Supplemental Discussion

Below we provide a more extensive discussion on structural and AXIN1-related functional aspects of our manuscript:

Deleting the GSK3β binding domain from AXIN1 had the biggest impact on β-catenin signaling. This is not surprising given that the GSK3β kinase is not only required for directly phosphorylating the N-terminal residues of β-catenin resulting in its proteolytic breakdown, but also for phosphorylating APC and AXIN1/2 residues, leading to enhanced stability of the breakdown complex and stronger binding to β-catenin(1). The GSK3β domain of AXIN1 adopts an α-helical structure that binds to GSK3β through a hydrophobic interface. Most tumor-associated variants apparently do not seriously affect this structure and retain normal GSK3β binding. Among 18 tested variants, the only exception appears to be R395P, which will disrupt the hydrophobic packing of the interface over a larger region because the proline cannot be properly accommodated inside the α-helix due to steric constraints.

The N-terminal amino-acids of the GSK3β domain also bind to SIAH1/2 proteins(2). Besides the tankyrase enzymes that regulate AXIN1 stability at the N-terminus of AXIN1, SIAH proteins can independently ubiquitinate and breakdown AXIN1 by binding to the V383-X-P385 motif. The P385A variant investigated by us, was shown to disrupt this association(2). However, mutations that reduce binding to SIAH1/2 are not expected to contribute to tumorigenesis, as they would theoretically lead to a more stable AXIN1 variant that in fact will be more efficient in β-catenin regulation. A similar effect is expected for missense variants in the N-terminal 80 amino-acids that would reduce tankyrase binding, as these would also result in a more stable AXIN1 protein. Accordingly, none of 10 investigated variants within this domain clearly affected β-catenin signaling.

Deletion of the β-catenin binding domain of AXIN1 also led to strongly increased signaling, in addition to 4 out of 15 tested variants. Apparently, this increase is not directly resulting from a complete loss of β-catenin associating with the destruction complex, as β-catenin can still co-precipitate when the entire β-catenin domain is lost (**Fig. 4E**). Most likely, this results from β-catenin binding to co-precipitated GSK3β and APC. Accordingly, when we used a C-terminal AXIN1 construct lacking GSK3β and APC binding domains, we observed that the D461N, N466Y and V478G variants all showed reduced β-catenin binding. The β-catenin domain is located within close proximity of the GSK3β binding region. By failing to bind to this

domain of AXIN1, β-catenin most likely cannot interact properly with the kinase domain of GSK3β, resulting in an inefficient phosphorylation and breakdown of β-catenin.

In our hands, deletion of the entire RGS/APC domain (P81_R212del) significantly increased signaling, which is in apparent contrast to another report that unknowingly extended the deletion into the tankyrase domain(3). An accurate removal of the RGS/APC domain leads to increased signaling and fails to efficiently form degradasomes, visible as intracellular puncta. This was also the case for 13 out of 37 investigated variants (**Fig. 5A**). At large, we observe two classes of variants within the RGS/APC domain affecting β-catenin signaling. The first class are missense variants that affect directly the interaction with APC, either by their location directly within the binding interface (A120D, A143D), or being located in adjacent structural elements that support the AXIN1 interface (D113N/D113Y and R125W). The primary consequence of these variants is loss of APC binding. The second class consists of mutations that occur within the hydrophobic core of the RGS/APC domain, and are predicted to destabilize the entire RGS/APC domain, indirectly also leading to loss of APC binding. An additional feature of a subset of these core variants has been reported by Anvarian et al., showing that they induce the formation of nanoaggregates that affect β-catenin signaling in a dominant-negative manner by binding to a new repertoire of proteins(3). Nevertheless, restoring APC binding results in a partial rescue of activity even in those stronger variants, indicating that their effect on β-catenin signaling is at least in part resulting from lost APC binding. The exact role of APC in the destruction complex is still debated, but it has been shown to promote the capture, phosphorylation and ubiquitination of β-catenin(4). It also strongly facilitates puncta formation in cells as reviewed by Schaefer and Peifer(5), thereby increasing the local concentrations of proteins involved in β-catenin breakdown. In line with this, all tested variants that lost APC binding failed to efficiently form these puncta.

We tested all missense variants using overexpression in HEK293T cells. Quantitative PCR experiments showed that this leads to a more than 3000-fold overexpression on RNA level. Assuming that this also translates into a similar overexpression on protein level, it means that basically all AXIN1 protein incorporated into the breakdown complex is represented by the missense variants that we transfected. If that AXIN1 variant cannot bind to GSK3β, it strongly interferes with β-catenin regulation through the mechanism described above. Variants that lose APC binding have a more modest effect, most likely because APC can also

be incorporated into the breakdown complex indirectly by binding to β-catenin, albeit less efficiently.

This line of reasoning may also explain why the R395P variant when introduced into mouse livers through hydrodynamic transfection in combination with cMET, was basically the only variant that efficiently induced the formation of liver cancers. This method makes use of the Sleeping Beauty transposase to introduce several expression constructs in approximately 2-10% of the hepatocytes(6). Expression levels will be somewhat higher than endogenous levels, but are unlikely to reach the same levels obtained in the transient transfection experiments. As such, more of the breakdown complexes are expected to have retained endogenous wild-type Axin1. This implies that only when expression levels of transfected mutant AXIN1 are sufficiently high and of sufficient defective nature, it will effectively lead to an impairment of the breakdown complex below a critical threshold level. At that stage it can be regarded as a near-knockout of Axin1, which has been shown to effectively induce HCC formation when combined with cMET(7, 8). As the R395P and V478G variants appear to be more defective in β-catenin regulation than RGS/APC domain variants, it is more likely to bring overall destruction complex activity below this threshold.

References

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