Overexpression of the SETD2 WW domain inhibits the phosphor-IWS1/SETD2 interaction and the oncogenic AKT/IWS1 RNA splicing program.

Georgios I. Laliotis\textsuperscript{1,2,3,8,*}, Evangelia Chavdoula\textsuperscript{1,2}, Vollter Anastas\textsuperscript{1,2,4}, Satishkumar Singh\textsuperscript{5}, Adam D. Kenney\textsuperscript{6,7}, Samir Achaya\textsuperscript{1,2,9}, Jacob S. Yount\textsuperscript{6,7}, Lalit Sehgal\textsuperscript{2,5} and Philip N. Tsichlis\textsuperscript{1,2,*}

\textsuperscript{1}The Ohio State University, Department of Cancer Biology and Genetics, Columbus, OH, 43210, \textsuperscript{2}The Ohio State University Comprehensive Cancer Center-Arthur G. James Cancer Hospital and Richard J. Solove Research Institute, Columbus, OH, 43210, \textsuperscript{3}University of Crete, School of Medicine, Heraklion Crete, 71500, Greece, \textsuperscript{4}Tufts Graduate School of Biomedical Sciences, Program in Genetics, Boston, MA, 02111, USA, \textsuperscript{5}College of Medicine, Department of Hematology, The Ohio State University, Columbus, OH 43210, \textsuperscript{6}Department of Microbial Infection and Immunity, The Ohio State University, Columbus, OH, 43210

Running Title: SETD2 WW domain inhibits the IWS1 RNA splicing program

Present address:

\textsuperscript{8}Department of Oncology, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins School of Medicine, Baltimore, MD, 21231
\textsuperscript{9}Nationswide Children's Hospital, Columbus, OH, 43205

*Correspondence should be addressed to: Philip N. Tsichlis (Lead contact) (Philip.tsichlis@osumc.edu) and Georgios I. Laliotis (glaliot1@jhmi.edu)
Abstract

Our earlier studies had shown that AKT phosphorylates IWS1, and that following phosphorylation, IWS1 recruits the histone methyltransferase SETD2 to an SPT6/IWS1/ALY complex, which assembles on the Ser2-phosphorylated CTD of RNA Pol II. Recruited SETD2 methylates histone H3 at K36, during transcriptional elongation of target genes, and this regulates multiple steps in RNA metabolism. By regulating the RNA splicing of U2AF2, it controls cell proliferation. Importantly, pathway activity correlates with grade, stage and metastatic potential of lung adenocarcinomas, especially those with EGFR mutations. By regulating nucleocytoplasmic mRNA transport of intronless genes, including those encoding type I IFNs, it regulates sensitivity to viral infection. Here, we show that SETD2 interacts with IWS1 via its WW domain, that the interaction is IWS1 phosphorylation-dependent and that WW domain overexpression blocks the interaction and inhibits the pathway and its biological outcomes. We conclude that blocking the phosphor-IWS1/SETD2 interaction is feasible and has significant therapeutic potential in human cancer.
Introduction

Earlier studies had shown that IWS1 interacts with, and recruits SETD2 to an
SPT6/IWS1/ALY complex, which assembles on the Ser2-phosphorylated CTD of RNA Pol II
(Yoh et al., 2007) and that the SETD2 recruitment depends on the phosphorylation of IWS1
at Ser720/Thr721 by AKT (Sanidas et al., 2014, Laliotis et al., 2021). The binding of SETD2
to the SPT6/phospho-IWS1 complex results in trimethylation of histone H3 at K36 during
transcriptional elongation. Whereas the phosphorylation of IWS1 regulates the recruitment of
SETD2, it does not affect the interaction between IWS1 and the other components of the
SPT6/IWS1/ALY/SETD2 complex.

The recruitment of SETD2 to the SPT6/IWS1/ALY complex and the trimethylation of
histone H3 in the body of genes targeted by this complex, has profound effects on the biology
of NCI-H522 and other NSCLC cell lines. Our earlier studies had shown that it regulates the
alternative RNA splicing of FGFR2, favoring the FGFR2 IIIc splice variant (Sanidas et al.,
2014), which is primarily expressed in mesenchymal cells (Luco et al., 2010), and which, in
cancer cells, promotes epithelial to mesenchymal transition and cell migration, invasiveness
and metastasis (Thiery et al., 2006). RNA-Seq studies, comparing the gene expression
profiles of NCI-H522 cells transduced with shControl, or shIWS1 constructs, and shIWS1-
transduced NCI-H522 cells rescued with wild type IWS1 (WT-R) or with the phosphorylation
site IWS1 mutant (MT-R), revealed genome wide IWS1 and IWS1 phosphorylation-dependent
changes in gene expression and RNA processing (Laliotis et al., 2021). Importantly, many of
the genes whose expression and/or RNA processing were deregulated in shIWS1 and
shIWS1/MT-R cells, were genes encoding proteins involved in epigenetic regulation and RNA
processing.

One of the genes undergoing IWS1 phosphorylation-dependent alternative RNA
splicing, is the U2AF2 gene, which encodes the core RNA splicing factor U2AF65. The
predominant U2AF2 mRNA in empty vector (shControl) and shIWS1/WT-R cells contain exon
2, which is absent from the predominant U2AF2 mRNA in shIWS1 and shIWS1/MT-R cells.
Exon 2 encodes part of the N-terminal RS domain of U2AF65, which interacts with several
proteins involved in the regulation of RNA processing. One of these proteins is Prp19, a member of an RNA splicing complex with ubiquitin ligase activity (Prp19C), which is composed of four core and three accessory polypeptides and plays a critical role in spliceosomal activation (Chanarat et al., 2013). Prp19 is recruited to the phosphorylated CTD of RNA Pol II, via its interaction with U2AF65. In vitro RNA splicing experiments have shown that the U2AF65/Prp19 binding promotes the splicing of exons located downstream of weak polypyrimidine tracts (David et al., 2011). Our more recent studies provided evidence that by regulating U2AF2 RNA splicing, IWS1 phosphorylation promotes the expression of CDCA5, which encodes Sororin, a member of the Cohesin complex, and that by regulating this pathway, IWS1 phosphorylation plays a critical role in cell cycle regulation and cell proliferation (Laliotis et al., 2021). In addition, IWS1 phosphorylation-dependent RNA splicing promotes the nucleocytoplasmic transport of the mRNAs of intronless genes that harbor Cytoplasmic Accumulation Region Elements (CAR-E). This set of genes includes the genes encoding type I interferons, whose expression is impaired when the pathway is disrupted (Laliotis et al., 2021). Most important, the IWS1 phosphorylation pathway correlates positively with the grade, stage and metastatic potential of lung adenocarcinomas, especially those with activating mutations or amplification of the gene encoding EGFR. As a result, patients with EGFR mutant lung adenocarcinomas exhibit higher relapse rates after treatment, and shortened survival (Laliotis et al., 2021).

Based on the preceding observations, we hypothesized that targeting this pathway may have significant therapeutic implications in lung adenocarcinomas with EGFR mutations, and perhaps in other types of human cancer. Inhibiting AKT3, the AKT isoform primarily responsible for the activation of the pathway, should block this pathway. However, we do not currently have clinical grade AKT3 specific inhibitors and even if we did, inhibition of AKT3 would have multiple off target effects, because AKT3 does not only regulate this pathway. Based on these considerations, we proceeded to investigate whether inhibiting the interaction between IWS1 and SETD2 is feasible, and what would be the biological consequences of blocking this interaction. The IWS1 domain interacting with SETD2 had been mapped earlier...
to a sequence which extends from amino acid 522 to amino acid 698 and includes part of the TFIID homologous IWS1 domain (Yoh et al., 2008). As a first step therefore, we mapped the SETD2 domain interacting with phosphorylated IWS1, and we showed that it is limited to a 40 amino acid peptide, which includes the 30 amino acid WW domain of SETD2. Following mapping of the interacting domains, we showed that the WW domain of SETD2 interacts preferentially with the phosphorylated form of IWS1, and that when overexpressed, it blocks the interaction of the two proteins and inhibits the pathway activated via this interaction. More important, overexpression of the WW domain of SETD2, inhibits tumor cell proliferation in culture and tumor growth in xenograft models in animals, suggesting that blocking the interaction of SETD2 with phosphorylated IWS1 is feasible and has significant therapeutic potential in human cancer.

Results

The interaction between SETD2 and phosphorylated IWS1 is mediated by the WW domain of SETD2.

Our first task prior to developing strategies to block the AKT phosphorylation-dependent interaction between IWS1 and SETD2, was to map the SETD2 domain interacting with phosphorylated IWS1. To this end, we generated a series of Hemagglutinin (HA)-tagged deletion mutants of SETD2 (Fig. 1a), which we expressed via transient transfection in the NCI-H522 and NCI-H1299 lung adenocarcinoma cell lines and we used co-immunoprecipitation to determine their interaction with endogenous IWS1. Based on these experiments we mapped the IWS1-interacting SETD2 domain to a 40 amino acid peptide, which includes the 33 amino acid WW domain (aa 2388-2422) and surrounding sequences (Fig. 1b and 1c).
The WW domain of SETD2 binds selectively IWS1 phosphorylated at Ser720/Thr721 and disrupts the interaction of IWS1 with SETD2

Our earlier studies had shown that SETD2 is recruited to an SPT6/IWS1/ALY-REF complex, following phosphorylation of IWS1 at Ser720/Thr721, by AKT1 or AKT3. This observation raised the question whether the binding of the WW peptide of SETD2 to IWS1 also depends on IWS1 phosphorylation. To address this question, we expressed Flag-tagged constructs of wild type IWS1 or its phosphorylation site mutant in the lung adenocarcinoma cell lines NCI-H522, NCI-H1299, A549 and NCI-H1975. Following this, we transduced the cells with a V5-tagged construct of the SETD2 WW domain. Probing V5 (WW domain) immunoprecipitates with the Flag-tag (IWS1) antibody and Flag-tag immunoprecipitates with the V5 tag antibody, revealed that the WW domain of SETD2 indeed interacts with IWS1, but the interaction is significantly more robust with wild type than with mutant IWS1 (Fig. 2a). These observations strongly suggest that the interaction of the WW domain of SETD2 with IWS1 continues to be IWS1 phosphorylation-dependent and therefore physiologically relevant.

Following the demonstration that the WW domain of SETD2 interacts selectively with phosphorylated IWS1, we examined whether overexpression of this domain inhibits the IWS1/SETD2 interaction. First, we addressed the interaction of exogenous Flag-tagged wild type IWS1, or IWS1-S720A/T721A with HA-SETD2, and following this, the interaction of the endogenous proteins. Co-immunoprecipitation experiments confirmed that the overexpression of the WW domain of SETD2 inhibits the interaction of both the exogenous and the endogenous proteins (Fig. 2b and 2c).

Overexpression of the SETD2 WW domain inhibits the IWS1 phosphorylation-dependent RNA splicing program.

The observation that overexpression of the SETD2 WW domain blocks the interaction between phosphorylated IWS1 and SETD2, suggested that it would also interfere with IWS1
phosphorylation-dependent alternative RNA splicing. Our earlier studies identified several IWS1 phosphorylation-dependent RNA splicing targets, including \textit{U2AF2} (exon 2), \textit{SLC12A2} (exon 21), \textit{IFT88} (exon 8), \textit{C1qTNF6} (exon 3), \textit{STXB1} (exon 18) and \textit{FGFR2} (exon 8) (Laliotis et al., 2021\textsuperscript{3}, Sanidas et al., 2014\textsuperscript{2}). Transduction of the four lung adenocarcinoma cell lines used for the experiments in figure 2 with a pLx304-based lentiviral construct of the SETD2 WW domain revealed that overexpression of this domain reproduces the alternative RNA splicing pattern of the IWS1 knockdown in all six genes (Fig. 3a, 3d). To determine whether the WW domain-induced changes in alternative RNA splicing are due to inhibition of the IWS1 phosphorylation pathway, we examined the binding of SETD2 and the abundance of H3K36me3 marks, in selected regions of the \textit{U2AF2} and \textit{FGFR2} genes in NCI-H522 and NCI-H1299 cells transduced with the pLx304-WW domain construct. The \textit{U2AF2} and \textit{FGFR2} Transcription Start Sites (TSS) and \textit{GAPDH} Exon 3 (E3) were used as controls. This was addressed with chromatin Immuno Cleavage (ChIC) experiments, which showed that the WW domain of SETD2 dramatically inhibits the binding of SETD2 and the abundance of histone H3K36me3 marks (Fig. 3c, 3d). These experiments provided proof of principle for the feasibility of blocking the IWS1/SETD2 interaction and for the ability of the block to inhibit the IWS1 phosphorylation-dependent regulation of RNA processing. Based on our earlier findings on the biology elicited by the IWS1 phosphorylation pathway, these data also suggested the blocking this pathway by overexpressing the SETD2 WW peptide should have a major impact on the pathobiology of lung adenocarcinomas and perhaps other types of human cancer.

**Overexpression of the WW domain of SETD2 inhibits pathways regulated by the alternative RNA splicing of \textit{U2AF2}**.

The exon 2-deficient splice variant of \textit{U2AF2}, which is expressed in shIWS1 and shIWS1/MT-R cells, is defective in the processing of the \textit{CDCA5} pre-mRNA. This results in the downregulation of the \textit{CDCA5} protein product, Sororin. The latter is involved in the regulation of ERK phosphorylation, the expression of CDK1 and Cyclin B1, and the regulation of the G2/M phase of the cell cycle (Laliotis et al., 2021\textsuperscript{3}). Cells expressing the exon 2-deficient
U2AF2 are also defective in the nucleocytoplasmic transport of the mRNAs of a set of intronless genes that harbour CAR-Elements, including JUN, HSBP1 (encoding HSP27), IFNA1 and IFNB1 (encoding IFNα1 and IFNβ1 respectively) (Laliotis et al., 2021⁸). We therefore asked whether, by promoting the skipping of U2AF2 exon 2, the overexpression of the WW domain of SETD2 also inhibits the phosphorylation of ERK and the expression of CDK1 and Cyclin B1, as well as the expression and ERK-dependent phosphorylation of JUN, and the expression of HSP27 and type I IFNs. The results confirmed the prediction by showing that the WW domain of SETD2 reproduces the IWS1 knockdown ERK phosphorylation, cell cycle and RNA transport phenotypes (Fig. 4a, 4b). Type I IFNs in this experiment were induced by Sendai virus infection (Laliotis et al., 2021⁸) (Fig. 4b).

Collectively, these results show that overexpression of the SETD2 WW domain inhibits the endogenous IWS1/SETD2 interaction (Fig. 2), the binding of SETD2 and the abundance of H3K36me3 marks in select regions of target genes, the IWS1 dependent-alternative RNA splicing of the targets of the IWS1 phosphorylation pathway (Fig. 3), and signalling pathways controlled by these targets (Fig. 4).

**Overexpression of the SETD2 WW domain inhibits cell cycle progression and proliferation of lung adenocarcinoma cell lines, and anchorage-independent growth of Human Bronchial Epithelia Cells (HBECs) transduced with IWS1-S720D/T721E (IWS1/DE).**

Our earlier studies had shown that the IWS1 phosphorylation pathway is cell cycle-regulated, and that its inhibition profoundly affects the proliferation of lung adenocarcinoma cell lines. Given that the overexpression of the SETD2 WW domain inhibits this pathway, we examined its effects on the proliferation of four lung adenocarcinoma cell lines (NCI-H522, NCI-H1299, A549 and NCI-H1975) in culture. The same cells transduced with shIWS1 were used as controls. The results showed that the proliferation of 2D cultures of all four cell lines was significantly impaired when the cells were transduced with a lentiviral construct of the WW domain (Fig. 5a and Supplementary figure 1a). Flow-cytometric analysis of log phase
cultures of NCI-H522, NCI-H1299, A549 and NCI-H1975 cells transduced with shControl, shIWS1 or pLx304-SE TD2/WW constructs and stained with Propidium Iodide (PI), confirmed that the overexpression of the WW domain of SETD2 results in partial G2/M arrest, as expected (Fig. 5b).

To determine whether the partial G2/M arrest and the inhibition of cell proliferation by the overexpressed WW domain of SETD2 are because of the overexpression on the alternative RNA splicing of U2AF2, we examined the ability of the RS domain-containing U2AF65α and the RS domain-deficient U2AF65β to rescue the hypo-proliferative phenotype induced by the overexpression of the WW domain. The results confirmed that whereas U2AF65α rescues the phenotype, U2AF65β does not (Supplementary Fig. 1b and Fig. 5c).

However, the rescue was only partial, suggesting that overexpression of the WW domain of SETD2 may inhibit cell proliferation by targeting the alternative RNA splicing of U2AF2, in addition to other targets, which have not been identified to-date.

In parallel experiments, we overexpressed the SETD2 WW domain in human bronchial epithelial cells immortalized with the telomerase catalytic subunit hTERT, (hTERT-HBEC) and transformed with the constitutively active phosphomimetic mutant IWS1-DE. hTERT-HBECs transduced with the empty vector pLx304, were used as controls. The soft agar colony growth data in these cells showed that the WW domain inhibits anchorage-independent cell growth (Fig 5d). These data were consistent with the proliferation data in the lung adenocarcinoma cell lines, described in the preceding paragraph.

Overexpression of the SETD2 WW domain blocks the IWS1 phosphorylation pathway and inhibits the growth of NCI-H1299 mouse xenografts.

The data in the preceding paragraphs confirmed that the inhibition of cell proliferation and transformation by the SETD2 WW domain is due, at least in part, to the inhibition of the pIWS1/U2AF2/CDCA5 pathway, which based on our earlier observations, is strongly pro-oncogenic (Laliotis et al., 20213). This raised the question whether the SETD2 WW domain peptide inhibits the growth of lung adenocarcinoma mouse xenografts. The experiment addressing
this question was done, using two groups of NSG (NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ) mice (Fig. 6a). Group 1 was inoculated subcutaneously in the flanks with NCI-H1299 cells transduced with a lentiviral shIWS1 construct, or with the empty lentiviral vector (shControl) (7 mice each, 14 mice total). Group 2 was inoculated similarly with NCI-H1299 cells transduced with the same shIWS1 lentiviral vector used in group 1, or with a SETD2 WW domain construct in the lentiviral vector pLx304 (also 7 mice each, 14 mice total) (Fig 6a). The animals were monitored for tumor growth, and they were sacrificed 4 weeks later. The results confirmed that both the loss of IWS1 and the overexpression of the SETD2 WW domain, significantly reduce tumor growth. Notably, the inhibition of tumor growth in mice inoculated with shIWS1-transduced cells (Fig. 6b). This observation supports the hypothesis that the SETD2 WW domain may inhibit cell proliferation by targeting not only the alternative RNA splicing of <i>U2AF2</i>, but also other cell proliferation-promoting targets.

Based on the preceding data and the role of the IWS1 phosphorylation pathway in tumor cell proliferation in culture and in animals (Sanidas et al., 2014<sup>2</sup>, Laliotis et al., 2021<sup>3</sup> and this report), we hypothesized that the WW domain of SETD2 would inhibit the interaction of IWS1 with SETD2 in mouse xenografts, and by inhibiting the interaction, would also inhibit xenograft growth. This was confirmed with co-immunoprecipitation experiments, which showed that overexpression of the SETD2 WW domain indeed inhibits the interaction between IWS1 and SETD2 (Fig. 6c). The expression of the SETD2 WW domain and the abundance of IWS1 and phospho-IWS1 in these tumors, were confirmed by western blotting (Fig. 6d).

Using RT-PCR we also addressed the alternative RNA splicing of confirmed targets of the IWS1 phosphorylation pathway. The results showed that in mouse xenografts, like in cultured cells, overexpression of the SETD2 WW domain dramatically inhibits the IWS1 phosphorylation-dependent alternative RNA splicing pathway (Fig. 6d). Next, we probed western blots of tumor cell lysates with antibodies to Sororin, phospho-ERK (Y202/T204), CDK1, Cyclin B1, PCNA and Ki-67, all of which are regulated by the IWS1 phosphorylation-
dependent alternative RNA splicing of U2AF2 (Laliotis et al., 2021). The results confirmed that the WW domain also inhibits the phosphorylation of ERK and the expression of these regulators of the cell cycle (Fig. 6d). We conclude that overexpression of the SETD2 WW domain inhibits tumor growth, by inhibiting the IWS1/U2AF2/CDCA5 pathway and cell cycle progression.

IWS1 phosphorylation controls the alternative RNA splicing of FGFR2, favoring the FGFR2 mRNA splice variant IIIc. This variant is expressed primarily in mesenchymal cells, and it has been shown to promote epithelial to mesenchymal transition (EMT) in cancer cells. We therefore questioned whether the overexpression of the SETD2 WW domain inhibits the expression of EMT markers in the developing tumors. Immunohistochemistry experiments, addressing the expression of ZEB1, TWIST and Vimentin confirmed this prediction (Fig. 6d).

Representative examples of the histology of the tumors derived from shControl, shIWS1 and pLx304-SETD2 WW domain-transduced NCI-H1299 cells (Fig. 6e). The same figure also shows representative examples of immunohistochemistry experiments addressing the expression of the Ki-67 proliferation marker, ZEB1, TWIST and Vimentin in the same tumors. Whereas the histology of the tumors derived from the control cells was similar to the histology of the tumors derived from cells engineered to express the WW domain of SETD2, the results of the Ki-67 ZEB1, TWIST and Vimentin immunohistochemistry experiments revealed that overexpression of the WW domain of SETD2, resulted in the downregulation of all these markers (Fig. 6e). Quantification of the abundance of Ki-67 ZEB1, TWIST and Vimentin in all the same tumors confirmed the results (Fig. 6f) and was consistent with the western blot data (Fig. 6d).

Overall, the data in this report support the model in figure 7. According to this model, the SETD2 WW domain binds phosphorylated IWS1, and by doing so it antagonizes the interaction of phosphor-IWS1 with SETD2 and the recruitment of SETD2 to the RNA elongation complex assembling on the Ser2-phosphorylated CTD of RNA Pol II. Blocking the recruitment of SETD2 to the complex prevents histone H3K36 trimethylation in the body of target genes during transcriptional elongation. Given the importance of H3K36me3 marks in
RNA elongation and RNA splicing, the loss of these marks results in major shifts in RNA
splicing and other RNA processing events. This alters the IWS1 phosphorylation-dependent
H3K36me3 distribution and subsequent oncogenic AKT/IWS1 RNA splicing program, leading
to inhibition of tumor growth (Fig. 7).

In addition to showing that blocking the interaction of phosphor-IWS1 with SETD2 is
feasible and has profound effects on the biology of tumor cells, the data in this report also
provide information for the design of a strategy to screen for small molecules to inhibit the
interaction. Such molecules hold promise for the therapeutic targeting of the AKT/phosphor-
IWS1 pathway in lung adenocarcinomas and potentially other types of human cancer.

Discussion

Although AKT is known to play a major role in most types of human cancer, its
therapeutic targeting has been only minimally successful to-date. This may be due to several
reasons. First, there are three AKT isoforms which functionally overlap, but also have non-
overlapping, and in some cases opposing functions (Song et al., 201910, Wang et al., 201811).
This suggests that using inhibitors that target all AKT isoforms, which is the case for most of
the inhibitors available to-date, may have suboptimal results. Another reason is that AKT has
wide reaching effects in cell signalling, both in normal and in cancer cells (Manning et al.,
201712), and its inhibition may result in significant toxicity. One way to address these limitations
is to identify and selectively target AKT-regulated signalling pathways with critical roles in
human cancer. One such pathway is the IWS1 phosphorylation pathway, which is activated
by AKT in a cell cycle-dependent manner and epigenetically regulates transcription, RNA
metabolism and perhaps other epigenetically regulated processes, and has a major role in
human lung adenocarcinomas and most likely in other forms of human cancer (Sanidas et al,
20142, Laliotis et al, 20213). In this report we present our initial data on a strategy aiming to
selectively target this pathway.

The knockdown of IWS1 in human lung adenocarcinoma cell lines, and its rescue with
the S720A/T721A IWS1 mutant, which cannot be phosphorylated by AKT, result in significant
inhibition of cell proliferation in culture and tumor growth in xenograft models in immunocompromised mice. Our recent studies provided a mechanistic explanation for this observation, by showing that IWS1 is induced and undergoes phosphorylation by AKT during the transition from the G1 to the S phase of the cell cycle, and that following phosphorylation, it regulates the alternative RNA splicing of multiple genes, one of which is U2AF2, the gene encoding the core RNA splicing factor U2AF65. The predominant U2AF2 mRNA in cells with low abundance of phosphorylated IWS1 lacks exon 2, which encodes the RS domain of U2AF65, a domain that is required for the interaction of U2AF65 with additional RNA splicing regulators, including Prp19. The loss of the Prp19-interacting domain impairs the recruitment of the Prp19 complex (Prp19C) to the spliceosome, resulting in defects in the processing and nucleocytoplasmic transport of the mRNAs of multiple genes. One of these genes is CDCA5, which encodes Sororin, a member of the Cohesin complex (Zhang et al., 2012). The IWS1-regulated Sororin is phosphorylated by ERK and the phosphorylated Sororin enhances ERK phosphorylation by an unknown mechanism. This Sororin/ERK feedback loop promotes progression through the G2/M phase of the cell cycle and cell proliferation (Laliotis et al., 2021).

As indicated above, the IWS1 phosphorylation-dependent alternative RNA splicing of U2AF2 also regulates the nucleocytoplasmic transport of the mRNAs of a set of intronless genes, which harbor Cytoplasmic Accumulation Region Elements (CAR-E). The absence of IWS1 phosphorylation therefore, lowers the nuclear export of these mRNAs and the abundance of the proteins they encode. Genes, whose regulation by this mechanism has been confirmed, include JUN, HSPB1 (encoding Hsp27), IFNA1 and IFNB1 (encoding IFNα1 and IFNβ1 respectively). The abundance of these proteins may affect cell proliferation and survival in response to external signals, in addition to regulating the sensitivity to viral infection (Laliotis et al., 2021).

FGFR2 is also known to undergo alternative RNA splicing and our earlier studies had shown that its RNA splicing is also regulated by IWS1 phosphorylation. The latter promotes the skipping of exon 8, giving rise to the IIIc mRNA transcript, which is observed in
mesenchymal cells and is associated with Epithelial to Mesenchymal Transition (EMT). The loss of IWS1 phosphorylation on the other hand, promotes the inclusion of exon 8, giving rise to the IIIb mRNA transcript of FGFR2, which is observed in epithelial cells. By regulating the alternative RNA splicing of FGFR2, the IWS1 phosphorylation pathway also promotes EMT, cell migration and tumor cell invasiveness. By regulating these pathways, IWS1 phosphorylation contributes to cell transformation in culture and to tumor growth in animals. More important, this pathway is active in human lung adenocarcinomas, especially those with EGFR mutations, and the activity of the pathway correlates with tumor stage, histologic grade, metastasis, relapse after treatment, and poor prognosis.

The first step in the activation of the IWS1 pathway is the recruitment of SETD2 to an SPT6/IWS1/ALY-REF complex, which assembles on the Ser2-phosphorylated CTD of RNA Pol II. The signal for the recruitment of SETD2 is the phosphorylation of IWS1 by AKT. The work presented in this report focuses on the question whether we can block the interaction between phosphorylated IWS1 and SETD2 and whether blocking this interaction will result in the reversal of the known phenotypic effects of the pathway in cancer cells. Using deletion mutants of SETD2 and co-immunoprecipitation assays in HEK293 cells we mapped the IWS1-interacting domain of SETD2 to its WW domain, a short, 33 amino-acid peptide. The SETD2 WW domain is preceded by a proline-rich stretch and the same is observed in the WW domains of other proteins, including Huntingtin. NMR studies of the Huntingtin WW domain have shown that it interacts with the proline-rich stretch, and that the interaction between the two domains results in a closed conformation (Gao et al., 201414). If the interaction between these domains is conserved in SETD2 as expected, it may have an auto-inhibitory role. In this case, the binding to phosphorylated IWS1, may allosterically activate SETD2. This finding is significant because it suggests that the binding of SETD2 to the CTD complex does not only link SETD2 to the machinery that will allow it to methylate chromatin during transcriptional elongation, but that it may also activate it.

The WW domain is defined by the presence of two tryptophan (W) residues 20-23 aa apart (Vargas et al., 201915, Jäger et al., 200616). WW domains bind proline-rich protein...
regions, including proline-rich phospho-tyrosine and phospho-serine-threonine sites (Ingham et al., 2005\textsuperscript{17}, Sudol et al., 2010\textsuperscript{18}, Salah et al., 2012\textsuperscript{19}). By binding to their targets, they transduce signals along signalling cascades and they regulate the cytoskeleton and cell polarity (Lin et al., 2019\textsuperscript{20}, Ilsley et al., 2002\textsuperscript{21}). Changes in the interaction between WW domains and their targets have been linked to genetic disorders, such as the Liddle syndrome (Pucheta-Martinez et al., 2016\textsuperscript{22}, Chang et al., 2019\textsuperscript{23}, Sudol et al., 2012\textsuperscript{24}) muscular dystrophy, Huntington’s chorea, Alzheimer’s disease (Kunkle et al., 2019\textsuperscript{25}) and cancer (Salah et al., 2011\textsuperscript{26}, Lee et al., 2012\textsuperscript{27}, Hung et al., 2020\textsuperscript{28}). Based on the motifs they bind, five classes of WW domains have been identified to-date. The most common is Class I, which recognizes the sequence (L/P)Pp(Y/poY), where L and P stand for Leucine and Proline respectively, identifies a phosphorylated residue and lower case letters represent favoured, but not conserved residues (Salah et al., 2012\textsuperscript{19}). The IWS1 domain interacting with SETD2 extends from amino acid 522 to amino acid 698 (Yoh et al., 2008\textsuperscript{9}). Analysis of this sequence identified several Class I and Class II motifs, which may be recognized by the WW domain of SETD2 (Supplementary Fig. 2). However, the role of these motifs in the phospho-IWS1/SETD2 interaction remains to be determined.

Data in figure 5 showed that the proliferation defect induced by the overexpression of the WW domain of SETD2 in lung adenocarcinoma cell lines is rescued, but only partially, by the RS domain containing U2AF65 isoform, U2AF65\textalpha. Moreover, the data in figure 6, showed that the growth inhibition of tumor xenografts engineered to overexpress the WW domain of SETD2, is more robust than the growth inhibition induced by the IWS1 knockdown. These data combined, suggest that the overexpression of the WW domain of SETD2 may inhibit cell proliferation by targeting the alternative RNA splicing of U2AF2, in addition to other targets that have not been identified to-date. p53, which interacts with the WW domain of SETD2 via its N-terminal transactivation domain (aa 1-45) is unlikely to be one of these targets, because its interaction with SETD2 stimulates the expression of many of the anti-proliferative and pro-apoptotic genes it regulates (Xie et al, 2008\textsuperscript{29}). Therefore, blocking its interaction with SETD2 should inhibit the expression of these genes. In addition, p53 should not be relevant to some
of the cell lines we used in this study, including NCI-H1299, which is p53-null, and perhaps NCI-H522 and NCI-H1975, which harbor point mutations in p53. Another protein known to interact with the WW domain of SETD2 is Huntingtin (Gao et al., 2014, Seervai et al., 2020).

SETD2 bound to Huntingtin and the actin-binding adaptor HIP1R, trimethylates Actin at K68. ActK68me3 localizes to the F-actin cytoskeleton and regulates actin polymerization/depolymerisation. Blocking actin methylation, which is expected to occur in cells overexpressing the WW domain of SETD2, inhibits cell migration (Seervai et al., 2020) and perhaps cell survival (Cisbani et al., 2012).

In summary, data presented in this report, provide robust evidence that selective inhibition of the IWS1 phosphorylation pathway has a strong therapeutic potential. In addition, they show that blocking the interaction between phosphorylated IWS1 and SETD2 is feasible and it can effectively inhibit the pathway. Finally, they provide a roadmap for strategies that can be employed to identify small molecule inhibitors of the pathway.
Methods

Cells and Culture conditions.
NCI-H522, NCI-H1299, A549, NCI-H1975 and HBEC-hTERT cells were grown in Roswell Park Memorial Institute 1640 medium (Sigma-Millipore, Cat No. D8758) and HEK-293T cells were grown in Dulbecco’s modified Eagle’s medium (Sigma-Millipore, Cat No. D5796) supplemented with penicillin/streptomycin (Corning, Cat No. 30-002-CL), nonessential amino acids (Corning, Cat No. 25-025-CL), glutamine (Corning, Cat No. 25-005-CL), plasmocin 2.5ng/uL (Invivogen, Cat No. ant-mpp) and 10% fetal bovine serum. Cells were used for up to 5 passages. Cell lines were also periodically checked for mycoplasma, using the PCR mycoplasma detection kit (ABM, Cat No. G238). All experiments were carried out in mycoplasma-free cultures.

shRNAs and expression constructs
The origin of the shRNAs and expression constructs is described in Supplementary Table 3. The SETD2 deletion mutants were cloned in pENTR/D-TOPO cloning vector (Invitrogen, Cat. No. 45-0218), with PCR-based techniques, using pENTR SETD2-HA wt clone as substrate (Open Biosystems MHS6278-211691086, Clone ID : 40125713, corresponding to NM_014159.6, Sanidas et al., 20142, Laliotis et al., 20213). To ensure successful amplification and separation, the PCR products were run in 1% agarose gel and were gel-purified using the NucleoSpin Gel and PCR Clean-Up kit (M&N, Cat. No. 740609.50). Following cloning in the entry vector, the two clones were recombined with Gateway™ pcDNA™-DEST40 Vector (Thermofisher, Cat No #12274015) for transient transfection or with the pLx304 V5-DEST (Addgene #25890) for stable expression, using standard Clonase II LR mix (Thermofisher, Cat No 11791100). The retroviral expression vector for the two U2AF65 isoforms was created by recombining pENTR U2AF65 (α or β isoform) (Laliotis et al., 20213), with pMSCV N-Flag-HA IRES puro (Addgene #41033). Using Clonase II LR as well. The primers used for the cloning are listed in Supplementary Table 3. All constructs were sequenced in Genomic Shared Resource of The Ohio State University, prior to use.
Transfections and infection

Retroviral constructs were packaged by transient transfection of these constructs in HEK-293T cells, in combination with ecotropic (Eco-pac) or amphotropic (Ampho-pac) packaging constructs. Lentivirus constructs were also packaged in HEK-293T cells by transient transfection of the constructs in combination with the packaging constructs psPax2 (Addgene #12260) and pMΔ2.G (Addgene #12259). Transfections were carried out using 2x HEPES Buffered Saline (Sigma, Cat. No 51558) and CaCl₂ precipitation. After 48 hours of transient transfections of HEK-293T cells, the supernatant were collected and filtered. Infections were carried out in the presence of 8 μg/ml polybrene (Sigma, Cat. No. 107689). Depending on the selection marker in the vector, 48 hours after the infection, cells were selected for resistance to puromycin (Gibco, Cat. No. A11138) (10 μg/ml), G-418 (Cellgro, Cat. No. 30-234) (500μg/ml), or blasticidin (Gibco, Cat. No A1113903) (5 μg/ml). Cells infected with multiple constructs, were selected for infection with the first construct, prior to the next infection. The transient transfection of the HA-SETD2 deletion mutants in NCI-H522 and NCI-H1299 were performed using 2x HEPES Buffered Saline and CaCl₂ precipitation. After 48 hours the cells were harvested.

Virus propagation, titering and infections

For the stimulation of the type I IFN, the NCI-H522 and NCI-H1299 were subjected to infection with Sendai-GFP (SeV-GFP) (Yount et al., 2006³²), as previously described (Laliotis et al., 2021⁸). Briefly, the cells were infected by using an MOI=0.5 for 16h and MOI of 0.25 for 24h, respectively. Sendai virus expressing GFP (SEV-GFP) was propagated in 10-day-old embryonated chicken eggs at 37°C for 40 hours and titered on Vero cells.

Cell Proliferation assay

The cell proliferation assay was performed as previously described (Laliotis et al., 2021⁸). Briefly, the cells were harvested by trypsinization, counted and plated evenly in 12 well tissue
culture plates in 3 biological replicates for each condition. For all the cells and conditions 5,000 cells/well were plated. Photomicrographs were taken every 6 hours using an Incucyte live cell imager (Essen Biosciences, Ann Arbor, MI) depending on growth parameters of each cell line (NCI-H522 7 days, NCI-H1299 7 days, A549 7 days and NCI-H1975 12 days total acquisition). Images were taken and analyzed using the Incucyte confluence masking software (Essen Biosciences, Ann Arbor, MI)

**Cell Transformation assay**

The cell transformation assay was performed and analyzed as previously described (Laliotis et al., 2021), using the Cell Transformation Assay Kit-Colorimetric (Abcam Cat No. ab235698). Briefly, 1x10^4 HBEC hTERT cells per condition were mixed with top agarose layer in 10x DMEM solution and plated in 96-well plate, in triplicates along with three blank wells. The cells were then plated for 7 days in 37°C and monitored for colony formation. After 7 days, the cells were imaged in the Incucyte live cell imager using the 20x lens. Then, the cells were incubated for 4 hours on WST working solution at 37°C. The absorbance at 450nm was determined with a plate reader.

**FACS analysis**

The FACS assays was performed and analysed as previously described (Laliotis et al., 2021). Briefly, the cells were plated in equal numbers and they were harvested from semi-confluent cultures 48 hours later. The cellular pellet was resuspended in 700uL ice-cold 1x PBS and fixed in 2.8mL ethanol, overnight at -20°C. Following two washes with 1x PBS, the fixed cells were stained with Propidium Iodide mix (Propidium Iodide (1:2500) (Invitrogen, Cat. No. P3566), 0.1 mg/mL RNAse A (Invitrogen, Cat. No. 12091-039), 0.05% Triton-X) and incubated in the dark at 37°C for 30 minutes. Subsequently, the cells were analysed on the BD FACSCalibur (BD Biosciences, San Jose, CA). The analysis was performed in the Flow Cytometry Shared Resource (FCSR) of the Ohio State University.
RT-PCR and qRT-PCR

Total cell RNA was extracted using the PureLink RNA Kit (Invitrogen, Cat. No 12183018A), as previously described (Laliotis et al., 2021). Briefly, cDNA was synthesized using oligo-dT priming and the QuantiTect Rev. Transcription Kit (QIAGEN, Cat No. 205310) and the gene and exon expressions were quantified by quantitative real time RT-PCR using the iTaq™ Universal SYBR® Green Super mix (Biorad, Cat No. 1725121) and a StepOne Plus qRT-PCR machine (Thermofisher). Data was normalized to hGAPDH or human 18S rRNA, which was used as an internal control. The primer sets used for all the real time PCR assays throughout this report are listed on the Supplementary Table 2.

Western Blotting

Western blotting was performed as previously described (Laliotis et al., 2021). Briefly, the cells were lysed using a RIPA lysis buffer (50 mM Tris (pH 7.5), 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 0.5% Sodium deoxycholate, 1% NP-40 and fresh 1x Halt™ Protease and Phosphatase Inhibitor Cocktails (Thermofisher, Cat. No 78444). The clarified lysates were electrophoresed (20μg protein per lane) in SDS-PAGE. Electrophoresed lysates were transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore Cat No. IPVH00010) in 25 mM Tris and 192 mM glycine. The membranes were probed with antibodies (at the recommended dilution), followed by horseradish peroxidase-labeled secondary antibodies (1:2500), and they were developed with Pierce ECL Western Blotting Substrate (Thermo Scientific, cat. no 32106). The antibodies used are listed in Supplementary Table 1.

Immunoprecipitation

Immunoprecipitation assays was performed as previously described (Laliotis et al., 2021). Briefly, cells were lysed using a cytosolic Lysis Buffer 1 (LB1), Lysis Buffer 2 (LB2) and nuclear
Lysis Buffer 3 (LB3). Following overnight incubation with the immunoprecipitating antibody (Supplementary Table 1) or the rabbit Isotype Control (Thermofisher, Cat. No 10400C) at 4°C, the conjugates were subjected to multiple washes with LB3 and 300uL of the clarified lysates were added in the Magnetic beads-Antibody conjugates, followed by overnight incubation at 4°C. Following several washes with LB3, they were electrophoresed (20μg protein per lane) in SDS-PAGE, as described in Western Blotting section.

Image acquisition and figure preparation

For the western blotting images, the acquisition was performed in Li-Cor Fc Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE) using the 700 nm (protein ladder detection), 800 nm (reduced background and increased sensitivity) and chemi luminescent (protein bands) detection using a linear acquisition method. For the DNA agarose gels, the acquisition was performed in Li-Cor Fc Odyssey Imaging System using the 600 nm channel using a linear acquisition method. Identical approach was followed for all the images presented in this report to ensure unbiased analysis. In both conditions, the images were exported in high-quality image files (600 dpi png files) and further imported in Adobe Illustrator 2021 (Adobe, San Jose, CA) for figures preparation. The summary figures were designed in Bio Render (https://biorender.com).

Chromatin Immuno-Cleavage (ChIC)

In order to address the SETD2 and H3K36me3 abundance of IWS1-dependent targets, we utilized ChIC assays as previously described (Laliotis et al., 2021, Skene et al 2013). Briefly, 2.5x10^5 cells were washed several times with wash buffer. Magnetic Biomag Plus Concanavalin A Beads (Bangs Laboratories, Cat. No. BP531) were activated with multiple washes using a binding buffer. Prior to use, the immunoprecipitation antibodies (Supplementary Table 1) or the Rabbit Isotype Control (Thermofisher, Cat. No 10500C), were diluted in 1:50 dilution in 50 uL antibody buffer]. Then, the activated beads were resuspended with the antibody buffer, containing the immunoprecipitated antibody, and mixed with the cell
fraction. Following overnight incubation at 4°C, the immunoprecipitates were subjected to multiple washes with the wash buffer. Similarly to the primary immunoprecipitating antibody, the Guinea Pig anti-Rabbit IgG (Heavy & Light Chain) secondary antibody (Antibodies-Online, Cat. No. ABIN101961) was diluted in 1:50 dilution in 50uL antibody buffer, was mixed with the immunoprecipitates. Subsequently, the immunoprecipitates were subjected to multiple washes and mixed with the CUTANA™ pAG-MNase (EpiCypher, Cat No. SKU: 15-1116) at 700 ng/mL. The targeted digestion was activated with 100mM CaCl₂ and occurred by incubation on ice for 30 minutes. The reaction was terminated with addition of 2x stop buffer and the chromatin fragments were released by incubation at 37°C for 10 minutes. Subsequently, the chromatin fragments were extracted with DNA Purification Buffers and Spin Columns (Cell Signaling, Cat. No 14209). Real-time PCR using different sets of primers to amplify the genomic loci was carried out as described in the qRT-PCR section. The data were analysed using the analysis substrate file provided online by Sigma-Aldrich, calculating the fold enrichment. (https://www.sigmaaldrich.com/technical-documents/articles/biology/chip-qpcr-data-analysis.html).
Tumor xenografts

*Ethics statement:* All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Ohio State University. IACUC protocol number 2018A0000126; P.I.: Philip Tsichlis.

*Procedure:* The mouse xenograft engraftment was performed as previously described (Laliotis et al., 2021). 2x10⁶ NCI-H1299 cells per injection were mixed with 30% Matrigel (Corning, Cat. No. 356231) in PBS for a total volume of 200 µl and implanted subcutaneously into either side (left side for the shControl and right side for the shIWS1 group 1 and lift side for the shIWS1 and right side for the pLx304 SETD2 WW group 2) of immunocompromised 6 week old NSG (NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl/SzJ</sup>) female mice. The mice were monitored every 3 days and the size of the tumors was measured using a digital caliper. The tumor volume was calculated by use of the modified ellipsoid formula: \[ V = \frac{1}{2} \times 1 \times s^2 \] (1/2 Length × Width²). The mice were sacrificed 4 weeks after the injection. The tumors were removed and their weights were measured. One part of the dissected tumors was immediately snap frozen in liquid nitrogen prior to RNA and protein isolation, and a second part was fixed overnight in 10% (v/v) formalin (Sigma, Cat. No. HT501640), transferred to 70% EtOH and then embedded in paraffin at the Comparative Pathology & Mouse Phenotyping Shared Resource of the Ohio State University Comprehensive Cancer Center, prior to immunohistochemistry (IHC) staining.

**RNA and protein isolation from the mouse xenograft tumors**

The RNA and protein extraction from the mouse tumors was performed as previously described (Laliotis et al., 2021). Briefly, 50-100 mg of the frozen mouse xenograft samples were homogenized using 1mL Trizol reagent (Thermofisher Scientific, Cat. No. 15596026). RNA and protein were extracted according to the manufacturer’s instructions. Both protein and RNA materials from the tumors were further processed with immunoblotting and qRT-PCR, respectively, for the expression of various targets described in this report.
The IHC staining from the mouse tumors was performed as previously described (Laliotis et al., 2021). Briefly, sections of 5 μm from the paraffin embedded mouse tumors were heated to 55°C for 20 min prior to deparaffinization in xylene (Fisher scientific, Cat. No. X3F-1GAL). The slides were then rehydrated through graded ethanol concentrations up to distilled water. The endogenous peroxidase activity was blocked at RT by a 10 min incubation in the final developmental 3% H$_2$O$_2$ (Fisher Scientific, Cat. No. H325500) in PBS (pH 7.4), followed by antigen retrieval at 80 °C for 30min using the Citrate Buffer, pH 6.0, Antigen Retriever (Sigma, Cat. No. C9999). The Vectastain Elite ABC Universal kit peroxidase (Horse Anti-Mouse/Rabbit IgG) (Vector Laboratories, Cat. No. PK-6200) was used for blocking and incubation with the primary and secondary antibody according to the manufacturer’s instructions. After a 5 min wash with 1x PBS the slide was incubated for 30 min with the Vectastain Elite ABC reagent followed by a 2-10 min incubation with a DAB peroxidase substrate solution (Vector Laboratories, Cat. No. SK-400) according to the manufacturer’s instructions. The slide was then washed in tap water and covered with the DPX mounting medium (Sigma, Cat. No. 06522).

All images were captured on the Nikon eclipse 50i microscope with attached Axiocam 506 color camera using the ZEN 2.6 blue edition software (Zeiss). Image processing and analysis was further performed using the ImageJ software, as described in the following section.

Imaging files were imported to ImageJ (Schneider et al., 2012) and analyzed as previously described (Laliotis et al., 2021). For each slide, at least 5 different areas of the tumor were scanned. The average value of the signal divided to its different area, was the final value of
the analysis. Identical approach and settings were followed for all the images to ensure unbiased analysis.

**Data availability**

All the raw data underlying figures 1-6 (uncropped gel images, qPCR, FACS, plates reader and proliferation data) and microscope images derived from this report have been deposited in the Mendeley Dataset in a publicly available dataset (Laliotis et al., 2021). Specific P values are also included in these datasets.

**Statistics and reproducibility**

The experiments in Fig. 1b-c, 2a-2c, 4a-4b were performed twice. The experiments in Fig. 3a-d and 5a-5d were performed at least in 3 independent biological experiments. The data in figure 6 (mouse xenografts) were performed once, using 7 mice/group. The Western blot analysis, IP experiments, RT-PCR assays and IHC staining of xenografts derived tumors was performed once with the techniques outlined in the methods section. All the statistical analysis was performed in GraphPad Prism 9.1, as described in the corresponding section. All the statistical analysis reports can be found in the Mendeley dataset where the source data of this report were deposited. (Laliotis et al., 2021)

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**Author Contributions**

**G.I.L.** Conceptualization, overall experimental design. Performed experiments, analysed the data, prepared the figures and wrote the manuscript. **E.C.** Designed and performed the mouse xenografts, protein and RNA extraction of mouse tumours, performed the mouse xenografts staining studies and picture acquisition and edited the manuscript. **V.A.** Designed, performed, analysed the proliferation experiments and edited the manuscript. **S.S.** Assisted in FACS experiments. **A.D.K.** Designed, optimized and performed the infection with Sendai virus and edited the manuscript. **A.K.K.** Performed RT-PCR experiments and assisted in cloning. **K.A.N.** Performed RT-PCR experiments and assisted and assisted in cloning. **S.A.** Assisted in the design for SETD2 deletion mutants and edited the manuscript. **J.S.Y.** Advised on the viral strain infection and edited the manuscript. **L.S.** Advised on the design of experiments and edited the manuscript. **P.N.T.** Conceptualization, overall experimental design, project supervision, manuscript writing and editing.

**Competing Interests**

The authors declare no competing interests.

**Corresponding authors**

Correspondence to: Philip N. Tsichlis (lead contact) and Georgios I. Laliotis

**References**


screen reveals akt isoform-specific signals linking RNA processing to lung cancer.


https://doi.org/10.1038/s41467-021-24795-1


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**Figure Legends**

**Figure 1. The interaction between SETD2 and phosphorylated IWS1 is mediated by the WW domain of SETD2.**

A. Graphical representation of the SETD2 deletion mutants (1-8) used in this study. AWS: Associated with SET, SET: Su(var)3–9, Enhancer-of-zeste, Trithorax, PS: post-SET, LCR: Low Charge Region, WW, SRI: Set2 Rpb1-interacting.

B. and C. NCI-H522 and NCI-H1299 cells were transiently transfected with the SETD2 deletion mutants. Anti-HA, anti-IgG rabbit isotype control immunoprecipitates and input lysates were probed with the indicated antibodies.

**Figure 2. SETD2-WW domain binds phosphorylated IWS1 and disrupts the endogenous IWS1-SETD2 interaction.**

A. NCI-H522, NCI-H1299 (upper panel), A549 and NCI-H1975 (lower panel), were transduced with the indicated constructs. Anti-V5, anti-IgG rabbit isotype control immunoprecipitates and input lysates were probed with the indicated antibodies.

B. NCI-H522, NCI-H1299 (upper panel), A549 and NCI-H1975 (lower panel), were transduced with the indicated constructs. Anti-Flag, anti-IgG rabbit isotype control immunoprecipitates and input lysates were probed with the indicated antibodies.

C. NCI-H522, NCI-H1299 (upper panel), A549 and NCI-H1975 (lower panel), were transduced with the indicated constructs. Anti-IWS1, anti-IgG rabbit isotype control immunoprecipitates and input lysates were probed with the indicated antibodies.
Figure 3. The overexpression of SETD2 WW domain inhibits the downstream IWS1 phosphorylation-dependent RNA splicing program.

A. Overexpression of the SETD2 WW domain inhibits the IWS1-dependent RNA splicing program. (Upper lanes) NCI-H522, NCI-H1299, A549 and NCI-H1975, were transduced with the indicated constructs and their lysates probed with the indicated antibodies. (Lower lanes) RT-PCR examining the exon usage of the known IWS1 RNA splicing targets U2AF2 (exon 2), SLC12A2 (exon 21), IFT88 (exon 8), STXBP1 (exon 18) and C1qTNF6 (exon 3). GAPDH was used as control.

B. Quantitative RT-PCR showing the E2/E3 and IIIb/IIIc ratio in the U2AF2 (upper panel) and FGFR2 (lower panel) mRNA transcripts in shControl, shIWS1 and pLx304 SETD2 WW-V5 NCI-H522, NCI-H1299, A549 and NCI-H1975 cells. Bars show the E2/E3 or IIIb/IIIc ratio (mean ± SD) in all the cell lines, relative to the shControl.

C. and D. ChIC assays of SETD2 and H3K36me3 abundance in the indicated regions of U2AF2, FGFR2 and GAPDH genes, in the same cells as in 3A. Bars show the mean fold enrichment (anti-SETD2 IP and anti-H3K36me3 IP vs anti-IgG IP) ±SD. Data were normalized relative to the input (2%). All assays were done in triplicate, on three biological replicates. n.s : non-significant *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (one-side unpaired t-test)

Figure 4. The overexpression of SETD2 WW domain inhibits the downstream IWS1 phosphorylation-dependent pathways.

A. The indicated cells as in 3A, were probed with the indicated antibodies of the Sororin/ERK phosphorylation pathway.

B. Western blots of lysates of the same cells, were probed with the indicated antibodies. For type I IFN induction SeV-GFP infection was performed (NCI-H522 : MOI=0.5 for 16h, NCI-H1299 : MOI of 0.25 for 24h of SeV-GFP virus).
Figure 5. The overexpression of SETD2 WW domain inhibits cell proliferation, cell cycle progression and cell transformation in lung adenocarcinoma.

A. Growth curves of the indicated NCI-H522, NCI-H1299, A549 and NCI-H1975 cells in media supplemented with 10% FBS. Cell proliferation was measured every 6 hours in three independent cultures and expressed as confluence mean percentages±SD. For simplicity, 12h points are shown. P values were calculated for the endpoint measurements, using the one-side unpaired t-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

B. Cell cycle profiles of the indicated propidium iodide (PI)-stained cell lines. Figure shows one representative, out of three biological replicates. Numbers in red show the mean percentage of cells in different phases of the cell cycle and the P value using unpaired one-sided t test between shCon vs shIWS1 or vs pLx304 WW.

C. Growth curves of the indicated NCI-H522, NCI-H1299, A549 and NCI-H1975 cells in media supplemented with 10% FBS. Live-cell numbers (mean ±SD) were measured with the alamarBlue™ Cell Viability Reagent in three independent cultures for each cell type. The expression of V5-U2AF65 isoforms is shown in Supplementary Figure 1A.

D. (Upper panel) HBEC hTERT cells transduced with the indicated constructs, were subjected to cell transformation assay for 7 days. At that time point the cells were imaged. Scale bar in the right corner of each image. (Lower left panel) Validation of the cells used in the transformation assay by probing western blot lysates with the indicated antibodies. (Lower right panel) After imaging, the cells were incubated for 4 hours in WST solution as described in the Cell Transformation Assay Kit (Abcam). Bars show the number of transformed cells as measured in absorbance 450nm ±SD. All assays in this figure were done in triplicate, on three biological replicates. n.s : non-significant *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (one-side unpaired t-test).
Figure 6. The overexpression of SETD2 WW domain inhibits tumor growth and the IWS1 phosphorylation pathway in vivo.

A. Summary of the experimental design. Schematic of the mouse groups used in this study (Upper panel), along with their downstream applications (Lower panel). Specifically, 2 mice from each group were used for IP experiments, while 5 were used for RNA and protein extraction. The NCI-H1299 shIWS1-derived tumors of group 1 were stored for future use.

B. NSG mice were injected subcutaneously with shControl or shIWS1 (group 1) or shIWS1 or pLx304 SETD2 WW (group 2) 2x10^6 NCI-H1299 cells. (N=7 mice/group). Images of the induced tumors, harvested at 4 weeks from the time of inoculation. Scatter plots showing the tumor weight and volume of the tumors. The horizontal lines indicate mean tumor weight or volume. Statistical analyses were done using one-sided paired t-test.

C. The overexpression of the SETD2 WW domain inhibits the IWS1/SETD2 interaction in vivo. The indicated tumors from the xenografts model in 6a were harvested and subjected to IP experiments. The anti-IWS1 IP and anti-IgG immunoprecipitation and input lysates were probed with the indicated antibodies.

D. The overexpression of the SETD2 WW domain inhibits the IWS1-dependent pathways in vivo. Cell lysates derived from the indicated mouse xenograft tumors, were probed with the indicated antibodies. RT-PCR of U2AF2 E2, SLC12A2 E21, IFT88 E8, C1qTNF6 E3, STXBP1 E18 from the same tumors.

E. Formalin-fixed, paraffin-embedded tumor samples from the experiment in Figure 6a were stained with the indicated antibodies. Secondary antibody was HRP-labelled. Scale in the right lower corner of each image.

F. Scatter plots showing IHC signal of the indicated marker relative to the section area in the mouse xenograft tumors. The horizontal line shows the mean signal in the indicated
groups of xenografts. Statistical analyses were performed, using the one-sided paired t-test.

**Figure 7. Graphical abstract of the findings.**

The overexpression of the SETD2 WW domain antagonizes the interaction of phosphorylated IWS1 with SETD2 and its recruitment in IWS1-dependent RNA splicing targets. This affects the H3K36me3 distribution and subsequent oncogenic AKT/IWS1 RNA splicing program, inhibiting tumor growth. This interaction and chemical affinity of the WW domain can be used as a drug screening platform for the IWS1/SETD2 complex in lung adenocarcinoma.
Figure 1

a

SETD2

AWS SET PS LCR WW SRI

HA 260 kDa 1

HA 125 kDa 2

HA 117 kDa 3

HA 105 kDa 4

HA 23 kDa 5

HA 29 kDa 6

HA 8 kDa 7

HA 10 kDa 8

b

IWS1

HA-tag

IWS1

Lamin A/C

NCI-H522

IP : HA-tag

10% input

NCI-H1299

β-actin
Figure 2
Figure 4

(a) Western blot analysis of Sororin, p-ERK (Y202/T204), ERK, Cyclin B1, CDK1, and α-actinin in NCI-H522, NCI-H1299, A549, and NCI-H1975 cells transfected with shCon, shIWS1, pLX304 WW, or pLX304 WW + SeV-GFP.

(b) Western blot analysis of p-c-Jun (S73), c-Jun, Hsp27, β-actin, and IFNβ1 in NCI-H522 and NCI-H1299 cells transfected with shCon, shIWS1, pLX304 WW, or pLX304 WW + SeV-GFP.
Figure 7

AKT/IWS1 oncogenic RNA splicing program

Target genes

SETD2

IWS1

Spts

CTD

RNA Pol II

Lung Adenocarcinoma Tumor Growth

Inhibition of the AKT/IWS1 oncogenic RNA splicing program

Target genes

SETD2

IWS1

Spts

CTD

WW SETD2

RNA Pol II

Inhibition of Tumor Growth

blocking the IWS1/SETD2 interaction

S720/T721