7f-h and Figure S11c**. Similar results were also found in U2OS cells, a cell type in which expression of FOXK1, but not FOXK1 O-GlcNAcylation defective mutant, promotes BAP1 recruitment and cell proliferation (**new Figure S11a**). This indicates that O-GlcNAcylation is critical for FOXK1's role in gene regulation and cell cycle control.

We also provide additional data to strengthen the molecular and functional link between FOXK1-O-GlcNAcylation and BAP1 (**new Figure 7e, new Figure 8 and new Figure S12**)

In conclusion, the previous and new data altogether strongly support the functional role of FOXK1 O-GlcNAcylation in regulating cell cycle progression, BAP1 recruitment, E2F gene expression and tumorigenesis.

(Please see also **Response R1-4 and Response E-1**).

Major points

1. In Fig. 2, the authors state the number of E2F target genes regulated by FOXK1, including analysis of “the 200 E2F target genes.” The number of target genes vastly exceeds this number, and it is not clear how the authors arrived at this group of genes. More importantly, these genes may be regulated as a consequence of cell cycle perturbations caused by over-expression of FOXK1 rather than by FOXK1 directly, and further functional studies are lacking. The existence of a binding site by ChIP-seq does not guarantee a functional impact.

Response R2-2: We included additional data to support our conclusions and added additional clarifications in the result and discussion sections. First, by analyzing differential gene expression between FOXK1 and FOXK2, we identified 286 genes that were significantly up-regulated in the FOXK1 condition. Among these, 60 genes were E2F target genes. According to the MSigDB database, 200 genes are associated with the E2F target pathway. We represented the transformed counts for each condition from our RNAseq of these 200 E2F target genes to highlight FOXK1's capacity to promote the expression of E2F target genes, with 60 out of 200 genes (45%) being up-regulated (**Figure 2 and Figure S3**). We note that our genomics approaches are widely used to ensure that the selected genes are relevant and significant for studying the biological significance of a given gene expression program.

On the other hand, ChIP-Seq and Cut&Run studies demonstrated that FOXK1 directly and markedly binds to the promoters of these E2F target genes, supporting its role in transcriptional regulation (**Figure 3, Figure 4 and Figure S4**). Additionally, FOXK1 also binds enhancers that are located near FOXK1 bound promoters, and are characterized by H3K27ac and BRD4 which, independently of RNA-seq, further link FOXK1 binding to E2F gene activity (**new Figure 4b and Figure 4c**).

Moreover, we now exploited Hi-C 3D chromatin organisation data to show the interaction of FOXK1 enhancers with E2F promoters. Hi-C analysis revealed that the promoters of E2F target genes bound by FOXK1 also interact with distal enhancer regions, further supporting the role of FOXK1 in regulating E2F gene expression through chromatin interactions (**new Figure 4d,e**).

We also synchronized IMR90 cells using contact inhibition to induce cell cycle exit, followed by replating at low density to observe cell cycle re-entry. FACS analysis and EdU incorporation assays showed that FOXK1-overexpressing cells rapidly engage the S phase, further supporting its role in driving E2F gene expression and cell cycle (**Figure 1d**). We also now show that FOXK1 binding to gene regulatory regions is dynamic and strongly increases at the G1/S transition, time at which E2F target genes are induced. Similar results were also observed in U2OS cells following cell cycle synchronization (**new Figure 3i,j and new Figure S4g**).

We also point out that FOXK1 mutants deficient in O-GlcNAcylation (FOXK1^{7A} and FOXK1^{11A}) exhibited a significantly reduced ability to induce E2F1 expression and other E2F targets) and we now established a strong functional link between FOXK1 and BAP1, which is known to regulate E2F target genes (**new Figures 7e, new Figure 8**).

Finally, we utilized Palbociclib, a clinically approved CDK4 and CDK6 inhibitor targeting the E2F pathway to suppress tumor cell growth. Notably, IMR90 cells overexpressing FOXK1 exhibited a significantly greater reduction in proliferation following Palbociclib treatment (**new Figure 2g**). This additional finding suggests that cells with FOXK1 overexpression are more sensitive to this inhibitor, likely due to enhanced activation of the E2F pathway in these cells.

In conclusion, our study now provides substantial evidence that FOXK1 directly regulates E2F target genes and set the stage for further mechanistic studies on this important cell proliferation regulatory network.

(Please see also **Response E-1 and E-4**).

2. FOXK1 and FOXK2 bind to nearly identical regions (Fig. 3), yet the authors make the claim that the two factors function differently, namely, that the former activates E2F target genes, but the latter does not. The authors ascribe this to OGlcNAcylation, which is regulated both by cell cycle and metabolic cues, which the authors have not distinguished in this study. Both cell cycle state and metabolism is altered by the perturbations used to synchronize cells.

Response R2-3: We have provided initial evidence that FOXK1 and FOXK2, despite binding to nearly identical regions, function differently. We showed that FOXK1, but not FOXK2, promote cell proliferation, oncogenic transformation and expression of E2F target genes. We also showed that the O-GlcNAcylation FOXK1, which is important for activating E2F target gene expression, is not present in FOXK2. We showed that O-GlcNAcylation of FOXK1 is increased at the G1/S transition. This is consistent with previous cell cycle synchronization studies showing a peak of O-GlcNAcylation just before the G1/S transition⁷. We also note that metabolic changes and cell cycle signaling are highly interconnected. We believe that uncovering the precise molecular mechanism responsible for the increase in FOXK1 O-GlcNAcylation during the cell cycle is beyond the scope of this study, as it warrants an investigation in its own right. Instead, we focused on further distinguishing the functions of FOXK1 and FOXK2 as well as the impact of FOXK1 O-GlcNAcylation in cell cycle regulation. We consolidated our results with additional new

experiments. Notably, we now show that FOXK1 and FOXK2 do not simultaneously bind regulatory regions, i.e., do not bind regulatory regions at the same time (**new Figure 3g,h**), and interact differently with chromatin during cell cycle progression (**new Figure 3i,j**), further explaining their functional divergence. We strengthened the link of FOXK1 with BAP1 (**new Figures 7e, new Figure 8 and new Figure S12**). Our data altogether also explain previous studies on the specific functions of these transcription factors in cell proliferation and other processes (see discussion).

(Please also see **Response E-1** and **E-4**)

3. Major questions remain about the FOXK1-11A mutant used in key studies in the paper. For example, it is not known whether the structure and activity of this protein is altered by the mutations. Does the mutant protein bind to the genome normally and recapitulate the chromatin modifications seen with the wild-type protein? According to Fig. 6 and extended Fig. 8, there are no obvious differences between the proteins. Does the mutant normally regulate target genes, and does it directly recruit BAP and OGT? In short, it is hard to determine the mechanistic impact of the FOXK1-11A mutation on gene regulation. Importantly, Fig. 6e, shows a quantitative reduction in binding of the mutant to the genome, and this could reflect the modest diminution of other histone marks in a subsequent figure panel (fig. 6g). Altogether, this series of key experiments falls short of presenting a convincing case for the mechanistic defects associated with loss of the O-GlcNAcylation modification on FOXK1.

Response R2-4: We performed additional experiments to strengthen our data. As outlined above, we provided further evidence to demonstrate the functional importance of FOXK1 O-GlcNAcylation. We show that while FOXK1^{11A} mutant binds to the genome similarly to the wild-type protein, it shows significant functional differences. FOXK1^{11A} mutant fails to induce the expression of key E2F target genes, highlighting its compromised transcriptional regulatory function. The FOXK1^{11A} mutant exhibits a reduced ability to recruit BAP1 to E2F target genes, as evidenced by Cut&Run and in vitro interaction assays. This effect is specific to BAP1, but not another major FOXK1-interacting partner, SIN3A. This diminished recruitment leads to altered chromatin modifications, particularly reduced H3K4me1 and increased H2Aub levels at specific genomic regions, indicating a shift towards a repressive chromatin state. We believe that the previous and new findings collectively now provide a convincing case that outline the mechanistic defects associated with the FOXK1^{11A} mutation and its impact on gene regulation and cell proliferation.

(Please also see **Response E-1, Response E-4** and **Responses R1-1, R1-3 and R1-4**)

4. Another major shortcoming of the study is that nearly all experiments rely on over-expression of FOXK1 and other proteins. Another drawback is that multiple, unrelated normal and cancer cell lines (IMR90, U2OS, K562, HLF, 3T3L1, PC3, HCT116) are used throughout the study, rather than using a single line to systematically perform all experiments.

Response R2-5: We recognize that the use of multiple cell lines and overexpression models might question the relevance and be perceived as lacking uniformity. However, we have performed overexpression studies of FOXK1, as this approach is highly relevant to elucidating the functional consequences of FOXK1 overexpression in cancer. This situation is comparable to that of other well-established oncogenes whose overexpression promotes cancer progression. Importantly, overexpression of FOXK1 is found in a wide variety of cancers of various developmental origins, and such overexpression is associated with a poor cancer prognosis, highlighting its importance

and significance. The expression levels of overexpressed FOXK1 in our experiments are similar to those found in cancers that overexpress FOXK1 (**Figure S3**), making our study highly relevant to cancer pathogenesis. Moreover, we believe that using a variety of cell lines helps to demonstrate that the effects are consistent across different genetic and epigenetic backgrounds. Indeed, we have used multiple normal and cancer cell lines, including IMR90, HLF, 3T3L1 (relevant to FOXK1 function in normal cell cycle regulation) and U2OS, K562, PC3, and HCT116 (relevant to FOXK1 function in cancer) to enhance the generalizability of the findings. We also try to diversify our cells to cover for sex considerations. For instance, U2OS and K562 are female cells and PC3 and HCT116 are male cells. Overall, while sometimes it is difficult to make direct comparisons, we try to increase cell diversity to ensure that the observed effects are not cell-line specific, but are broadly applicable across different cellular contexts, thereby strengthening our conclusions.

We also note that as we progressed in our investigation into the mechanisms of O-GlcNAcylation, we incorporated additional cell types to address specific questions. For example, we included IMR90 and HLF cells, which are commonly used to study normal cell cycle, senescence and oncogenic transformation. Both IMR90 and K562 are valuable for genomic studies and have existing datasets that helped interpret our findings. 3T3L1 cells, which can effectively exit the cell cycle and differentiate, provided an excellent model for analyzing FOXK1 O-GlcNAcylation in both proliferating and differentiated cells. We also included mesothelioma cells, a cancer characterized by a high frequency of BAP1 mutations, as FOXK1-BAP1 interaction has been proven to be important in BAP1-positive mesothelioma tumor cells (**new Figure 8 and new Figure S12**).

We also emphasize that key experiments were systematically performed in IMR90 cells and U2OS cells to ensure consistency and validate the primary findings. For instance, the functional impact of FOXK1 O-GlcNAcylation on cell cycle regulation and gene expression was thoroughly investigated in IMR90 cells, as shown by RNA-seq, ChIP-seq, and functional assays. Thus, for the main figures, we focused primarily on IMR90 and U2OS cells, with data from additional cell lines presented mostly in the supplemental Figures (Please also see **Response E-3**).

Reviewer #3 (Remarks to the Author):

Mascelf et al.

O-GlycNAcylation of FOXK1 orchestrates the E2F pathway and promotes oncogenesis

This manuscript explored the nuanced differences between FOXK1 and FOXK2 transcription factors in cancer. FOXK1 expression alone correlated with increased proliferation, delayed senescence, and tumor initiation in transformed fibroblast cells. FOXK, but not FOXK2, overexpression promotes increased proliferation of non-transformed fibroblasts and can increase E2F1 gene expression and tumor growth in vivo when combined with known oncogenes. Authors show that FOXK1, but not FOXK2, is O-GlcNAcyated and mutating eleven O-GlcNAc sites reduced FOXK1 O-GlcNAcylation and reduced effect on proliferation and tumor growth in vivo. Lastly, author show that FOXK1 O-GlcNAcylation regulates BAP1 recruitment to E2F target gene promoters.

Noteworthy: This study identified a novel regulation of E2F1-regulated genes by FOXK1. It also

identifies FOXK1 as an O-GlcNAcylated protein.

Not clear if work will be of significance: FOXK1 and FOXK2 are both known to have tumor promoting activity. Also, its relevance in cancer and oncogenesis is not well studied here as nearly all experiments were performed in fibroblast cells. In addition, many of the main conclusions are not well supported by the data.

Possible flaws: Also, major model to compare difference between FOXK1 vs FOXK2 is problematic since model used overexpressed FOXK1 nearly 5 folds over FOXK2 in fibroblasts.

Response R3-1: We addressed concerns regarding potential issues with protein overexpression and provided additional data to clarify that the effects of FOXK1 and FOXK2 can be compared and that only FOXK1 promotes cell proliferation. In IMR90 cells, FOXK2 expression was observed to decrease over time relative to FOXK1 during prolonged culture (**Figure 2f**). IMR90 cells expressing FOXK1 or FOXK2 show approximately four-fold and ten-fold overexpression of FOXK1 and FOXK2, respectively, relative to their endogenous levels (**new Figure S2a**). This demonstrates that FOXK2 is even more highly overexpressed than FOXK1, yet it does not promote proliferation. Instead, FOXK2 appears to have a slight inhibitory effect on cell proliferation, likely leading to the selection of cells with reduced FOXK2 expression over time. This inhibitory effect of FOXK2 is consistent with our findings that FOXK2, but not FOXK1, increases senescence-associated PML bodies (**Figure 1g**) and induces the expression of the cell cycle inhibitor p21 (**Figure 2e,f**). Importantly, RNA-seq experiments performed immediately after viral transduction and selection confirmed that FOXK1 and FOXK2 were expressed at comparable protein levels (**new Figure S3a**). Under these conditions, FOXK1 upregulated cell cycle genes, including E2F targets (**Figure 2b-f**), while FOXK2 activated differentiation programs and pathways that negatively regulate the cell cycle, such as the p53 pathway (**Figure S3f**). To further support our findings, we performed viral transduction experiments with reduced FOXK1 viral titers and extended selection periods, ensuring similar FOXK1 and FOXK2 protein expression levels. Even under these conditions, FOXK1, but not FOXK2, promoted cell proliferation (**new Figure S2b**). On the other hand, while, genome occupancy studies revealed that FOXK1 and FOXK2 bind similar regulatory regions, ChIP-ReChIP experiments demonstrated that they do not simultaneously occupy the same DNA sites, indicating distinct chromatin contexts, likely associated with specific protein interactions (**new Figure 3g, h**). Additionally, Cut&Run analysis in synchronized cells showed that FOXK1 recruitment to gene regulatory regions strongly increases during the G1/S transition, comparatively to FOXK2 (**new Figure 3i, j**). Notably, FOXK1 O-GlcNAcylation peaks at the G1/S transition (**Figure 5e, Figure S6c,d**). HiC-based chromatin interaction data further indicated significant promoter-enhancer interactions for FOXK1-associated genes compared to non-differentially expressed genes (**new Figure 4d, e**).

~~We also provide additional genetic data supporting the functional link between FOXK1 and BAP1 in regulating the E2F pathway. Using a mouse model with a Bap1^{T492A} mutation, which disrupts FOXK1/FOXK2 interaction by inhibiting BAP1 phosphorylation, we observed partial embryonic lethality, developmental retardation, and growth abnormalities in homozygous mice (**panels b-d, figure page 5 of this rebuttal**). Genome-wide BAP1 recruitment analysis in MEFs revealed disrupted binding at regions critical for viability and the E2F pathway (**panel f, g, figure page 5 of this rebuttal**). These phenotypes closely resemble those of FOXK1 knockout mice, which also exhibit embryonic lethality and proliferation defects, unlike FOXK2 knockout mice, which lack a reported phenotype. These results strongly support our conclusions on the distinct and critical roles of the FOXK1-BAP1 interaction in cell cycle regulation and the E2F pathway.~~

Collectively, these data confirm that FOXK1 uniquely drives cell proliferation, while FOXK2 seems to exert opposing effects. This highlights the distinct functional roles of FOXK1 and FOXK2 in regulating cell cycle and proliferation pathways.

We also emphasize that key findings were validated in other cancer cell lines, including U2OS, HCT116, PC3 to demonstrate the relevance of our observations across different cellular contexts. We also showed that BAP1-expressing mesothelioma and lung cancer cells are responsive to FOXK1-mediated increase of cell proliferation (**new Figure 8h and Figure S12**). We also exploited the genome cancer drug dependency database (<https://depmap.org/portal/>) to reveal that FOXK1, but not FOXK2, exhibit a similar dependency pattern to BAP1 (**new Figure 8a**).

We believe that altogether with the new data, our study identifies a novel regulation of the E2F network by FOXK1 and establishes FOXK1 as an O-GlcNAcylated protein. This post-translational modification distinguishes FOXK1 from FOXK2, explaining their differing roles in promoting cell proliferation and tumor progression. Our data show that while FOXK1 and FOXK2 bind to nearly identical genomic regions, only FOXK1's activity is regulated by O-GlcNAcylation, which enhances its ability to recruit co-factors such as BAP1, thereby promoting the expression of E2F target genes critical for cell proliferation and tumor growth.

(please see above and notably **Responses E1, E2 and E4**)

Major Points

1. Figure 1: Need to show expression levels between FOXK1 vs FOXK2 via western blot and to also compare overexpression to normal levels (see issue below).
2. Figure 2F: IMR90 cells used here (also used in Fig. 1?) have very high amounts of FOXK1 levels compared to FOXK2 levels. This could mean the changes in phenotypes seen in Fig. 1 and gene expression in Fig. 2 could be result of nearly 4-5 fold higher levels of FOXK1. Authors should overexpress similar levels of FOXK1 and FOXK2 and examine effects on cell phenotypes & E2F1 genes to support conclusion of differential effects of these transcription factors.

Response R3-2: We have demonstrated that FOXK1, but not FOXK2, promotes cell proliferation, and this selective effect of FOXK1 is not attributable to differences in protein expression levels. Briefly, in the previous version of the manuscript, we reported that exogenous FOXK1 protein levels were higher than those of FOXK2 (**Figure 2f**). However, these cells had undergone multiple passages prior to western blot analysis, and we believe that cells expressing low levels of FOXK2 are preferentially selected over time, at the expense of cells with high expression of FOXK2, which appear to inhibit cell proliferation, albeit modestly. This hypothesis is supported by trends observed in cell counts, PML body formation, and FACS analysis, all indicating that FOXK2 exerts a mild inhibitory effect on cell proliferation. Also, only FOXK2 expression leads to a consistent increase in the expression of the negative regulator of cell cycle p21 (**Figure 2e, f**).

We also observed that IMR90 cells expressing FOXK1 or FOXK2 exhibit approximately a four-fold and ten-fold increase, respectively, in their expression levels compared to their corresponding endogenous proteins (**new Figure S2a**). This indicates that FOXK2 is overexpressed to an even greater extent than FOXK1 relative to its endogenous counterpart.

For RNA-seq, we have harvested cells rapidly after infection and also ensured that exogenous FOXK1 and FOXK2 are expressed at comparable levels. Under these conditions, FOXK1, but not FOXK2, promotes cell proliferation, and global gene expression analysis reveal that FOXK1 only promotes the E2F pathway (**Figure 2a-e and Figure S3a-d**). Moreover, FOXK2 overexpression

is associated with stress responses and even the p53 pathway, which is a negative regulator of the cell cycle (**Figure S3f**).

(Please see also **Response E2**, **Response R1-3** and **Response R3-1**).

3. Line 77 & 124 authors conclude FOXK1 “..constitutes a potent oncogene.”. Data presented shows FOXK1 is a potent tumor promotor in fibroblasts. Authors would need to add more data to support conclusion or alter conclusion.

Response R3-3: We thank the reviewer for this insightful comment. We agree and have revised the text to state that “FOXK1 displays potent oncogenic promoting activities,” which more accurately reflects the data presented. Furthermore, we have included additional experimental data to strengthen our conclusions regarding FOXK1's oncogenic properties, providing a more comprehensive characterization of its role in tumor promotion.

4. Figure 4, 5 & 6 was nearly all done in fibroblasts and thus its relevance to cancer and oncogenesis is preliminary and needs to be verified in cancer cells.

Response R3-4: While it is true that many experiments were performed in fibroblasts (IMR90), the conclusions of our study about FOXK1's role in cancer and oncogenesis are well validated in other cancer cells, and we have now added additional data in the revised form of our manuscript to support our conclusions. First, the primary reason for using IMR90 or HLF fibroblasts was to study normal cellular processes (e.g., cell proliferation and senescence), and identify the molecular mechanisms governed by FOXK1. Moreover, this model allows performing oncogenic transformation. However, to address the broader applicability of our findings, we have also conducted critical experiments in various cancer cell lines. These include U2OS (osteosarcoma), HCT116 (colon Cancer), PC3 (prostate cancer) and other cancer cells including mesothelioma cell lines (**new Figure 8**, **Figure S2**, **Figure S9** and **new Figure S12**). In these cancer cell models, overexpression of FOXK1 led to significant increases in cell proliferation, colony formation, and tumor growth, compared to controls. We now also included additional data to further validate the role of the BAP1-FOXK1 interaction in cancer. We overexpressed FOXK1 in both BAP1-proficient and BAP1-deficient cell lines, primarily derived from mesothelioma tumors, a cancer characterized by high BAP1 mutation frequency. Our results indicate that FOXK1, but not its O-GlcNAcylation-deficient mutant, promoted cell proliferation in BAP1-expressing cells, with no effect in BAP1-deficient cells (**new Figure 8h**). This finding is particularly noteworthy, as BAP1 expression has been associated with poor prognosis in mesothelioma. Additionally, gene dependency analyses revealed that FOXK1 dependency closely aligned with BAP1, whereas FOXK2 displayed a distinct dependency profile (**new Figure 8a**). Finally, consistent with our data in various cell types, we found that FOXK1 amplification in human tumors is associated with poor prognosis and a striking upregulation of E2F target genes (**Figure 2h-j**).

(Please see also **Response E-3**).

5. The data linking FOXK1 O-GlcNAcylation to BAP1 recruitment was not very convincing. There is no functional data showing BAP1 play a role in FOXK1-regulation of E2F1 regulated genes and that FOXK1 O-GlcNAcylation play a role.

Response R3-5: We thank the reviewer for this comment. We conducted extensive additional experiments to confirm and expand upon the functional role of FOXK1 O-GlcNAcylation in

recruiting BAP1 and regulating E2F target genes. Our initial data revealed that wild-type FOXK1, but not the O-GlcNAcylation-defective FOXK1-11A mutant, enhances BAP1 binding at 2,130 genomic regions, including enhancers and promoters of E2F target genes (**Figure 7f**). Gene ontology analysis confirmed that E2F-related genes are the primary targets showing differential BAP1 recruitment depending on FOXK1 O-GlcNAcylation (**Figure 7g**). We now performed in vitro interaction assays, which further demonstrated that FOXK1 O-GlcNAcylation specifically promotes interaction with BAP1 while not affecting binding to other repressors, such as SIN3A, NCOR1, or HDAC3 (**new Figure 7e**). Moreover, we performed additional Cut&Run experiments in U2OS cells, and consistent with data obtained in IMR90, we show decreased BAP1 recruitment at E2F-regulatory regions when the O-GlcNAcylation-defective mutant was expressed (**new Figure S11a**). Importantly, SIN3A recruitment was unaffected by the FOXK1 O-GlcNAcylation state in IMR90 cells (**new Figure S11b**). Functionally, we found that FOXK1 mutants lacking either the FHA domain (required for BAP1 interaction) or DNA-binding ability failed to enhance cell proliferation and even displayed dominant-negative effects, reducing cell growth (**new Figure 8b**). Further experiments in U2OS cells with stable BAP1 knockdown demonstrated that FOXK1 overexpression does not restore proliferation in the absence of BAP1, underscoring its dependence on this interaction (**new Figure 8c,d**). Further, a reanalysis of our previous microarray data from BAP1-depleted cells revealed significant downregulation of 43 E2F target genes, with FOXK1 directly binding to 33 of these promoters or distal regions (**new Figure 8e-g**). Using mesothelioma cancer cells, a cancer with frequent BAP1 mutations, we show that FOXK1 overexpression increased proliferation in BAP1-proficient pleural mesothelioma and lung cancer cell lines, but not in BAP1-deficient cells, while the O-GlcNAcylation-defective mutant failed to promote growth in either context (**new Figure 8h**). On the other hand, genetic dependency mapping further revealed that FOXK1-dependent cancer cells are also dependent on BAP1 for survival, with no dependency is observed for FOXK2 (**new Figure 8a**).

~~On the other hand, as part of another manuscript under preparation, we studied a mouse model carrying the Bap1^{T492A} mutation, which disrupts BAP1's interaction with FOXK1/FOXK2. Homozygous Bap1^{T492A/T492A} mice displayed partial embryonic lethality, with severe developmental retardation observable at E16.5 (**panel c, figure page 5 of this rebuttal**). The few surviving mice showed significant growth defects and developmental abnormalities (**panels c and d, figure page 5 of this rebuttal**). Genome-wide BAP1 recruitment analysis in murine embryonic fibroblasts (MEFs) revealed reduced binding at E2F-regulated genes critical for viability and cell cycle progression (**panel f, g, figure page 5 of this rebuttal**). Notably, these phenotypes closely resemble those of FOXK1 knockout mice⁴, which also exhibit embryonic lethality and impaired cell proliferation, further distinguishing FOXK2, which lacks a reported phenotype (<https://www.mousephenotype.org/data/genes/MGI:1916087>).~~

Taken together, these results strongly confirm the critical role of FOXK1 O-GlcNAcylation in BAP1 recruitment and regulation of the E2F pathway, establishing its functional relevance in cell proliferation and cancer progression.

(Please see also **Response E1** and **Response E4**).

Minor Points

1. Fig 1D: Fox2 graph does not look like 59%, looks way lower than the control 60%

Response R3-6: We used the G0 propidium iodide FACS profile to define a somewhat arbitrary proportion of resting cells versus cycling cells (S and G2 phases), which may not have precisely distinguished the S and G2 phases (**Figure 1d**). However, this approach allowed us to compare experimental conditions and monitor cell progression toward S and G2 phases. Importantly, we employed an additional method (EdU incorporation) to evaluate cell cycle progression, which yielded supporting results (**Figure 1e**). These findings collectively reinforce our initial conclusion that FOXK1 promotes the G1/S transition. Details of our gating strategy are provided in the supplementary information.

2. Fig 1I: Statistical significance? Explain how HDM2/FOXK1 seemed sufficient for growth, but this was not seen with the addition of the third oncogenic factor RASG12V.

Response R3-7: We appreciate the comment of the reviewer on this point. The observed lack of growth stimulation in the FOXK1/HDM2/RASG12V combination can be attributed to the impact of oncogene-induced senescence (OIS) in this setting^{8,9,10}. RASG12V is a potent oncogene that, when overexpressed, can induce a senescence response in primary cells such as IMR90 fibroblasts. This phenomenon is well-documented and serves as a cellular defense mechanism against tumorigenesis. However, when FOXK1 was combined with a minimal set of oncogenes such as HDM2 or E1A, there was a significant increase in colony formation and proliferation, demonstrating the oncogenic potential of FOXK1 in these contexts. We also note that the combination of only E1A and FOXK1 can overcome the oncogene-induced senescence by RAS. Furthermore, additional data provided in the manuscript supports this explanation. For instance, cells expressing FOXK1 in combination with RASG12V and E1A exhibited a rapid generation of tumors with high penetrance and lower latency compared to controls. This suggests that while RASG12V alone can induce senescence, the combined presence of FOXK1 and E1A can overcome this barrier, promoting oncogenic transformation and tumor growth. We have included additional discussion in the text with appropriate references on this aspect. Finally, all statistics have been revised

3. Figure 1N image should be a representative of Figure 1M. These figures not well labeled thus not clear what is being shown.

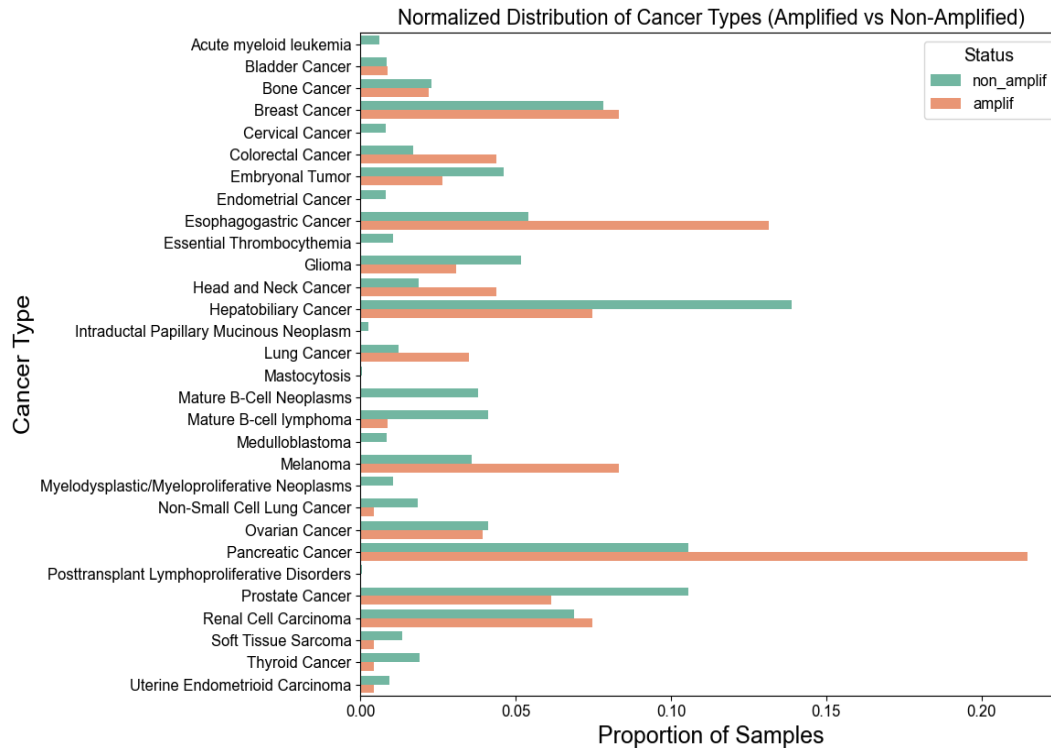
Response R3-8: We apologize for the oversight and have improved the labeling of panels in **Figure 1**, including panels m and n, to enhance the clarity of the figure.

4. Figure 2D: Vector1 seemingly has significantly different E2F target expression (authors should explain).

Response R3-9: We acknowledge the reviewer's observation regarding variations in E2F target expression in **Figure 2d**, specifically for Vector1. We believe that these differences reflect inherent biological variations, which are not uncommon in experimental systems. Primary IMR90 have been subjected to viral transduction and puromycin selection. Importantly, as shown in **Figure 2d** and **Figure S3d**, the overall pattern of E2F target expression remains consistent across replicates, with a clear and reproducible increase following overexpression of FOXK1, but not FOXK2. Importantly, the difference observed in Vector1 for these E2F target genes were not found to be differentially expressed by FOXK1 overexpression, thus were not used in following figures.

5. Figure 2G: Not clear what type of cancer is being evaluated. Just solid tumors? All cancers?

Response R3-10: This figure shows all cancers presenting FOXK1 amplification compared to no amplifications (CNA) from TCGA Pan cancer (ICGC/TCGA, Nature 2020)¹¹ which includes solid and blood cancers. We included the list of these cancers in the raw data excel file. Below is a figure recapitulating cancer type (normalized by sample size). We also updated the survival graph using more recent data.



6. Line 162 & 164 figures are mislabeled in text.

Response R3-11: We have made the correction

7. Figure 3C, F : reads mTROC1. Is this mTORC1?

Response R3-12: We regret this oversight. We have made the correction and should be mTORC1

8. Figure 3D: the dotted lines do not directly align with columns – slightly confusing to interpret.

Response R3-13: We have improved the figure.

9. Figure 4A-D: Consider putting (-) signs in for clarity, and aligning the (+) signs

Response R3-14: We appreciate the excellent suggestion. We have made these changes (Now Figure 5a-d) and in other western blots across the manuscript.

10. Figure 4C: Input FOXK1 expression decreased upon OSMI-4, interferes with IP interpretation.

Response R3-15: We agree with the reviewer. We replaced this panel with an improved version that includes results using the OGA inhibitors (PUGNAc and Thiamet G) and the OGT inhibitor (OSMI-4). The revised panel clearly shows that inhibition of the O-GlcNAc-removing enzyme OGA results in an increase in FOXK1 O-GlcNAcylation, while inhibition of OGT strongly decreases

FOXK1 O-GlcNAcylation (**new Figure 5c**). These findings are consistent with our siRNA results (**Figure 5b**) and our data using OGA and OGT inhibitors in **Figure S6a**.

11. Figure 4F: Fraction F and E are either switched or mislabeled on the graph

Response R3-16: We have revised the panel (**Figure 5f**), and while E should have been loaded before F, we confirm that there is no error in labeling. E corresponds to the flow-through fraction, and F represents the WGA-bound FOXK1 fraction, as indicated in the schematic panel.

12. Figure 4H: Label C and N terminus

Response R3-17: This has been addressed, and the C and N termini have been labeled in **new Figure 5h** (previously **Figure 4h**)

13. Figure 5B: difficult to determine the alignment of the statistical markers, esp on the “10 days” column

Response R3-18: We have improved **Figure 6b** (previously **Figure 5b**) ensuring proper alignment of the statistical markers to enhance clarity.

14. Figure 5D: Appears to be n=2 for FOXK111A, based on number of squares visible and lack of statistical markers

Response R3-19: We appreciate the reviewer’s comment and have addressed this concern by improving **Figure 6d** (previously **Figure 5d**) with additional replicates to ensure robustness. We also expanded the analysis to now include additional E2F targets, such as Cyclin A, Cyclin E, and MCM3, further strengthening our findings.

15. Figure 5I: consider looking at overall O-GlcNAcylation levels within the transformed cells.

Response R3-20: We thank the reviewer for this insightful suggestion. Indeed, the global O-GlcNAcylation levels increase in transformed cells (**Figure 6i**), consistent with previous studies highlighting the role of O-GlcNAc in cancer¹²⁻¹⁴. This observation further supports the relevance of our IMR90 cell transformation system in studying the role of FOXK1 in oncogenesis. We accordingly added notes in the result and discussion sections.

Reviewer #4 (Remarks to the Author):

1. Overview and General Comments

The manuscript submitted by Affar and colleagues investigates the oncogenic properties observed in the FOXK1 transcription factor but not in FOXK2. The increased expression of FOXK1 is associated with the activation of E2F target genes. The study suggests a connection between FOXK1's oncogenic traits and its modification by O-GlcNAcylation. FOXK1 mutants lacking O-GlcNAcylation exhibit a reduced oncogenic capacity and decreased expression of E2F pathway genes. The glycosylation-impaired FOXK1 variants show decreased association with BAP-1 compared to their glycosylated counterparts. Furthermore, the glycosylation-impaired FOXK1 exhibits elevated histone H2AK119ub and reduced H3K4m1, leading to a shift towards a transcriptionally repressive state and a reduction in oncogenic properties.

While the manuscript is technically sound and presents novel findings, certain areas require clarification for a better understanding of the implications of these findings.

2. Key Observations and Findings

FOXK1's role as an oncogene has been previously reported, and this study provides additional experimental evidence showing that it promotes cell growth and tumorigenesis. This study reports the molecular mechanisms underpinning FOXK1's function, illustrating its association with the E2F pathway and the critical role of O-GlcNAcylation in modulating FOXK1's oncogenic capacity. O-GlcNAcylation is associated with BAP-1 recruitment to target genes. The tumor suppressor BAP-1 seems to stimulate the oncogenic properties of FOXK1.

3. Strengths

The manuscript is well-structured, offering extensive experimental data and its significance in the context of cancer biology.

The link of FOXK1 to E2F pathways and the identification of O-GlcNAcylation as a key post-translational modification influencing FOXK1's activity add a valuable dimension to the current understanding of transcription factor regulation in oncogenesis

4. Areas for Improvement and clarification

In Figure 1i, when comparing FOXK1/HDM2 and FOXK1/HDM2/RASG12V,

FOXK1/HDM2/RASG12V shows no growth stimulation. There is no discussion on this variance.

Response R4-1: We acknowledge and apologize for this omission. the absence of cell proliferation in the FOXK1/HDM2/RAS^{G12V} combination likely results from oncogene-induced senescence (OIS), as RAS^{G12V} overexpression can trigger a senescent response in primary cells⁸⁻¹⁰. In the absence of RAS^{G12V}, pairing FOXK1 with HDM2 or HDM2 and E1A, significantly promoted cell proliferation and colony formation, underlining FOXK1's oncogenic potential. Importantly, the minimal combination of FOXK1 and E1A was able to bypass RAS^{G12V}-induced senescence. Our data also show that cells expressing FOXK1 with RAS^{G12V} and E1A rapidly developed tumors with high penetrance and reduced latency, indicating that FOXK1 and E1A can override RAS^{G12V}-induced senescence to promote tumor growth (**Figure 1**). We have included additional details in the manuscript.

(Please also see **Response R3-7**).

In Figure 1n, the negative control should be E1A+RASG12V+Vector instead of Vector.

Response R4-2: We apologize for this oversight and have now corrected in the revised manuscript that in Figure 1n, the negative control should be indeed labeled as E1A+RAS^{G12V}+Vector instead of Vector alone.

In Figure 2f, the protein expression level of FOXK2 is significantly lower than FOXK1, albeit its high RNA level is reported in Extended Figure 2b. This makes it difficult to determine if the observed elevation of E2F1, CDC6, and cyclin A levels with FOXK1 overexpression is specific to FOXK1 or can be attributed to variations in protein expression.

Response R4-3: We acknowledge the reviewer's concern regarding the differences in protein expression levels between FOXK1 and FOXK2. We believe that we now have promptly addressed this important concern about protein overexpression by providing additional data to support the comparison of FOXK1 and FOXK2 effects, showing that only FOXK1 promotes cell proliferation. In IMR90 cells, FOXK2 is occasionally expressed at lower levels than FOXK1, particularly during prolonged culture (**Figure 2f**). However, FOXK2 and FOXK1 are overexpressed by approximately ten-fold and four-fold, respectively, relative to their endogenous levels (**Figure S2a**). We believe that despite higher FOXK2 overexpression, it slightly reduces proliferation, likely leading to selection for cells with reduced FOXK2 expression over time. Consistent with this, FOXK2, but not

FOXK1, induces senescence-associated PML bodies (**Figure 1g**) and the cell cycle inhibitor p21 (**Figure 2e,f**). RNA-seq performed immediately post-selection confirmed that, at comparable protein levels, FOXK1 upregulates cell cycle genes, including E2F targets, whereas FOXK2 induces differentiation and cell cycle inhibitory pathways, such as the p53 pathway (**Figure S3a,f**). Further experiments with adjusted FOXK1 viral titers and extended selection confirmed that even at matched expression levels, FOXK1, but not FOXK2, promotes proliferation (**Figure S2b**). These findings validate that the observed effects are independent of overexpression artifacts. (Please see also **Response E2** and **Response R3-2**).

Figure 4b, align the gel figure + signs.

Response R4-4: We thank the reviewer for pointing this out. In response, we have aligned the "+" signs and revised all panels (a, b, c, and d) for improved clarity and consistency.

P9, line 204: catalytically dead form of OGT data are not shown?

Response R4-5:

The term "Catalytic dead" (CD) (D925A) used in Figure 5a has been clarified in the revised manuscript to ensure clarity and avoid any potential confusion.

P9, line 207: OGA is not defined previously.

Response R4-6: The term "OGA" which stands for O-GlcNAcase, an enzyme responsible for removing O-GlcNAc modifications from proteins, has now been defined in the revised manuscript to ensure clarity and completeness.

Reference

- 1 Yu, H. *et al.* The ubiquitin carboxyl hydrolase BAP1 forms a ternary complex with YY1 and HCF-1 and is a critical regulator of gene expression. *Mol Cell Biol* **30**, 5071-5085 (2010). <https://doi.org/10.1128/MCB.00396-10>
- 2 Farzin, M. *et al.* Loss of expression of BAP1 predicts longer survival in mesothelioma. *Pathology* **47**, 302-307 (2015). <https://doi.org/10.1097/PAT.0000000000000250>
- 3 Baumann, F. *et al.* Mesothelioma patients with germline BAP1 mutations have 7-fold improved long-term survival. *Carcinogenesis* **36**, 76-81 (2015). <https://doi.org/10.1093/carcin/bgu227>
- 4 Garry, D. J. *et al.* Myogenic stem cell function is impaired in mice lacking the forkhead/winged helix protein MNF. *Proc Natl Acad Sci U S A* **97**, 5416-5421 (2000). <https://doi.org/10.1073/pnas.100501197>
- 5 Balasubramani, A. *et al.* Cancer-associated ASXL1 mutations may act as gain-of-function mutations of the ASXL1-BAP1 complex. *Nat Commun* **6**, 7307 (2015). <https://doi.org/10.1038/ncomms8307>
- 6 Wang, L. *et al.* Epigenetic targeted therapy of stabilized BAP1 in ASXL1 gain-of-function mutated leukemia. *Nat Cancer* **2**, 515-526 (2021). <https://doi.org/10.1038/s43018-021-00199-4>
- 7 Drougat, L. *et al.* Characterization of O-GlcNAc cycling and proteomic identification of differentially O-GlcNAcylated proteins during G1/S transition. *Biochim Biophys Acta* **1820**, 1839-1848 (2012). <https://doi.org/10.1016/j.bbagen.2012.08.024>
- 8 Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D. & Lowe, S. W. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* **88**, 593-602 (1997). [https://doi.org/10.1016/s0092-8674\(00\)81902-9](https://doi.org/10.1016/s0092-8674(00)81902-9)

- 9 Mallette, F. A., Gaumont-Leclerc, M. F. & Ferbeyre, G. The DNA damage signaling pathway is a critical mediator of oncogene-induced senescence. *Genes Dev* **21**, 43-48 (2007). <https://doi.org:10.1101/gad.1487307>
- 10 Moiseeva, O., Bourdeau, V., Roux, A., Deschenes-Simard, X. & Ferbeyre, G. Mitochondrial dysfunction contributes to oncogene-induced senescence. *Mol Cell Biol* **29**, 4495-4507 (2009). <https://doi.org:10.1128/MCB.01868-08>
- 11 Consortium, I. T. P.-C. A. o. W. G. Pan-cancer analysis of whole genomes. *Nature* **578**, 82-93 (2020). <https://doi.org:10.1038/s41586-020-1969-6>
- 12 Taparra, K. *et al.* O-GlcNAcylation is required for mutant KRAS-induced lung tumorigenesis. *J Clin Invest* **128**, 4924-4937 (2018). <https://doi.org:10.1172/JCI94844>
- 13 Liu, Y. *et al.* FOXA1 O-GlcNAcylation-mediated transcriptional switch governs metastasis capacity in breast cancer. *Sci Adv* **9**, eadg7112 (2023). <https://doi.org:10.1126/sciadv.adg7112>
- 14 Yi, W. *et al.* Phosphofructokinase 1 glycosylation regulates cell growth and metabolism. *Science* **337**, 975-980 (2012). <https://doi.org:10.1126/science.1222278>

RESPONSE TO REVIEWER'S COMMENTS

We sincerely thank the reviewers and the editor for their insightful comments and constructive suggestions, which have helped us to improve the clarity and strength of our manuscript. Below are the responses to the reviewer's comments.

Reviewer #1 (Remarks to the Author): The authors conducted sufficient experiments and have addressed most of my concerns.

Response: We sincerely thank the reviewer for the consideration and are pleased that there are no further concerns.

Reviewer #2 (Remarks to the Author): The authors have largely responded adequately to my concerns with new experiments. However, the Palbociclib and HiC experiments do not substantively add to the conclusions. They are add-ons that should be removed from the manuscript.

Response: Regarding the palbociclib data, we believe its inclusion significantly strengthens the evidence for FOXK1's role in regulating the E2F pathway. Specifically, the remarkable decrease of cell proliferation in response to palbociclib further substantiates our results indicating a gain-of-function effect driven by elevated FOXK1 expression, thus highlighting its direct impact on cell cycle progression.

On the other hand, our Hi-C experiments reveal the distinct specificity of FOXK1 compared to FOXK2 by revealing unique promoter–enhancer interactions that govern differentially expressed genes, particularly E2F targets.

We concluded that keeping these data will further enhance the quality of the paper.

We sincerely thank the reviewer for the critical revision of this work.

Reviewer #3 (Remarks to the Author): Authors have addressed all major & minor issues.

Response: We are delighted that there are no further concerns. We sincerely thank the reviewer for the consideration and are pleased that there are no further concerns.

Reviewer #4 (Remarks to the Author): The revised manuscript submitted by Affar and colleagues addresses the reviewers' comments and incorporates additional data to strengthen the conclusions.

The study provides substantial evidence linking the well-established oncogenic properties of FOXK1 to E2F pathways. While this association is not unexpected given the oncogenic nature of FOXK1, the findings strengthen this connection with additional data. Moreover, the study identifies O-GlcNAcylation as a key post-translational modification that regulates FOXK1's activity and mediates its association with BAP-1. The newly added Figure 7A highlights the direct link between FOXK1 glycosylation and its association with BAP-1.

One of this reviewer's comments was on the difference in expression levels between FOXK1 and FOXK2. This issue has been addressed by incorporating half-titer expression vectors where applicable in the revised manuscript.

Regarding concerns about the cross-reactivity of FOXK1 and FOXK2 antibodies, Supplementary Figures 4a, b, and c demonstrate the specificity of the FOXK1 antibody to FOXK1 and the FOXK2 antibody to FOXK2. However, since the blot does not display both molecular weight ranges for the FOXK1 and FOXK2 for each probe, it remains difficult to fully assess potential cross-reactivity between endogenous FOXK1 and FOXK2. Including both molecular weight ranges in the blot would help resolve this issue and effectively rule out cross-reactivity concerns.

We included molecular weight markers in supplementary Figures 4a, b, and c. Our data indeed demonstrates the specificity of the FOXK1 and FOXK2 antibodies. Thus, we confidently exclude the possibility of any cross-reactivity. We sincerely thank the reviewer for the critical revision of this work.