IWS1 phosphorylation by AKT kinase controls the nucleocytoplasmic export of type I IFNs and the sensitivity of lung adenocarcinoma cells to oncolytic viral infection, through *U2AF2* RNA splicing.

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Type I IFNs orchestrate the antiviral response. Interestingly, *IFNA1* and *IFNB1* genes are naturally intronless. Based on previous work, the splicing factor U2 Associated Factor 65 (U2AF65), encoded by *U2AF2*, and pre-mRNA Processing factor 19 (Prp19) function on the Cytoplasmic Accumulation Region Elements (CAR-E), affecting the nuclear export of intronless genes. We have previously shown that the loss of IWS1 phosphorylation by AKT3, promotes the alternative RNA splicing of *U2AF2*, resulting in novel transcripts lacking exon 2. This exon encodes part of the Serine-Rich (RS) domain of U2AF65, which is responsible for its binding with Prp19. Here, we show that IWS1 phosphorylation and the *U2AF2* RNA splicing pattern affect the nuclear export of introless mRNAs. We also demonstrate that the same axis is required for the proper function of the CAR-Es. Mechanistically, whereas both U2AF65 isoforms bind CAR-E, the recruitment of Prp19 occurs only in cells expressing phosphorylated IWS1, promoting intronless genes' export. Moreover, analysis of Lung adenocarcinoma patients showed that high p-IWS1 activity correlates with the assembly of the U2AF65/Prp19 complex and export of intronless genes, *in vivo*. Accordingly, the expression of type I IFNs was decreased in cells deficient in IWS1 phosphorylation and the viral infection was increased. Furthermore, following infection with oncolytic virus, we observed reduced activation of p-STAT1 and expression of Interferon Stimulated Genes (ISG), in cells stimulated by shIWS1-derived supernatant, or cells treated with the pan-AKT inhibitor, MK2206. Consistently, killing curves and apoptosis assays after infection with oncolytic viruses, revealed increased susceptibility upon the loss of IWS1, with subsequent activation of Caspase-mediated death. The treatment of the lung adenocarcinoma cells with MK2206, phenocopied the loss of IWS1 phosphorylation. These data identify a novel mechanism by which the AKT/p-IWS1 axis, by hijacking the epigenetic regulation of RNA splicing and processing, contributes to the resistance to oncolytic viral infection, suggesting that combined inhibition of the splicing machinery and AKT/p-IWS1 signals would sensitize tumors to oncolytic viral treatment.

Introduction

Type I interferons (IFNs) are members of a large signaling family of proteins, known for their robust antiviral response. Specifically, type I IFNs are encoded by 13 *IFNA* genes and a single *IFNB* gene (Frisch et al., 2020[1]). These genes are rapidly induced by Pattern Recognition Receptors (PRRs), the sensors of innate immunity (Acosta et al., 2020[2]). These receptors recognize molecules presented by pathogens (pathogen-associated molecular patterns, PAMPs), such as double-stranded RNA (dsRNA), bacterial lipopolysaccharides, flagellin, bacterial lipoproteins, and cytosolic DNA. (Amarante-Mendes et al., 2018[3]). Signals from PRRs converge upon IKK-family kinases to phosphorylate and activate two transcription factors, IRF3 and NF-kB; these factors directly transactivate the *IFNB1* gene (Ablasser et al., 2020[4]). IFNβ in an autocrine or paracrine manner and activates the IFNAR1/2 receptors, resulting in Janus kinase (JAK)-mediated phosphorylation of STAT1 and STAT2, forming a canonical complex of STAT1/STAT2/IRF9, known as the Interferon Stimulated Gene Factor 3 (ISGF3) complex. This activated complex transcribes target genes (interferon-stimulated genes, ISGs) containing ISRE (ISGF3 response elements), that limit viral replication (Aleynick et al., 2019[5]).

Activation of the type I IFN pathway is critical in antiviral immunity and in mediating a wide array of innate immune responses. Modulating this pathway is not only critical for controlling antiviral and inflammatory responses, but it also offers translational applications. One of the most important translational applications of the activation of this pathway is the usage of oncolytic viruses (OV). OVs have gained momentum in recent years because of their immune-stimulating effects, both systemically and in the local tumor microenvironment (Park et al., 2020[7]). The first clinically approved OV, Talimogene laherparepvec (TVEC), is a genetically modified type I herpes simplex virus (HSV) that expresses granulocyte-macrophage colony-stimulating factor (GM-CSF) (Rehman et al., 2016[7]). While

TVEC is routinely used in select patients with melanoma, most other OVs have not shown robust antitumor efficacy in clinical trials, especially when used as monotherapy (Martinez-Quintanilla et al., 2019[8]). Thus, deeper comprehension of the molecular pathways involved in the efficacy of these therapeutic approaches is a necessity for the proper design of antitumor oncolytic therapies.

One way to improve the response to OV therapy is through the manipulation of multiple signaling pathways regulating the type I IFN response. Among them, the AKT pathway regulates the IFN response at multiple levels, with many of its activities being isoform-specific. Early studies have shown that by activating the mechanistic target of rapamycin (mTOR), AKT promotes the translation of ISGs (Kroczynska et al., 2014[9]). Subsequent studies revealed that AKT1 activates β-catenin, promoting the transcriptional activation of *IFNB1* (Gantneret al., 2012[10]). In addition, we have shown that AKT1 selectively phosphorylates EMSY at S209, relieving the EMSY-mediated repression of ISGs (Ezell et al., 2012[11]).

Interestingly, genes encoding *IFNA1* and *IFNB1* are naturally intronless (de Padilla et al., 2014[12]). In contrast to splicing-dependent mRNA export, little is known regarding the nuclear export of intronless mRNAs. Based on previous reports, components of the Transcription-Export (TREX) complex, the pre-mRNA Processing Factor 19 (Prp19) complex and the splicing factor U2 Associated-Factor 2 (U2AF2) associate with designated Cytoplasmic Accumulation Region Elements (CAR-E), a 10-nt consensus element on intronless genes, and function in their mRNA export (Lei et al., 2013[13]). These data suggest the presence of an additional layer in the regulation of type I IFN expression.

Based on our previous observations, IWS1 (Interacts With Spt6) is an RNA processing factor and phosphorylation target of AKT kinase at S720/T721 (Sanidas et al., 2014[14]). More recently we revealed that IWS1 phosphorylation affects genome-wide the alternative RNA splicing program in human lung adenocarcinoma (Laliotis et al., 2020[15]). In

one example, the loss of phosphorylated IWS1 resulted in a novel, exon 2 deficient splice variant of the splicing factor *U2AF2.* This exon encodes part of the U2AF65 Serine-Rich (RS) Domain, which is required for its binding with Prp19, further affecting the downstream splicing machinery. We also provided evidence that the *U2AF2* exon 2 inclusion depends on phosphorylated IWS1, by promoting histone H3K36 trimethylation by SETD2 and the assembly of LEDGF/SRSF1 splicing complexes, in a cell-cycle specific manner (Laliotis et al., 2020[16]).

Since IWS1 phosphorylation controls the U2AF65-Prp19 interaction through the epigenetic regulation of *U2AF2* alternative RNA splicing, we sought to investigate the effect of this pathway in the nucleocytoplasmic export of naturally intronless genes, including *IFNA1* and *IFNB1.* In this study, our goal was to elucidate the molecular mechanisms, following IWS1 phosphorylation and the subsequent hijacking of alternative RNA splicing program, that affect the nucleocytoplasmic export of intronless mRNAs and to highlight a novel role of AKT kinase signaling in the regulation of type I IFN response and sensitivity of lung adenocarcinoma cells to infection with oncolytic viral strains.

Results

IWS1 phosphorylation regulates the nucleocytoplasmic transport of intronless gene mRNAs, via *U2AF2* alternative RNA splicing.

Our earlier studies have shown that the knockdown of IWS1 and its replacement by the non-phosphorylatable mutant IWS1 S720A/T721A, altered the RNA splicing pattern of *U2AF2* (Laliotis et al., 2020[15]). The predominant novel splice variant of the *U2AF2* mRNA that we identified in these cells, lacks exon 2, which encodes part of the N-terminal RS domain of U2AF65. This domain is responsible for the interaction of U2AF65 with Prp19, a component of the seven-member ubiquitin ligase complex Prp19C (Laliotis et al., 2020[15], R.

Hogg et al., 2010[17], Chanarat S. et al., 2013[18]). More importantly, U2AF65 and Prp19C, along with components of the TREX complex, bind elements designated as Cytoplasmic Accumulation Region Elements (CAR-E) in naturally intronless mRNAs, promoting their nucleocytoplasmic transport (Lei et al., 2013[13]). Examples of mRNAs whose nucleocytoplasmic transport is regulated by this mechanism include *IFNA1, IFNB1, JUN and HSBP3* (Lei et al., 2013[13]).

Based on this knowledge, we hypothesized that IWS1 phosphorylation affects the nucleocytoplasmic export of these mRNAs, through the alternative RNA splicing of *U2AF2* and the U2AF65-Prp19 interaction. First, we engineered shControl, shIWS1, shIWS1/wild type IWS1 rescue (shIWS1/WT-R), shIWS1/phosphorylation site IWS1 mutant rescue (shIWS1/MT-R) and shIWS1/U2AF65α (exon 2-containing) or U2AF65β (exon 2-deficient) rescue NCI-H522 and NCI-H1299 lung adenocarcinoma cells. Notably, the IWS1 rescue clones used are engineered to be shRNA resistant (Sanidas et al., 2014[14]). Subsequently, we confirmed the significant down-regulation of IWS1 expression after the transduction of the cells with a lentiviral shIWS1 construct and the shRNA-resistant rescue of IWS1 knock-down with the Flag-tagged wild type protein and phosphorylation IWS1 site mutant (**Fig. 1A**). In the same cells, we performed RT-PCR investigating the inclusion of *U2AF2* E2. Consistent with our previous results, *U2AF2* exon 2 inclusion occurred in shControl and shIWS1/WT-R cells (**Fig. 1A**) (Laliotis et al., 2020[15]).

To further investigate our hypothesis, we fractionated the nuclear and cytosolic compartments of shControl, shIWS1, shIWS1/WT-R, shIWS1/MT-R, shIWS/U2AF65α, and shIWS1/U2AF65β NCI-H522 and NCI-H1299 cell lines, and calculated the Cytoplasmic to Nuclear (C/N) ratio of the set of intronless genes with qRT-PCR. Notably, in order to induce the transcription of type I IFNs (*IFNA1 and IFNB1*), we infected cells with the murine paramyxovirus, Sendai fused with GFP (SeV-GFP), which is a potent and commonly used tool for induction of type I IFNs (Yount et al., 2006[19], Bedsaul et al., 2016[20]). First, using

western blotting, we confirmed the validity of the fractionation by the detection of Lamin A/C and GAPDH only in the nuclear and cytosolic protein compartment, respectively (**Fig. S1A**). The results showed decreased Cytosolic/Nuclear ratio in shIWS1 and shIWS1/MT-R, due to nuclear retention of these mRNAs, which was rescued in the shIWS1/WT-R cells. More importantly, consistent with our hypothesis, the ectopic expression of U2AF65α, but not the U2AF65β, rescued the nuclear retention phenotype (**Fig. 1B**).

Next, we questioned whether IWS1 also affects the transcription of these intronless genes. To this extent, we examined their total RNA levels using qRT-PCR in shControl, shIWS1, shIWS1/WT-R, shIWS1/MT-R, shIWS1/U2AF65α and shIWS1/U2AF65β NCI-H522 and NCI-H1299 cells. Interestingly, we found that the total levels of type I IFN, *JUN* and *HSBP3* were upregulated in the shIWS1 and shIWS1/MT-R cells. Interestingly, the levels of type I IFN, but not *JUN and HSBP3*, were affected by the *U2AF2* RNA splicing pattern (**Fig. 1C**). We interpret these data to suggest that the upregulation of the mRNAs of IFN genes in shIWS1 cells may be due to different mechanisms than the upregulation of the mRNAs of other intronless genes. To expand the significance of these observations in human lung adenocarcinoma, analysis of the publicly available data of The Cancer Genome Atlas (TCGA), revealed a negative correlation of *IWS1* levels with the total RNA levels of intronless genes in patients with Lung adenocarcinoma (**Fig. S1B, left panel**). Notably, consistent with the *in vitro* results, analysis of the Reverse Phase Protein Assay (RPPA) in the same patients, revealed a significant positive correlation of p-c-Jun (S73) with p-AKT (S473) levels and the *IWS1* and *AKT3* RNA levels (**Fig. S1B**), further supporting the role of IWS1 in the nucleocytoplasmic export of RNAs transcribed from intronless genes, in human lung adenocarcinoma.

Given that only exported mRNAs are translated into protein products, we then examined the protein expression of c-Jun, Hsp27, and IFNβ1 with Western blotting in NCI-H522 and NCI-H1299 cells. Similarly, SeV-GFP infection was performed in order to

maximize the type I IFN induction. Consistent with the qRT-PCR data, the results showed reduced expression of the protein products of naturally intronless genes in shIWS1 and shIWS1/MT-R cells, compared to shControl cells, an effect rescued in shIWS1/WT-R. More importantly, the U2AF65α isoform rescues the shIWS1 phenotype, but the U2AF65β did not (**Fig. 1D**). These data support the hypothesis that IWS1 phosphorylation controls the nucleocytoplasmic RNA export transcribed from naturally intronless genes, through a process that depends on *U2AF2* alternative RNA splicing.

To provide additional support to our model, protein interaction analysis using the STRING database (Szklarczyk et al., 2019[21]) showed the expected interaction of U2AF65 and Prp19 with components of the TREX complex and export machinery, THOC2 and Ref/Aly (**Fig. S1C**). Furthermore, analysis of the previously performed RNA-Seq study in NCI-H522 shControl, shIWS1, shIWS1/WT-R, and shIWS1/MT-R cells, (Laliotis et al., 2020[15]), using Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005[21]), revealed significant enrichment of genes affecting nuclear export in cells expressing phosphorylated IWS1 (**Figure S1D**). Taken together, these results support a multi-layered role of IWS1 phosphorylation in the nuclear export machinery.

AKT3 kinase affects the nucleocytoplasmic export of intronless RNAs, through IWS1 phosphorylation.

IWS1 is phosphorylated by AKT3, and to a lesser extent by AKT1, at Ser720/Thr721 (Sanidas et al., 2014[14]). The preceding data show that IWS1 phosphorylation is required for the nucleocytoplasmic export of RNAs transcribed from a set of intronless genes. This raised the question of whether AKT, which phosphorylates IWS1 on Ser720/Thr721, is required for the activation of the pathway. To address that, we treated NCI-H522 and NCI-H1299 cells

with 5 μM of the pan-AKT inhibitor MK2206, a dose that fully inhibits all AKT isoforms (Sanidas et al., 2014[14], Laliotis et al., 2020[15]). The results confirmed that MK2206 inhibits AKT (S473) and IWS1 phosphorylation (S720) along with the *U2AF2* exon 2 inclusion, as expected (**Fig. 2A**). Following proper fractionation of these cells upon treatment with MK2206 (**Fig. S2A**), we examined the C/N ratio of *IFNA1, IFNB1* (induced by SeV-GFP infection), *JUN* and *HSBP3* RNA with qPCR and their protein expression. The results confirmed that inhibition of AKT kinase phenocopies the loss of phosphorylated IWS1 resulting in nuclear retention of intronless RNAs and subsequent downregulation of their protein products in both NCI-H522 and NCI-H1299 cell lines (**Fig. 2B, 2C**). To determine whether it is the AKT3 isoform, which is responsible for the observed effects of AKT inhibition, we transducted NCI-H522 and NCI-H1299 cells with lentiviral shAKT3 construct, along with shControl (**Fig. 2D**) and we examined its effects on the nucleocytoplasmic export and expression of the same set of RNAs, following successful compartment fractionation (**Fig. S2B**). The results phenocopied the inhibition with MK2206 (**Fig. 2E, 2F)**, providing evidence that the observed regulation of nucleocytoplasmic export depends on the phosphorylation of IWS1 by AKT3, through *U2AF2* RNA splicing.

IWS1 phosphorylation is required for the proper function of the Cytoplasmic Accumulation Region Elements (CAR-E).

It has been previously reported that the consensus of Cytoplasmic Accumulation Region Element (CAR-E) functions by promoting the cytoplasmic accumulation of mRNAs and the production of proteins (Lei et al., 2013[13]). To further study the role of IWS1 phosphorylation in the function of the CAR-E, we used a previously described construct, in which 16 tandem copies of the most conserved CAR-E (CCAGTTCCTG element of *JUN*) or its mutated version (CAR-E_{mut}), were inserted upstream of β-globin cDNA (**Fig. 3A**) (Lei et

al., 2013[13]). As controls, pCMV promoter-driven constructs encoding β-globin cDNA alone or β-globin gene containing its two natural introns were analyzed in parallel. Based on previous work, the β-globin gene mRNA, efficiently accumulates in the cytoplasm, whereas the β-globin cDNA transcript, is normally degraded in the nucleus (Dias et al., 2010[23], Lei et al., 2011[24], Valencia et al., 2008[25]).

Transient transfection of these constructs in shControl, shIWS1, shIWS1/WT-R, shIWS1/MT-R, shIWS1/U2AF65α, and shIWS1/U2AF65β NCI-H522 and NCI-H1299 cells (**Fig. 3B, S3A**), showed proper expression of the HA-β-globin gene in all conditions, independent of IWS1 phosphorylation. Furthermore, consistent with previous reports, β-globin cDNAs containing the inserted mutated CAR-E element (CAR-E_{mut}), employed a similar nucleocytoplasmic accumulation to the β-globin cDNA transcripts alone, with limited protein expression of β-globin detected in all conditions, due to nuclear retention. In addition, the insertion of 16 tandem CAR-E was efficient to induce the expression of HA-β-globin in shControl cells. Strikingly, the same construct was unable to promote the expression of CAR-E HA-β-globin in shIWS1 and shIWS1/MT-R cells, a phenotype rescued in shIWS1/WT-R cells. More importantly, the observed shIWS1 CAR-E HA-β-globin nuclear retention phenotype was rescued by U2AF65α, but not U2AF65β. These results suggest that IWS1 phosphorylation is required for the maintenance of CAR-E function, through *U2AF2* RNA splicing.

The splicing of the *U2AF2* mRNA downstream of IWS1 phosphorylation regulates the Prp19 recruitment on the CAR-E.

Based on our previous data, IWS1 phosphorylation is required for the function of the CAR-E (**Fig. 3**). As mentioned above, it has been previously demonstrated that U2AF65 and Prp19 affect the nucleocytoplasmic export of naturally intronless RNAs, by binding to the CAR-E (Lei et al., 2013[13]). Given that aberrant splicing of *U2AF2* in shIWS1 and

shIWS1/MT-R cells resulted in the loss of the interaction between U2AF65 and Prp19 (Laliotis et al., 2020[15]), we hypothesized that IWS1 phosphorylation affects the recruitment of the regulatory U2AF65-Prp19 complex to the CAR-E elements and, eventually, their function.

To address this hypothesis, we performed RNA Immunoprecipitation (RIP) assays in shControl, shIWS1, shIWS1/WT-R, shIWS1/MT-R, shIWS1/U2AF65α and shIWS1/U2AF65β NCI-H522 and NCI-H1299 cells, using a set of primers specifically amplifying the CAR-E or control regions on *IFNA1, IFNB1, JUN and HSPB3* mRNAs (**Fig. S4A**). The results confirmed that both spliced variants of U2AF65 bind equally well with the CAR-E, but not the control sequences (**Fig. 4A, 4B upper panels**). However, Prp19 binding to the same CAR-E regions was significantly impaired in shIWS1 and shIWS1/MT-R cells, which predominantly express the U2AF65β isoform (**Fig. 4A lower panels**). More importantly, the impaired Prp19 binding on the CAR-E was rescued by U2AF65α, but not U2AF65β (**Fig. 4B lower panels**). These results suggest that IWS1 phosphorylation controls the nucleocytoplasmic export of RNAs transcribed from intronless genes, by the regulation of the U2AF65-Prp19 interaction, through *U2AF2* alternative RNA splicing.

To further investigate the activity of this pathway in Lung Adenocarcinoma patients (LUAD), we used 3 high and 3 low p-IWS1 LUAD patients, as identified in our previous work (Laliotis et al., 2020[15]). Western blot and RT-PCR analysis of these samples confirmed the increased expression and phosphorylation of IWS1 in the high-expressing groups with parallel inclusion of *U2AF2* exon 2 (**Fig. 4C upper panels**). Following successful fractionation (**Fig. S4B**), qRT-PCR showed decreased Cytosolic/Nuclear ratio in the low p-IWS1 group, due to nuclear retention of intronless mRNAs (**Fig. 4C lower panel**). More importantly, consistently with the *in vitro* data, RIP experiments in the same tumor samples revealed increased binding of U2AF65 on the CAR-E in both patient groups, independent of its splicing pattern, with decreased recruitment of Prp19 on the CAR-E mRNA areas in the

low p-IWS1 group, which predominantly express the U2AF65β (**Fig. 4D**). These data suggest that the epigenetic complexes that are controlled by IWS1 phosphorylation and regulate the RNA export of intronless genes, are active in Lung Adenocarcinoma patients.

RNA-Pol II affects the splicing-independent nuclear export of intronless genes.

Based on previous reports, U2AF65 binds RNA Pol II, leading to an U2AF65-dependent recruitment of Prp19 to the newly-synthesized pre-mRNA and promoting proper co-transcriptional splicing activation (C.J David et. al., 2011[26]). In order to address the possible involvement of RNA Pol II in the splicing-independent RNA export, we cloned *IFNA1* and *IFNB1* cDNAs in the lentiviral vectors pLx304 and pLKO.1, which drive their expression through CMV (RNA Pol II-dependent) and U6 (RNA Pol III-dependent) promoters, respectively (**Fig. S5A**) (Schramm et al., 2002[27]). We then transduced NCI-H522 and NCI-H1299 cells with lentiviral shIFNα1 or shIFNβ1, in order to remove the endogenous mRNA products. Subsequently, we rescued their expression with the RNA Pol II (pLx304-R) or RNA Pol III-driven (pLKO.1-R) lentiviral construct and addressed the expression of *IFNA1* or *IFNB1* with qRT-PCR, following SeV-GFP infection. The results confirmed the significant downregulation of *IFNA1* and *IFNB1* expression in the shIFNα and shIFNβ cells, respectively, with robust expression in both pLx304-R and pLKO.1-R cells. (**Fig. 5A left panel**). To further investigate the role of RNA Pol II in the nucleocytoplasmic export of type I IFNs, we fractionated the same NCI-H522 and NCI-H1299 cells (**Fig. S5B**) and measured the C/N ratio with qRT-PCR, following SeV-GFP infection. Interestingly, the results showed nuclear retention and downregulation of IFNβ1 levels in the pLKO.1-R cells, an effect rescued in pLx304-R cells. (**Fig. 5A right panels, 5B**). These results suggest that RNA Pol II is involved in the regulation of the splicing-independent RNA export of naturally intronless RNAs.

The loss of IWS1 phosphorylation enhances viral infection through the aberrant p-STAT1 signaling and transcription of ISGs.

The preceding data suggest that IWS1 phosphorylation affects the nucleocytoplasmic export and expression of type I IFNs, through the regulation of the U2AF65-Prp19 interaction and their recruitment to CAR-E elements of *IFNA1 and IFNB1* mRNAs. Given that type I IFNs orchestrate the cellular antiviral response (Lazear et al., 2019[28]), we hypothesized that loss of IWS1 phosphorylation will accordingly increase viral replication, due to the downregulation of type I IFNs. To this extent, we infected NCI-H522 and NCI-H1299 shCon, shIWS1, shIWS1/WT-R and shIWS1/MT-R cells with a set of viral strains including Vesicular Stomatitis Virus (VSV-GFP), Sendai (SeV-GFP), Reovirus and Influenza A virus (IAV-GFP) and we monitored the levels of infections with flow cytometry (Chesarino et al., 2015[29], Kenney et al., 2019[30], Sermersheim et al., 2020[31]). To carry out these experiments we used short hairpin RNA constructs in a pGIPZ vector we modified by deleting the GFP cassette. Consistently, the results showed increased infection by all viral strains in shIWS1 and shIWS1/MT-R cells, a phenotype rescued in shIWS1/WT-R cells, which parallels the nucleocytoplasmic distribution and reduced expression of type I IFNs. (**Fig. 6A, S6A**).

Based on the latter data, we then questioned whether the observed downregulation of type I IFNs upon the loss of phosphorylated IWS1 affects the IFN downstream signaling pathways and the induction of Interferon Stimulated Genes (ISGs). To address this question, we infected NCI-H522 shControl and shIWS1 cells with VSV-GFP virus. After 16h of infection, the cells were harvested for RNA and the supernatant derived from each condition was used to stimulate NCI-H522 parental cells, for various time intervals (**Fig. 5B**). Western blot analysis of protein extracts from these intervals, revealed robust activation of p-STAT1 (Y701) after the stimulation of NCI-H522 cells with shControl-derived supernatant, with a

parallel expression of IFNβ1, phenomenon not triggered by the stimulation with the shIWS1-derived supernatant (**Fig. 5C, upper panel**).

The increase in the abundance of IFNβ1, within 10 minutes from the start of the stimulation, was surprising because it was too rapid to be due by the induction of the *IFNB1* gene. Previous studies had shown that IFNβ1 undergoes endocytosis and that it can be siloed in endosomes, where it can be detected for days following IFN treatment (Altman et al., 2020[32]). Based on this information, we hypothesized that IFNβ1 detected in this experiment was endocytosed from the supernatants of shControl cells. To address this hypothesis we treated parental NCI-H522 cells with recombinant human IFNβ1 and we probed the cell lysates harvested at sequential time points with antibodies to IFNβ1. The results confirmed the rapid accumulation of the recombinant IFNβ1 in the harvested cell lysates (**Fig. S6B**).

To further support our data, and given that p-STAT1 is a main component of the Interferon Stimulated Gene Factor 3 (ISGF3) for the transcriptional induction of ISGs (Wang et al., 2017[33]), we performed Chromatin Immuno Cleavage (ChIC) assays in NCI-H522 cells stimulated for 30' with shControl or shIWS1-derived supernatant, along with unstimulated cells. Consistent with the p-STAT1 activation pattern, the results showed increased binding of p-STAT1 (Y701) to the Interferon Stimulated Response Elements (ISREs) of the major ISGs *IRF1, IRF9, STAT1,* and *STAT2*, in the shControl-derived stimulated cells (**Fig. 6C, lower panel**). Notably, total RNA extracted from NCI-H522 shControl, shIWS1, shIWS1/WT-R, and shIWS1/MT-R cells following VSV-GFP infection, revealed robust downregulation of a set of 20 ISGs in shIWS1 and shIWS1/MT-R cells, a phenotype rescued in shIWS1/WT-R cells (**Fig. 6D**). More importantly, the treatment of NCI-H522 cells with the clinically used pan-AKT inhibitor MK2206 followed by VSV-GFP infection phenocopied the ISGs signature downregulation upon loss of IWS1 phosphorylation (**Fig. 6E**) and parallels the expression of type IFNs and p-STAT1 activation. Altogether, these data suggest that

IWS1 phosphorylation by AKT kinase, regulates viral replication, through the nucleocytoplasmic export of type I IFNs and the subsequent activation of p-STAT1 and induction of ISGs.

Inhibition of the AKT/p-IWS1 axis sensitizes lung adenocarcinoma cells to oncolytic viral killing.

Based on the preceding data, loss of IWS1 phosphorylation enhances viral replication by affecting the induction of p-STAT1 and ISGs, due to impaired type I IFNs expression. We then hypothesized that IWS1 would affect the sensitivity of lung adenocarcinoma cells to oncolytic viral infection, through the regulation of type I IFN response. To determine whether IWS1 phosphorylation affects the sensitivity of lung adenocarcinoma cells to infection with oncolytic viruses, we used the viral strains VSV-GFP and Reovirus, which are widely tested as potential virotherapy in a variety of solid tumors, including lung cancer (Schreiber et al., 2019[34], Villalona-Calero et al., 2016[35]). By infecting NCI-H522 and NCI-H1299 shControl, shIWS1, shIWS1/WT-R, and shIWS1/MT-R cells with increasing Multiplicity of Infection (MOI), we observed increased viral-induced killing of shIWS1 and shIWS1/MT-R cells by VSV and Reovirus, after 16h and 48h of infection respectively, compared to shControl and shIWS1/WT-R cells (**Fig. 7A, S7A**). It is worth mentioning that according to our previous observations, the NCI-H522 and NCI-H1299 shIWS1 cells do not exhibit proliferation deficits upon 48h, compared to the shControl cells, suggesting that the observed effect is solely due to the viral infection (Laliotis et al., 2020[15]). More importantly, the treatment of NCI-H522 and NCI-H1299 cells with the pan-AKT inhibitor MK2206, sensitized the cancer cells to viral killing by VSV and Reovirus, phenocopying the loss of IWS1 phosphorylation (**Fig. 7B, S7B**).

To further demonstrate the cellular events following the infection with the oncolytic viruses, NCI-H522 and NCI-H1299 shControl and shIWS1 cells were infected with VSV

(MOI=1) for several time point intervals, and cleavage of PARP, a hallmark of Caspase-mediated death, was examined by Western Blotting (Chaitanya et al., 2010[36]). Strikingly, the results revealed robust PARP-cleavage in the shIWS1 cells, observed at the early time points compared to the shControl cells, further supporting our hypothesis (**Fig. 7C, S7C**). These data come in agreement with the effect of IWS1 phosphorylation on the nucleocytoplasmic distribution of type I IFNs (**Fig. 1, Fig. 2**), the function of the CAR-E elements (**Fig. 3, Fig 4**), the pattern of viral infection and the activation of p-STAT1 and the downstream ISGs (**Fig. 5**).

Altogether, these data confirm our model in which, IWS1 phosphorylation by AKT kinase at S720/T721 controls the epigenetic regulation of *U2AF2* alternative RNA splicing, leading to the inclusion of exon 2. The exon 2-containing U2AF65α isoform binds and recruits Prp19 on the CAR-E of *IFNA1* and *IFNB1*, promoting their nucleocytoplasmic export. During oncolytic viral infection, the latter facilitates proper type I IFN expression, activation of the p-STAT1/ISGF3, and transcription of ISGs, enhancing the resistance of lung adenocarcinoma cells to oncolytic viral infection (**Fig. 8**)

Discussion

Our results implicate IWS1 phosphorylation as a regulator of the nucleocytoplasmic export of naturally intronless RNAs and the type I IFN response. We report a pathway initiated by the AKT3-mediated phosphorylation of IWS1 (**Fig. 1, Fig. 2**), which induces the alternative RNA splicing of *U2AF2* (Laliotis et al., 2020[37]). This shift in the alternative RNA splicing pattern controls the interaction of U2AF65 with yet another splicing factor, Prp19. These two factors interact and regulate the nucleocytoplasmic export and expression of naturally intronless genes *IFNA1, IFNB1, JUN* and *HSPB3,* by affecting the function of CAR-E and directly binding on these elements (**Fig. 3, Fig. 4**). Subsequently, our findings

suggest that inhibition of this pathway enhances viral replication in the infected cells, due to the downregulation of type I IFNs. Notably, this effect on viral replication is mediated by aberrant activation of p-STAT1 signals and active transcription of ISGs (**Fig. 6**). Finally, this manipulation of type I IFNs response through the inhibition of the AKT/p-IWS1 axis sensitizes lung adenocarcinoma cells to oncolytic viral killing mediated by Caspase signals (**Fig. 7**).

An important conclusion, based on the data presented in this report, is that the epigenetic regulation of alternative RNA splicing, through IWS1 phosphorylation, along with the downstream effect on splicing machinery, affects the splicing-independent nuclear export of naturally intronless mRNAs. AKT kinase-mediated signals, through IWS1 phosphorylation, affect *U2AF2* alternative RNA splicing and the U2AF65-Prp19 interaction and their function on the splicing-independent export. This is also supported by previous work showing that splicing factors are also associated with CAR-E (Lei et al., 2013[13]) and that many of them interact with adapter proteins of the export machinery (Müller-McNicoll et al., 2016[38]). These data provide insight into the explanation that splicing factors provide the backbone for protein-protein interactions with export machinery components, inducing the export of these genes. In addition, we provide evidence that IWS1 phosphorylation is necessary for the function of the CAR-E (**Fig. 3**). Given that these elements have been identified in the majority of intronless genes (Lei et al., 2011[24], Valencia et al., 2008[25], Lei et al., 2013[13]), our data suggest a global role of the AKT/p-IWS1 axis in the regulation of splicing-independent export, through *U2AF2* RNA splicing.

Interestingly, although IWS1 phosphorylation affects the export of intronless genes, our results also show a negative correlation of IWS1 with their RNA expression in cell lines and LUAD patients (**Fig. 1**). Notably, the *U2AF2* RNA splicing pattern affects the expression of type IFNs mRNA, but not the one of *JUN* and *HSBP3*. Based on our recent data, loss of IWS1 phosphorylation promotes genomic instability, through a process dependent on *U2AF2*

RNA splicing, leading to cytosolic DNA and transcription upregulation of type IFNs (Laliotis et al., 2020[39]). The exact role of this pathway in the transcriptional regulation of these genes will be addressed in future studies.

Our data also indicate that RNA Pol II contributes to the regulation of the nuclear export of intronless RNAs (**Fig. 5**). Nonetheless, components of the TREX complex have been shown to be efficiently loaded onto mRNAs even in the absence of splicing and regulate this process (Akef et al., 2015[40], Lee, E.S. et al., 2015[41], Lee, E.S. et al., 2020[42]). Given that the TREX complex is recruited to target genes by RNA Pol II co-transcriptionally (Sträßer et al., 2002[43]), this suggests that the observed effect of RNA Pol II in this process may be through the recruitment of TREX complex on these intronless genes. The exact role of RNA Pol II in this process will be elucidated in future studies.

In the present study, we also demonstrated that signals originating from AKT3 kinase regulate viral replication due to the regulation of type I IFNs, through IWS1 phosphorylation. Specifically, suppression of the AKT3/p-IWS1 axis disrupts the U2AF65-Prp19 interaction, leading to nuclear retention and downregulation of type IFNs (**Fig. 1, Fig. 2**). The latter enhances viral replication of a set of viral strains, due to impaired ISGs induction (**Fig. 6**). The PI3K/Akt pathway appears to be associated with the host cell immune response to counteract viral infection, in an isoform-specific manner (Diehl et al., 2013[44]). Based on our previous work, AKT1 selectively phosphorylates EMSY and stimulates the expression of ISGs (Ezell et al., 2012[11]). In the present work, we demonstrate the opposing role of the AKT3 isoform in the expression of ISG, through IWS1 phosphorylation. The fact that the selective inhibition of some of these pathways, such as the EMSY or the IWS1 pathway, had profound effects on the sensitivity of the cells to viral infection, suggests that these pathways may not function independently of each other and that their roles may not be additive, but synergistic. A recent report, consistent with our findings, found that PI3K/AKT blockade enhances the replication of Reovirus by repressing ISGs (Tian et al., 2015[45]). Importantly,

our data also implicate that alternative RNA splicing and RNA processing can regulate the immune and type I IFN response during viral infection. Given the fact that aberrant splicing is known to contribute to defects in IFN response and viral replication (Chauhan et al., 2019[46], Chang et al., 2017[47]), our data support another layer of this regulation through RNA export and processing.

Our findings support that inhibition of the AKT/p-IWS1 axis sensitizes the cells to oncolytic viral killing, through the manipulation of RNA processing and export. These results come in agreement with previous reports supporting that PI3K/AKT inhibition sensitizes cancer cells to oncolytic viral infection (Tong et al., 2015[48]). Interestingly, in the case of non-small cell lung cancer (NSCLC), several reports have demonstrated the synergistic role of Reovirus and VSV oncolytic viruses to the clinical outcome of lung cancer patients (Villalona-Calero et al., 2016[35], Bishnoi et al., 2018[49]), with several ongoing clinical trials to date (NCT03029871, NCT00861627). Notably, a preclinical *in vivo* lung cancer model with genetic ablation of *IFNAR1* (*IFNAR1*$^{-/-}$*),* demonstrates synergistic therapeutic effects of VSV (Schreiber et al., 2019[34]). Our data reveal that inhibition of the AKT/p-IWS1 axis downregulates the induction of ISGs following VSV infection, including *IFNAR1* (**Fig. 6**), further supporting the role of the AKT/IWS1 axis to the response to oncolytic viruses.

Another important clinical application of this pathway is that OVs often induce IFN release in the Tumor Microenvironment (TME), resulting in an upregulation of PD-L1 expression on tumor cells (Bellucci et al., 2015[50]). Previous studies have shown that the combination of Reovirus with PD-1 blockade enhanced the ability of NK cells to kill reovirus-infected tumor cells, increased CD8+T cells, and enhanced the antitumor immune response (Rajani et al., 2016[51]). Further studies proposed that a triple combination of anti–CTLA-4, anti–PD-1, and oHSV–IL-12 resulted in long-term durable cures in most of the mice treated in two syngeneic models of GBM by inducing a profound increase in the ratio of T effector to Tregs (Saha et al., 2017[52]). More importantly, oncolytic viruses preferentially

replicate in tumour cells because the antiviral responses in these cells are dysfunctional (Xia et al., 2016[53]). In healthy tissue, the production of interferons and interferon-related factors limits viral replication and boosts the rate of viral clearance, suggesting limited potential side effects (Bommareddy et al., 2018[54]). Driven by these strong evidences of the synergistic effect of OV with immune-checkpoint inhibitors, several clinical trials have actively investigated the possible synergy between these therapeutic approaches, with promising results (NCT02263508, NCT02307149, NCT03153085).

The data in this report may also be relevant for the design of strategies to prevent or overcome the resistance of *EGFR* mutant lung adenocarcinomas to Tyrosine kinase Inhibitors (TKI). Our recent studies had shown that the IWS1 phosphorylation pathway is active in human lung adenocarcinoma. More importantly, IWS1 phosphorylation and *U2AF2* exon 2 inclusion were shown to correlate positively with tumor stage, histologic grade and metastasis, and to predict poor survival in patients with *EGFR* mutant, but not *KRAS* mutant tumors (Laliotis et. al 2020[15]). A recent publication provided evidence, linking resistance to EGFR inhibitors to the upregulation of type I IFN signaling (Gong et al., 2020[55]). This suggests that by promoting type I IFN signaling, the IWS1 phosphorylation pathway may promote resistance to EGFR TKI, contributing to the poor prognosis of these tumors.

Our data also show that the U2AF65/Prp19 spliceosomal complex that controls the RNA export of intronless genes and recruited upon IWS1 phosphorylation, is active in Lung Adenocarcinoma patients, suggesting it as a possible therapeutic target for the manipulation of the IFN response in these patients. Based on the latter, we provide the rationale for three potential translation applications. First, the combination of AKT/IWS1 inhibitors with oncolytic viruses may enhance their lytic action in lung adenocarcinoma due to the suppression of the induction of ISGs. Second, IWS1 inhibition could enhance the response of lung adenocarcinoma patients treated with OV/PD-1 inhibitors, or the p-IWS1 levels may act as precision medicine marker for response to OV or the OV/PD-1 blockade combination,

through the regulation of IFN response in the TME. Third, given that the IWS1 phosphorylation-dependent effect on the response to oncolytic viruses is mediated through manipulation of RNA splicing and RNA processing and a specific *U2AF2* isoform, a process occurring in LUAD patients as well (**Fig. 4**), the oncolytic efficiency in lung adenocarcinoma patients may be enhanced with the use of highly isoform-specific antisense oligonucleotides and pharmacologic modulators of splicing machinery (Obeng et al., 2019[56]), which are currently under clinical trials (NCT03901469, NCT02711956, NCT02268552, NCT02908685).

Collectively, our results describe a novel pathway through IWS1 phosphorylation by AKT which, through the regulation of RNA processing and export machinery, may serve as a precision-medicine marker for response and important synergy target for the therapeutic outcome of oncolytic viral therapy in lung adenocarcinoma.

Materials and Methods

Cells and culture conditions, transfections and inhibitors

Cells, inhibitors and shRNAs, lentiviral constructs and experimental protocols are described in detail in the supplemental experimental procedures. Experimental protocols include use of inhibitors, transient transfections, lentiviral packaging, and cellular transduction with lentiviral constructs.

Expression Vectors and shRNAs

The origin of the expression clones and shRNAs are described in Supplementary Table S3. The cloning of *IFNA1* and *IFNB1* cDNA in the pLx304-V5 and pLKO.1 vectors is described in the Supplementary Experimental Procedures. The pGIPZ shIWS1 clone used in this report (Sanidas et al., 2014[14], Laliotis et al., 2020[15]), was subjected to modification in order to

remove the GFP cassette, are also described in the Supplementary Experimental Procedures.

Immunoblotting

Cells were lysed with RIPA buffer and cell lysates were resolved by electrophoresis in SDS-PAGE and analyzed by immunoblotting. Images were acquired and analyzed, using the Li-Cor Fc Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE). For the lists of antibodies used for immunoprecipitation and western blotting and for other details, see Supplemental Experimental Procedures.

Subcellular Fractionation

Cell pellets were fractionated into nuclear and cytoplasmic fractions, which were used to measure the relative abundance of proteins and RNAs in the nucleus and the cytoplasm, as described before (Laliotis et al., 2020[15]). For details, see Supplemental Experimental Procedures.

RT-PCR and qRT-PCR

Total RNA was isolated using the PureLink RNA Kit (Invitrogen, Cat. No 12183018A). Isolated RNA was analyzed by real-time RT-PCR for the expression of the indicated mRNAs. The mRNA levels were normalized based on the levels of GAPDH or 18S rRNA (internal control). The primer sets used are listed in the Supplemental Experimental Procedures.

Chromatin Immuno-Cleavage (ChIC)

The binding of p-STAT1 on ISRE of ISGs was addressed by chromatin Immuno-cleavage (Skene et al., 2018[57], Laliotis et al., 2020[15]). For details, for the ChIC protocols. see Supplemental Experimental Procedures.

Virus propagation and titering

Influenza virus A/PR/8/1934 (H1N1) expressing green fluorescent protein (PR8-GFP) was propagated in 10-day-old embryonated chicken eggs (Charles River Laboratories) for 48 hours at 37°C and titered in MDCK cells. 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. VSV expressing GFP (VSV-GFP) was propagated and titered in HeLa cells. Reovirus was propagated and titered in Vero cells.

Cell infections and flow cytometry

NCI-H522 cells were infected with PR8-GFP or Reovirus at an MOI of 1.0 for 24h, or with VSV-GFP or SEV-GFP at an MOI of 0.5 for 16h. NCI-H1299 cells were infected with PR8-GFP at an MOI of 1.0 for 24h, or with VSV-GFP or SEV-GFP at an MOI of 0.25 for 24h. Following infection, cells were fixed in 4% paraformaldehyde (Thermo Scientific), permeabilized with 0.1% Triton X-100 in PBS, and incubated with PBS containing 2% fetal bovine serum. Reovirus-infected cells were stained with anti-reovirus T3D sigma 3 antibody (DSHB, 10G10), followed by anti-mouse Alexa488-conjugated secondary antibody (Thermo Scientific, A-11029). PR8, VSV, and SEV infection rates were measured by detecting virus-encoded GFP. Flow cytometry was performed on a FACSCanto II flow cytometer (BD Biosciences) and analyzed using FlowJo software (DB, Ashland, OR).

RNA Immunoprecipitation

The binding of RNA binding proteins to regions of the IFNα, IFNβ, c-Jun and HSPB3 mRNAs was addressed by RNA Immunoprecipitation, as described before (Laliotis et al., 2020[15]). For details, see Supplemental Experimental Procedures.

Cellular Survival/Killing curves

For the determination of cell survival following oncolytic viral infections, NCI-H522 and NC-H1299 cells were plated in 24-well plates. The cells were exposed to increased MOI of VSV and Reovirus for 16h and 48h, respectively. After the incubation period, cell proliferation was quantified by fluorescent detection of the reduction of resazurin to resorufin by viable cells and normalized to DMSO-treated wells, using alamarBlue™ HS Cell Viability Reagent (Thermo Fisher Cat. No A50100), as described in the Supplemental Experimental Procedures.

TCGA/RPPA analysis

TCGA data were downloaded from https://portal.gdc.cancer.gov/ and analysed as described in the Supplemental Experimental Procedures.

Data availability

All the source data derived from this report have been deposited in the Mendeley Dataset. (Laliotis et al., 2020[58], doi: 10.17632/853gfbbx7m.1)

Statistical analysis

All the statistical analysis was performed in GraphPad Prism, 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., 2020[58], doi: 10.17632/853gfbbx7m.1)

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Author Contributions

G.I.L. Conceptualization, overall experimental design. Performed all the experiments except the viral infections in Figure 6, analyzed the data, prepared the figures and wrote the manuscript. **A.D.K.** Designed and performed all the infections with viral strains, optimized, performed and analyzed the flow cytometry experiments and edited the manuscript **E.C.** Designed and performed with G.I.L the time point interval experiment for p-STAT1 activation and Caspase-mediated death in Figure 6 and Figure 7, edited the manuscript **A.O.** Assisted to the time point interval experiment for Caspase-mediated death and edited the manuscript **A.K.K** Performed the cloning of the type I IFN vectors for RNA Pol II and III promoter expression, performed RT-PCR experiments **A.L.F.** Bioinformatics analyses of RNA-Seq and TCGA data, edited the manuscript. **V.A.** Assisted to the time point interval experiment for p-STAT1 activation and edited the manuscript **J.D.B** Advised on the design of experiments and edited the manuscript **C.T.** Advised on the design of experiments and edited the manuscript. **L.S.** Advised on the design of experiments and edited the manuscript **V.C.** Contributed to overall experimental design, edited the manuscript. **J.S.Y** Designed the

viral infection experiments and contributed to overall experimental design, edited the manuscript **P.N.T.** Overall experimental design, manuscript writing and editing.

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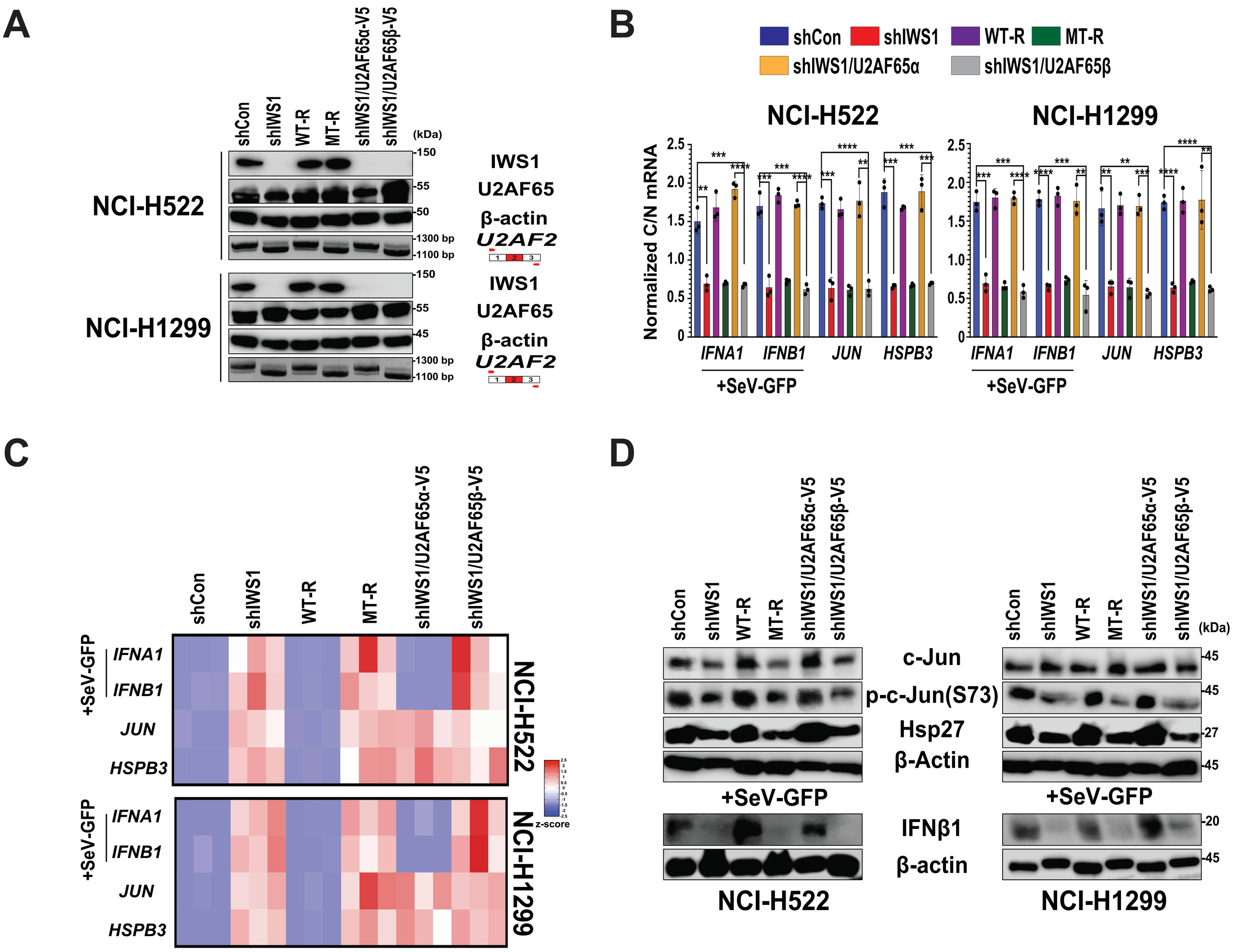

A
shCon
shIWS1
WT-R
MT-R
shIWS1/U2AF65α-V5
shIWS1/U2AF65β-V5
(kDa)
NCI-H522
NCI-H1299
IWS1
U2AF65
β-actin
U2AF2
150
55
50
45
1300 bp
1100 bp
B
shCon
shIWS1
WT-R
MT-R
shIWS1/U2AF65α
shIWS1/U2AF65β
NCI-H522
NCI-H1299
Normalized C/N mRNA
IFNA1
IFNB1
JUN
HSPB3
+SeV-GFP
C
z-score
D
c-Jun
p-c-Jun(S73)
Hsp27
β-Actin
IFNβ1
β-actin
+SeV-GFP
NCI-H522
NCI-H1299

Figure 1. IWS1 phosphorylation regulates the nucleocytoplasmic transport of mRNAs transcribed from a set of intronless genes, via *U2AF2* alternative RNA splicing.

A. Western blots of lysates of NCI-H522 and NCI-H1299 cells, transduced with the indicated constructs, were probed with anti-IWS1, anti-U2AF65 and anti-β-actin (control) antibodies. (first three lanes). RT-PCR of *U2AF2*, using oligonucleotide primers that map in exons 1 and 3 (fourth lane).

B. *IWS1 phosphorylation regulates the nucleocytoplasmic export of intronless mRNAs.* The same NCI-H522 (right panel) and NCI-H1299 (right panel) cells as in Fig. 1A, were fractionated into cytoplasmic and nuclear fractions. *IFNA1, IFNB1, JUN, HSPB3* and *GAPDH* mRNA levels in each fraction were determined using qRT–PCR. For each transcript, values are represented as cytoplasmic to nuclear ratios and were normalized to 18S rRNA. For type I IFN induction SeV-GFP infection was performed. The accuracy of the fractionation was validated with the experiment in Figure S1A. Bars show the mean normalized Cytosolic/Nuclear ratio ±SD

C. Total RNA was harvested from NCI-H522 (upper panel) and NCI-H1299 cells (lower panel), transduced with the indicated constructs. The expression of *IFNA1, IFNB1, JUN and HSPB3* mRNAs was determined by qRT-PCR and normalized to 18S rRNA. The results are represented as heatmap using z-scores from three individual biological experiments. For type I IFN induction SeV-GFP infection was performed. 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).

D. Western blots of lysates of NCI-H522 (left panel) and NCI-H1299 (right panel) cells, transduced with the indicated constructs, were probed with anti-c-Jun, anti-p-c-Jun (S73), anti-Hsp27, anti-IFNβ1 and anti-β-actin (control) antibodies. For type I IFN induction SeV-GFP infection was performed. Throughout this figure NCI-H522 and NCI-H1299 cells were infected with MOI of 0.5 for 16h and MOI of 0.25 for 24h of SeV-GFP virus, respectively.

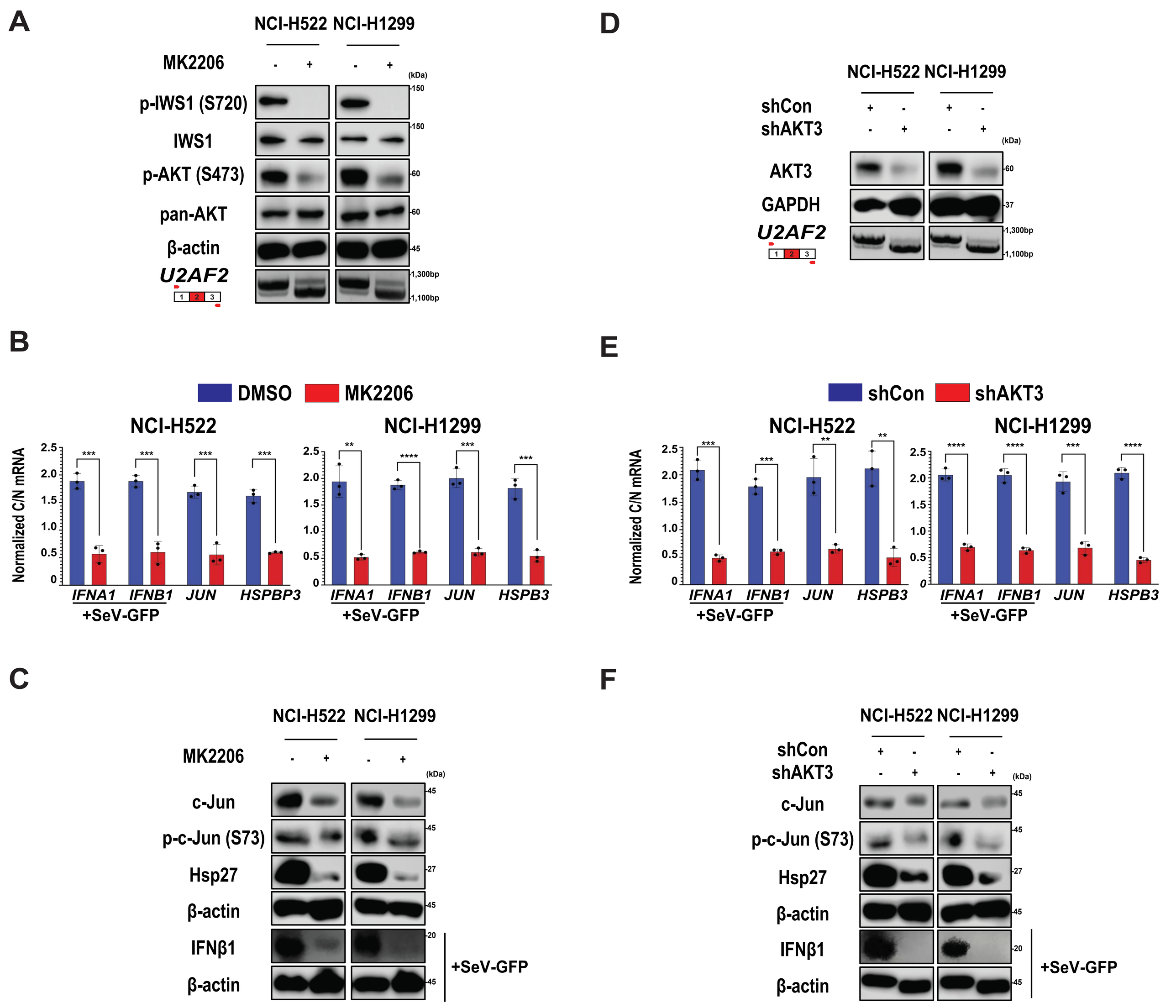

A
NCI-H522 NCI-H1299
MK2206
- + - +
(kDa)
p-IWS1 (S720)
IWS1
p-AKT (S473)
pan-AKT
β-actin
U2AF2
150
150
60
60
45
1,300bp
1,100bp
B
DMSO
MK2206
NCI-H522
NCI-H1299
Normalized C/N mRNA
IFNA1 IFNB1 JUN HSPBP3
IFNA1 IFNB1 JUN HSPB3
+SeV-GFP
C
NCI-H522 NCI-H1299
MK2206
- + - +
(kDa)
c-Jun
p-c-Jun (S73)
Hsp27
β-actin
IFNβ1
β-actin
45
45
27
45
20
45
+SeV-GFP
D
NCI-H522 NCI-H1299
shCon
shAKT3
+ - + -
- + - +
(kDa)
AKT3
GAPDH
U2AF2
60
37
1,300bp
1,100bp
E
shCon
shAKT3
NCI-H522
NCI-H1299
Normalized C/N mRNA
IFNA1 IFNB1 JUN HSPB3
IFNA1 IFNB1 JUN HSPB3
+SeV-GFP
F
NCI-H522 NCI-H1299
shCon
shAKT3
+ - + -
- + - +
(kDa)
c-Jun
p-c-Jun (S73)
Hsp27
β-actin
IFNβ1
β-actin
45
45
27
45
20
45
+SeV-GFP

Figure 2. AKT3 kinase affects the nucleocytoplasmic export of RNAs transcribed from intronless genes, thought IWS1 phosphorylation

A. NCI-H522 and NCI-H1299 cell lysates, harvested following a 4 hr treatment with MK2206 (5μM) or DMSO, were probed with the indicated antibodies. (Lower lane) RT-PCR reactions, using mRNA derived from the cells in the upper panel and oligonucleotide primers mapping in *U2AF2* exons 1 and 3 show that inhibiting Akt inhibits the inclusion of exon 2 in mature *U2AF2* mRNA transcripts in both cell lines.

B. *AKT kinase regulates the nucleocytoplasmic export of intronless mRNAs, through IWS1 phosphorylation.* Cells after similar treatment as Fig. 2A, were fractionated into cytoplasmic and nuclear fractions, *IFNα1, IFNβ1, c-Jun, HSPB3* and *GAPDH* mRNA levels in each fraction were determined using qRT–PCR. For each transcript, values are represented as cytoplasmic to nuclear ratios and were normalized to 18S rRNA. For type I IFN induction SeV-GFP infection was performed. The accuracy of the fractionation was validated with the experiment in Figure S2A. Bars show the mean normalized Cytosolic/Nuclear ratio ±SD

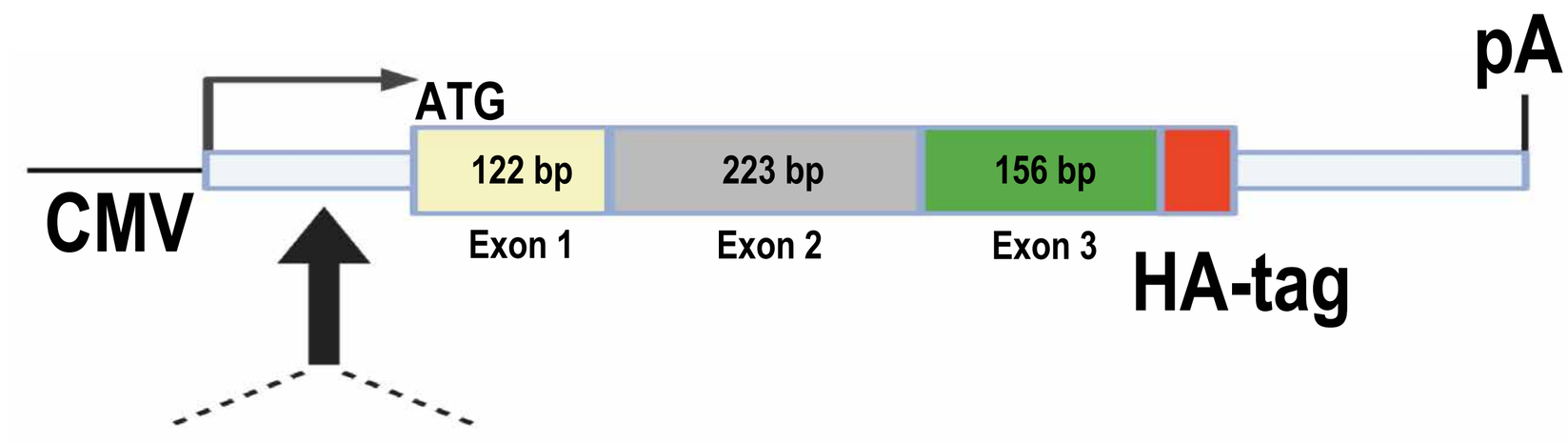
C. NCI-H522 and NCI-H1299 cell lysates as in Fig. 2A, were probed with the indicated antibodies. For type I IFN induction SeV-GFP infection was performed.

D. NCI-H522 and NCI-H1299 cells were transduced with lentiviral shAKT3 constructs along with shControl and their protein lysates were probed with the indicated antibodies

E. The indicated cells were fractionated into cytoplasmic and nuclear fractions, *IFNα1, IFNβ1, c-Jun, HSPB3* and *GAPDH* mRNA levels in each fraction were determined using qRT–PCR. For each transcript, values are represented as cytoplasmic to nuclear ratios and were normalized to 18S rRNA. For type I IFN induction SeV-GFP infection was performed. The accuracy of the fractionation was validated with the experiment in Figure S2B. Bars show the mean normalized Cytosolic/Nuclear ratio ±SD

F. NCI-H522 and NCI-H1299 cell lysates as in Fig. 2D, were probed with the indicated antibodies. For type IFN induction SeV-GFP infection was performed. Throughout this figure NCI-H522 and NCI-H1299 cells were infected with MOI of 0.5 for 16h and MOI of 0.25 for 24h of SeV-GFP virus, respectively. 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).

A



CAR-E (CCAGTTCCTG) x16

CAR-E mut (ggAGTTCCTG) x16

B

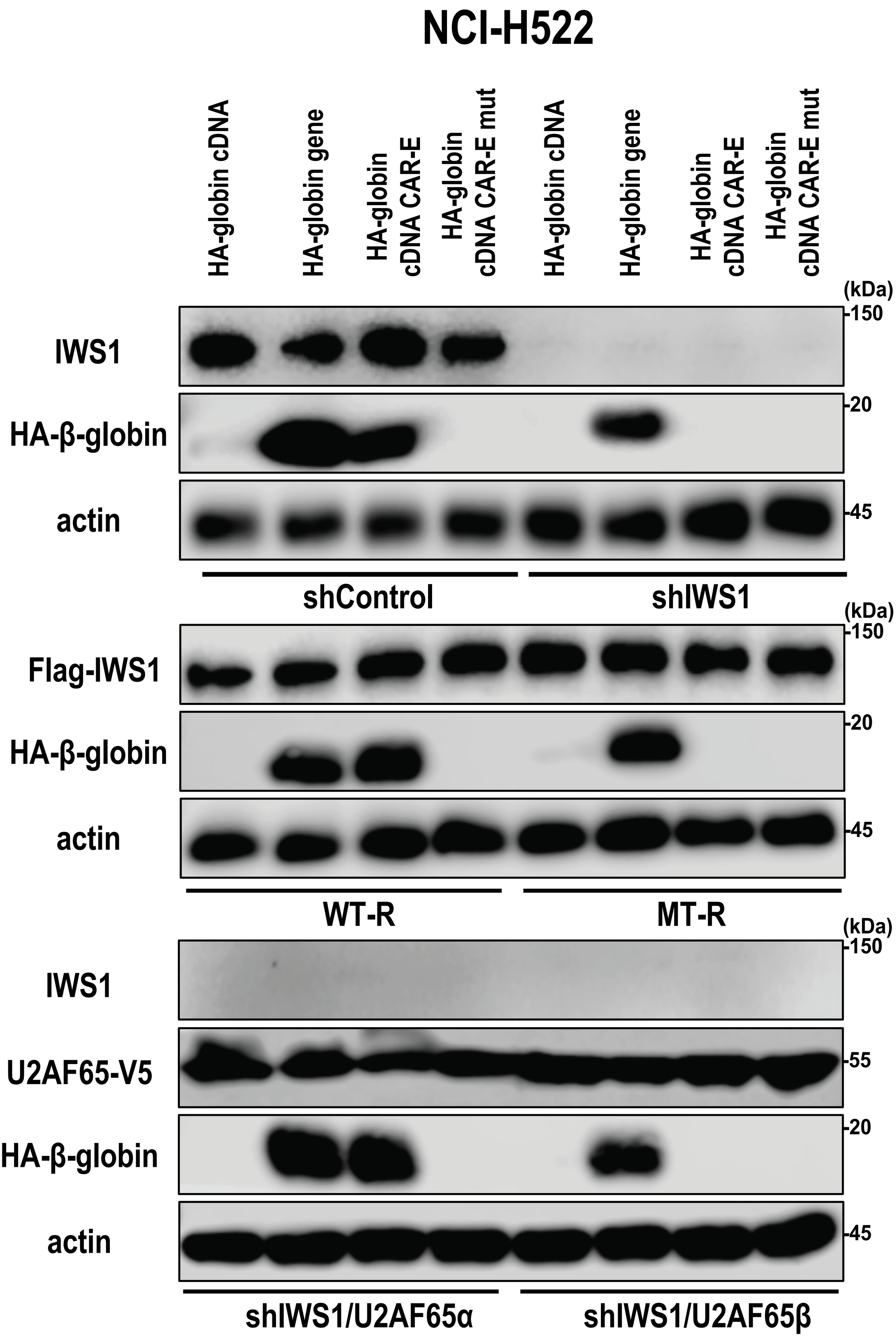


Figure 3. IWS1 phosphorylation is required for the proper function of the CAR-E elements.

A. Schematic representation of pCMV human β-globin cDNA construct indicating the position where the CAR-E and CAR-E_{mut} were inserted, as described before (Lei et al., 2013[13]). The start codon (ATG), HA tag and BGH polyA signal (pA) are indicated. The exon sizes (in bp) are shown.

B. *IWS1 phosphorylation is required for CAR-E function.* NCI-H522 cells transduced with the indicated constructs, were transfected transiently with pCMV HA-β-globin cDNA, HA-β-globin wt, HA-β-globin CAR-E and HA-β-globin CAR-E_{mut}. After 48h were harvested, and their lysates were probed with the indicated antibodies.

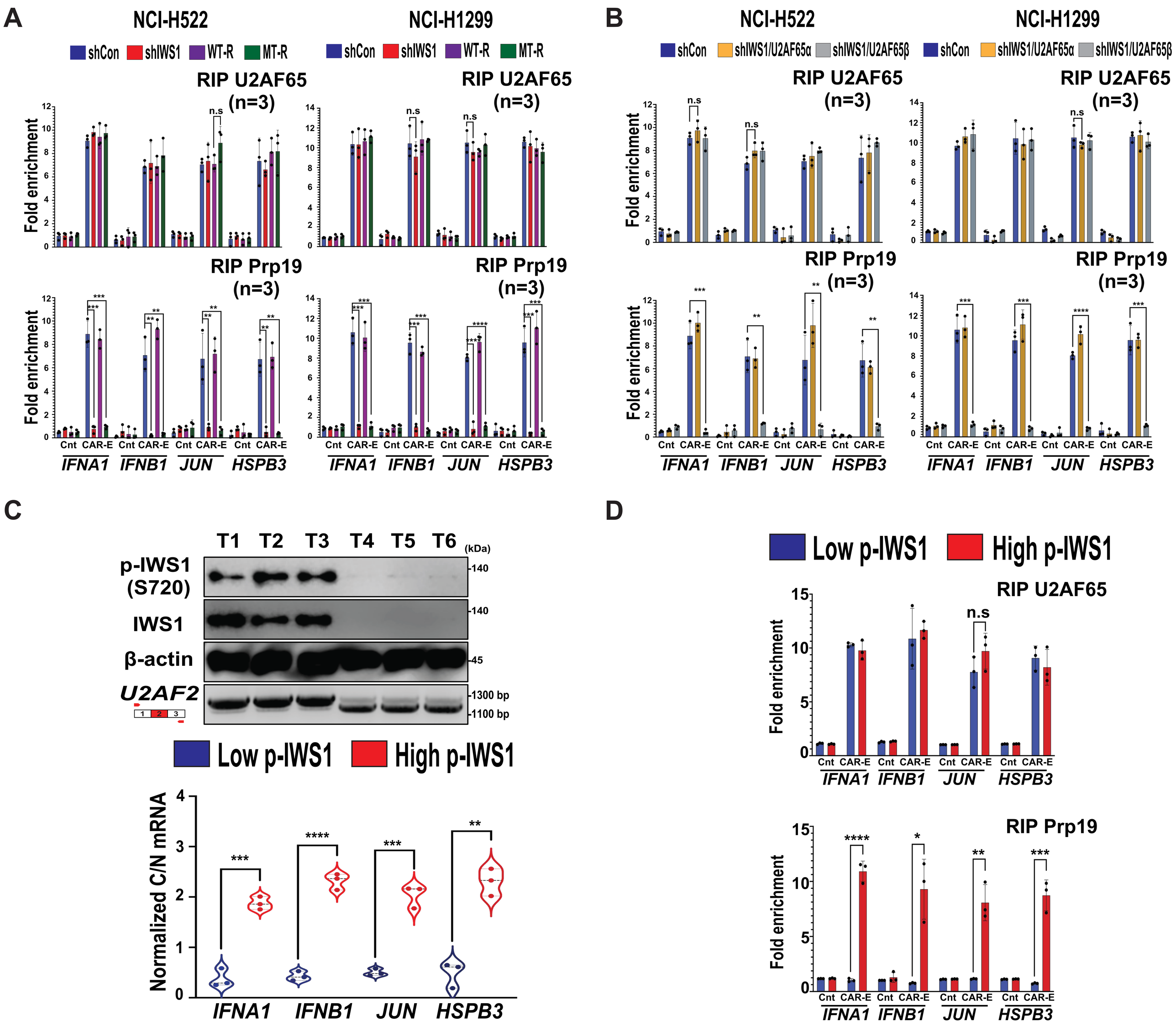

A
NCI-H522
NCI-H1299
shCon
shIWS1
WT-R
MT-R
RIP U2AF65 (n=3)
RIP Prp19 (n=3)
Fold enrichment
n.s
Cnt
CAR-E
IFNA1
IFNB1
JUN
HSPB3
B
shIWS1/U2AF65α
shIWS1/U2AF65β
C
T1
T2
T3
T4
T5
T6
(kDa)
p-IWS1 (S720)
IWS1
β-actin
U2AF2
140
45
1300 bp
1100 bp
Low p-IWS1
High p-IWS1
Normalized C/N mRNA
D

Figure 4. The splicing of the *U2AF2* mRNA downstream of IWS1 phosphorylation, regulates the Prp19 recruitment on the CAR-E elements.

A. and B. *IWS1 phosphorylation controls Prp19 recruitment on CAR-E elements.* RIP assays in NCI-H522 and NCI-H1299 cells transduced with the indicated constructs, show that whereas the binding of Prp19 to the CAR-E elements depends on the phosphorylation of IWS1, the binding of U2AF65 does not. The bars show the mean fold enrichment in U2AF65 (upper) and Prp19 (lower panel) binding in the indicated regions (anti-U2AF65 or anti-Prp19 IP, vs IgG control IP) ± SD. Data were normalized relative to the input (2%). The primer location within the intronless genes is described in Figure S4A.

C. *IWS1 phosphorylation regulates the nucleocytoplasmic export of intronless mRNAs in patients with Lung Adenocarcinoma. (*Upper panel) Western blots of lysates of 6 human LUAD samples with high and low IWS1 expression, as previously identified (Laliotis et al., 2020[15]), were probed with anti-IWS1, anti-p-IWS1 and anti-β-actin (control) antibodies. (first three lanes). RT-PCR of *U2AF2*, using oligonucleotide primers that map in exons 1 and 3 (forth lane). (Lower Panel) LUAD tumor samples were fractionated into cytoplasmic and nuclear fractions. *IFNA1, IFNB1, JUN, HSPB3* and *GAPDH* mRNA levels in each fraction were determined using qRT–PCR. For each transcript, values are represented as cytoplasmic to nuclear ratios and were normalized to 18S rRNA. The accuracy of the fractionation was validated with the experiment in Figure S4B. Bars show the mean normalized Cytosolic/Nuclear ratio ±SD

D. *IWS1 phosphorylation controls Prp19 recruitment on CAR-E in patients with Lung Adenocarcinoma.* RIP assays in the same high and low-IWS1 group, show that whereas the binding of Prp19 to the CAR-E elements depends on the phosphorylation of IWS1, the binding of U2AF65 does not. The bars show the mean fold enrichment in U2AF65 (upper) and Prp19 (lower panel) binding in the indicated regions (anti-U2AF65 or anti-Prp19 IP, vs IgG control IP) ± SD. Data were normalized relative to the input (2%).

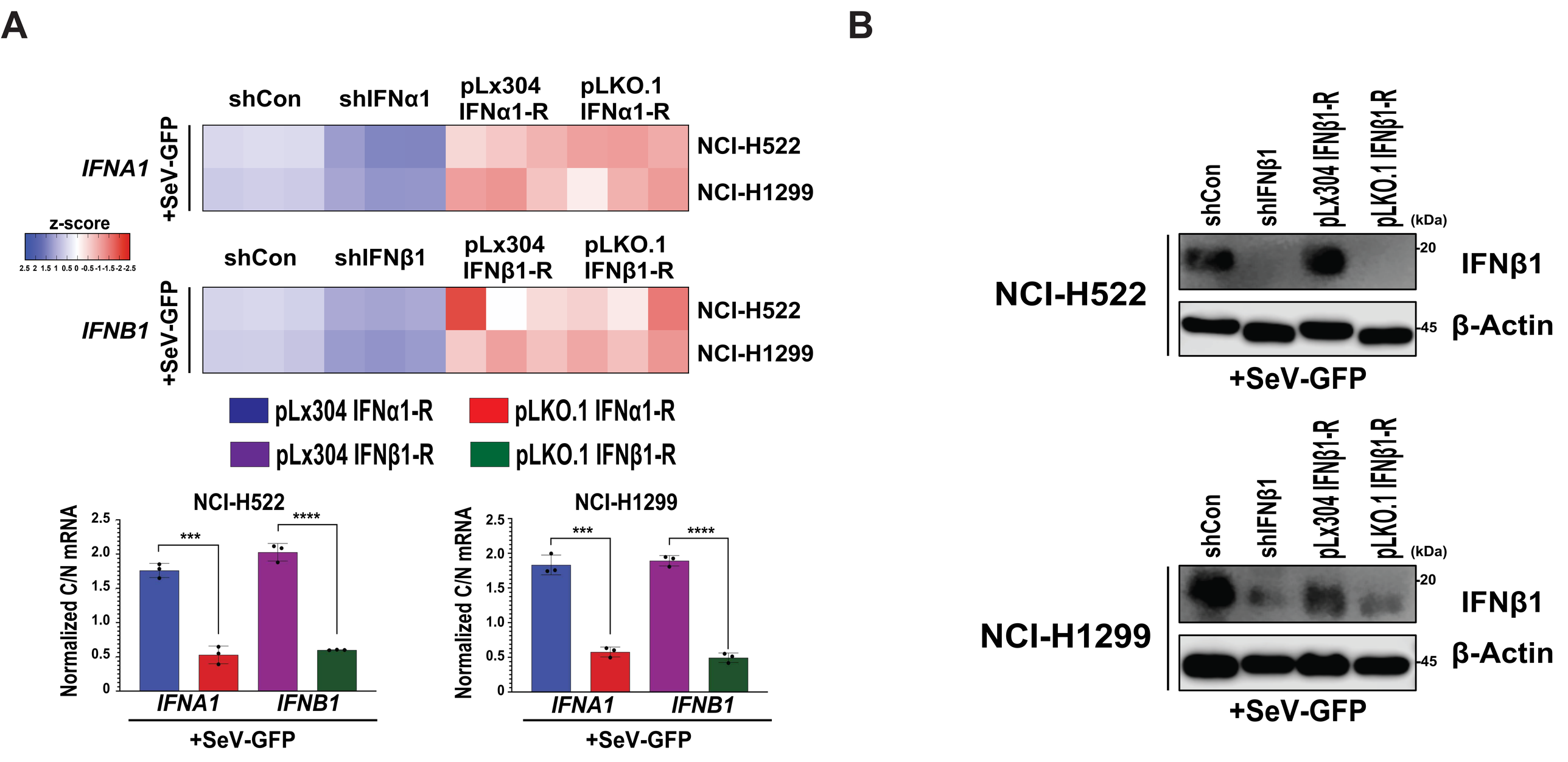

A
shCon
shIFNα1
pLx304 IFNα1-R
pLKO.1 IFNα1-R
IFNA1
+SeV-GFP
NCI-H522
NCI-H1299
z-score
2.5 2 1.5 1 0.5 0 -0.5 -1 -1.5 -2 -2.5
shCon
shIFNβ1
pLx304 IFNβ1-R
pLKO.1 IFNβ1-R
IFNB1
+SeV-GFP
NCI-H522
NCI-H1299
pLx304 IFNα1-R
pLKO.1 IFNα1-R
pLx304 IFNβ1-R
pLKO.1 IFNβ1-R
NCI-H522
Normalized C/N mRNA
2.5
2.0
1.5
1.0
0.5
0
***
****
IFNA1
IFNB1
+SeV-GFP
NCI-H1299
Normalized C/N mRNA
2.5
2.0
1.5
1.0
0.5
0
***
****
IFNA1
IFNB1
+SeV-GFP
B
shCon
shIFNβ1
pLx304 IFNβ1-R
pLKO.1 IFNβ1-R
(kDa)
NCI-H522
20
IFNβ1
45
β-Actin
+SeV-GFP
shCon
shIFNβ1
pLx304 IFNβ1-R
pLKO.1 IFNβ1-R
(kDa)
NCI-H1299
20
IFNβ1
45
β-Actin
+SeV-GFP

Figure 5. RNA-Pol II affects the splicing-independent nuclear export of intronless genes.

A. *RNA Pol II contributes to the nucleocytoplasmic export of intronless genes* (Upper panel) Total RNA was harvested from NCI-H522 and NCI-H1299 cells, transduced with the indicated constructs. The construct maps for the pLx304 (RNA Pol II-driven) or pLKO.1 (RNA Pol III-driven) can be found in Fig. S5A. The expression of *IFNA1 and IFNB1,* mRNAs was determined by qRT-PCR and normalized to 18S rRNA levels. The results are represented as heatmap using z-scores from three individual biological experiments. For type I IFN induction SeV infection was performed. (Lower panel) The same cells were fractionated into cytoplasmic and nuclear fractions, *IFNA1, IFNB1* and *GAPDH* mRNA levels in each fraction were determined using qRT–PCR. For each transcript, values are represented as cytoplasmic to nuclear ratios and were normalized to 18S rRNA. For type I IFN induction SeV-GFP infection was performed. The accuracy of the fractionation was validated with the experiment in Figure S5B. Bars show the mean normalized Cytosolic/Nuclear ratio ±SD

B. Western blots of lysates of NCI-H522 and NCI-H1299 cells, transduced with the indicated constructs, were probed with anti-IFNβ1 and anti-β-actin (control) antibodies. For type I IFN induction SeV-GFP infection was performed. In this figure, NCI-H522 and NCI-H1299 cells were infected with MOI of 0.5 for 16h and MOI of 0.25 for 24h of SeV-GFP virus, respectively. 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).

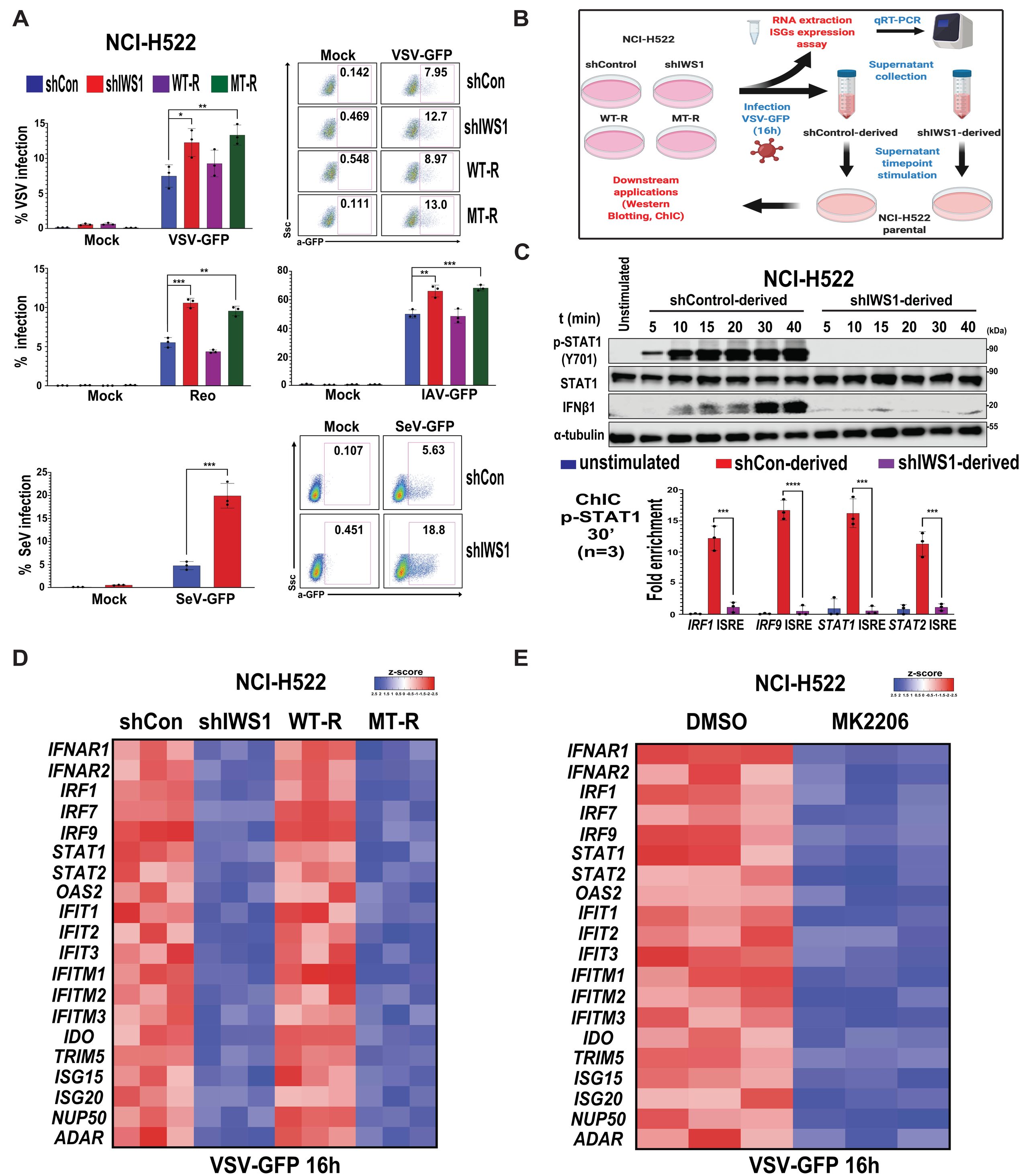

A
NCI-H522
shCon
shIWS1
WT-R
MT-R
% VSV infection
Mock
VSV-GFP
Mock
VSV-GFP
0.142
7.95
0.469
12.7
0.548
8.97
0.111
13.0
Ssc
a-GFP
% infection
Mock
Reo
Mock
IAV-GFP
% SeV infection
Mock
SeV-GFP
Mock
SeV-GFP
0.107
5.63
0.451
18.8
B
NCI-H522
shControl
shIWS1
WT-R
MT-R
RNA extraction
ISGs expression
assay
qRT-PCR
Supernatant
collection
Infection
VSV-GFP
(16h)
shControl-derived
shIWS1-derived
Supernatant
timepoint
stimulation
Downstream
applications
(Western
Blotting, ChIC)
NCI-H522
parental
C
NCI-H522
Unstimulated
shControl-derived
shIWS1-derived
t (min)
5 10 15 20 30 40
5 10 15 20 30 40
(kDa)
p-STAT1
(Y701)
STAT1
IFNβ1
α-tubulin
unstimulated
shCon-derived
shIWS1-derived
ChIC
p-STAT1
30'
(n=3)
Fold enrichment
IRF1 ISRE
IRF9 ISRE
STAT1 ISRE
STAT2 ISRE
D
NCI-H522
z-score
shCon
shIWS1
WT-R
MT-R
IFNAR1
IFNAR2
IRF1
IRF7
IRF9
STAT1
STAT2
OAS2
IFIT1
IFIT2
IFIT3
IFITM1
IFITM2
IFITM3
IDO
TRIM5
ISG15
ISG20
NUP50
ADAR
VSV-GFP 16h
E
NCI-H522
z-score
DMSO
MK2206
IFNAR1
IFNAR2
IRF1
IRF7
IRF9
STAT1
STAT2
OAS2
IFIT1
IFIT2
IFIT3
IFITM1
IFITM2
IFITM3
IDO
TRIM5
ISG15
ISG20
NUP50
ADAR
VSV-GFP 16h

Figure 6. The loss of IWS1 phosphorylation enhances viral infection, through the aberrant p-STAT1 signaling and transcription of ISGs.

A. *The loss of IWS1 phosphorylation enhances viral replication.* The indicated NCI-H522 cells were infected with VSV-GFP, SeV-GFP, Reovirus or Influenza A-GFP at an MOI of 0.5 for 16 hours (VSV-GFP and IAV-GFP), MOI of 1 for 24h hours (SeV-GFP and Reovirus), respectively. Cells were then fixed and were examined for GFP positivity (for VSV, SeV and Flu) to measure the percentage of cells infected using flow cytometry. For Reovirus, the fixed-infected cells were stained with anti-reovirus antibody, followed by anti-mouse Alexa488-conjugated secondary antibody, and analyzed as well with Flow cytometry. Results shown are representative of at least three independent experiments, each performed with triplicate samples. Bars show the % infection of each viral strain ±SD.

B. *Summary of the experimental design.* NCI-H522 shControl, shIWS1, shIWS1/WT-R and shIWS1/MT-R cells were infected with VSV-GFP at an MOI of 0.5 for 16h. Then the cells were harvested for RNA and their supernatant was collected. The RNA was used for downstream qRT-PCR applications. The supernatant derived from shControl and shIWS1 was used to stimulate NCI-H522 parental cells in time point intervals. These cells were used for Chromatin Immuno Cleavage (ChIC) or were harvested for protein.

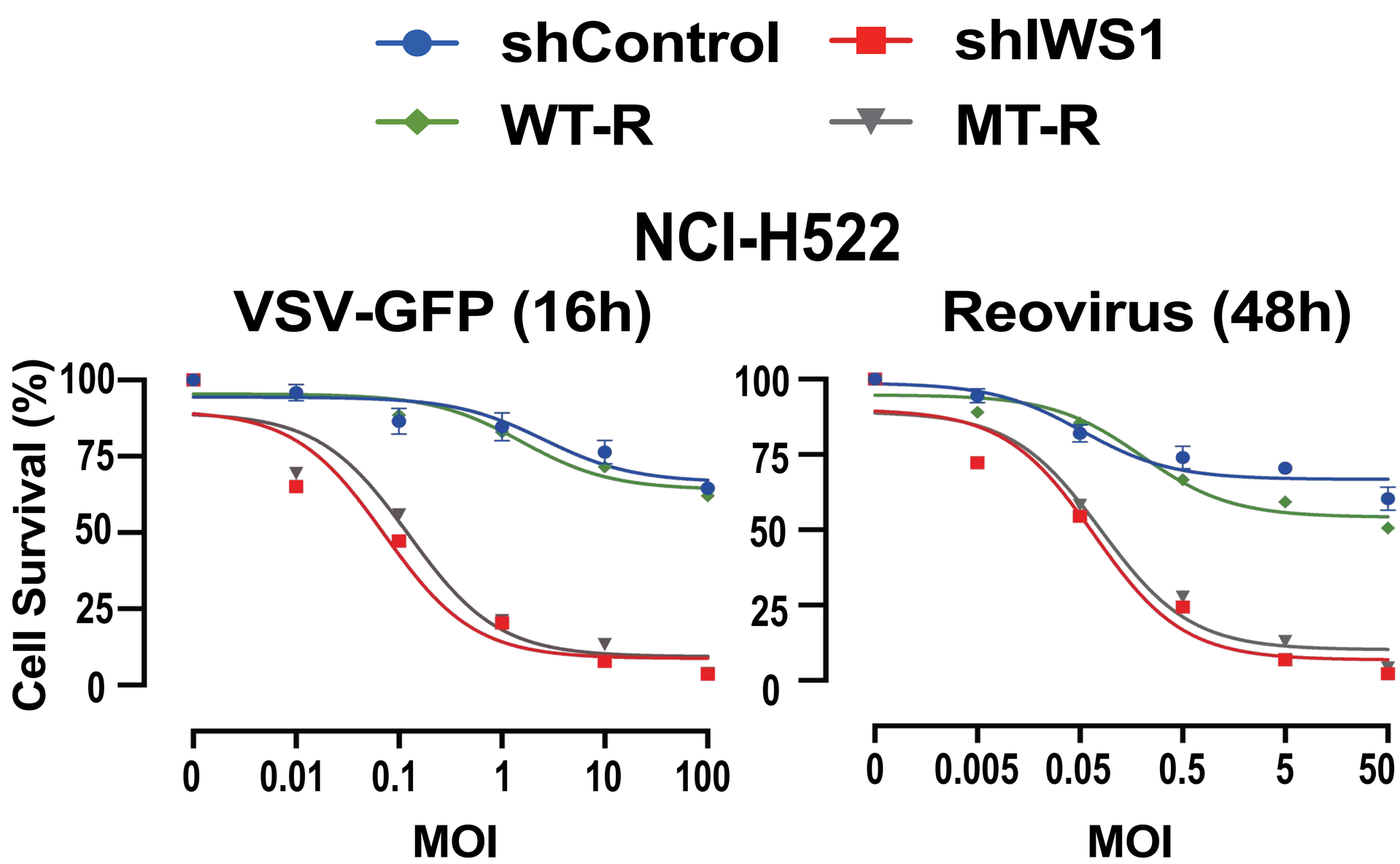
C. *Loss of IWS1 phosphorylation impairs p-STAT1 activation following viral infection.* (Upper panel) NCI-H522 parental cells were stimulated for the indicated time point intervals with shControl and shIWS1-derived supernatant and were harvested for protein. In parallel experiments, NCI-H522 unstimulated parental cells were harvested as control. The protein lysates were probed with the indicated antibodies. (Lower panel) ChIC assays showing the binding of p-STAT1 in the *ISRE of IRF1, IRF9, STAT1 and STAT2* genes, in NCI-H522 cells following stimulation for 30' with shControl and shIWS1-derived supernatant. In parallel experiments, NCI-

H522 unstimulated parental cells were proceeded as control. The bars show the mean fold enrichment in p-STAT1 binding in the indicated regions ±SD, relative to IgG IP.

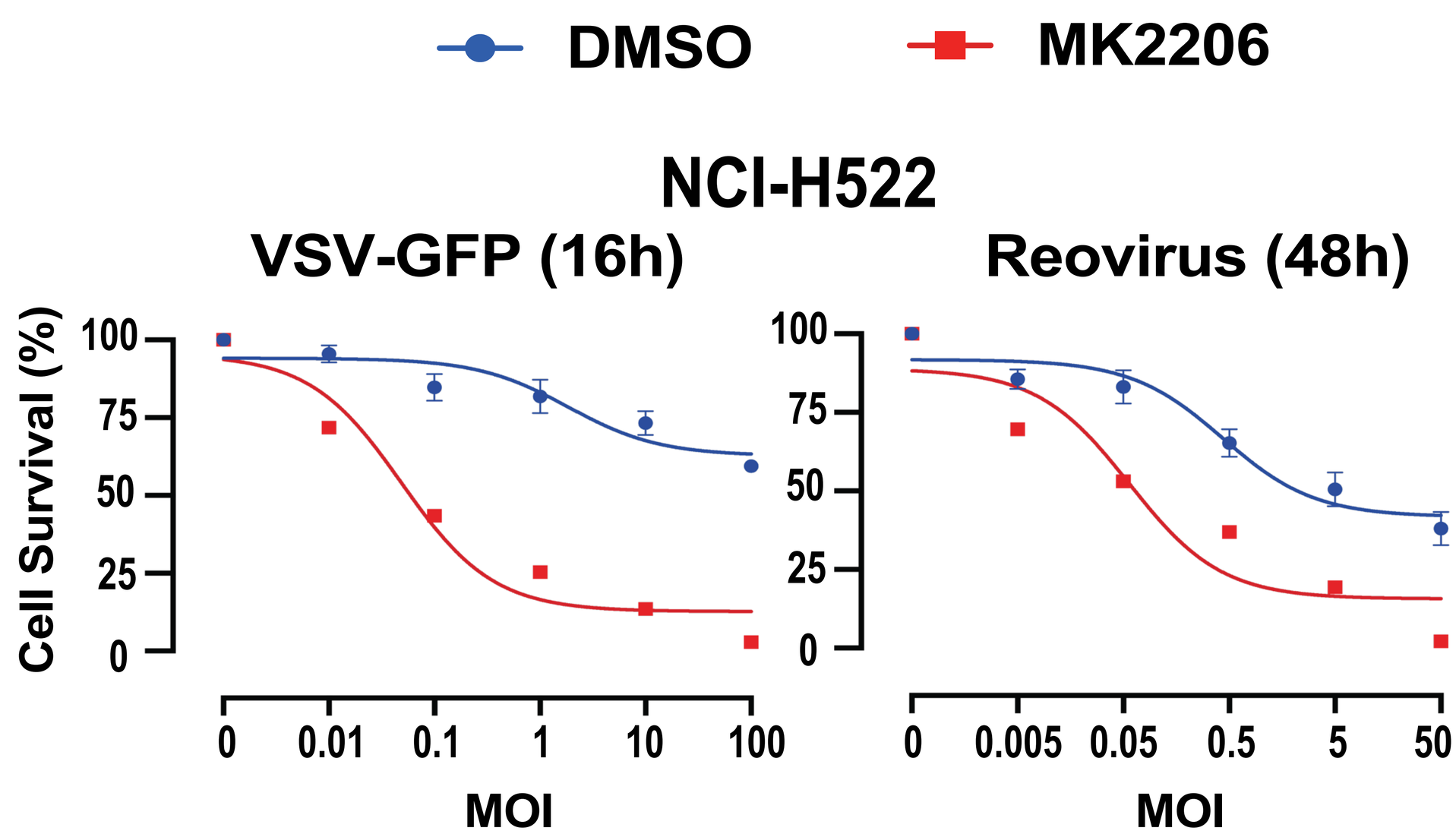
D. *Loss of AKT/p-IWS1 axis impairs the ISG transcription following viral infection.* Total RNA was harvested from NCI-H522 cells, transduced with the indicated constructs following VSV-GFP infection at MOI 0.5 for 16h. The expression of 20 ISGs mRNAs was determined by qRT-PCR and normalized to 18S rRNA. The results are represented as heatmap using z-scores from three individual biological experiments.

E. Total RNA was harvested from NCI-H522 cells, treated with DMSO or 5μM of the pan-AKT inhibitor MK2206, following VSV-GFP infection at MOI x for 16h. The expression of 20 ISGs mRNAs was determined by qRT-PCR and normalized to 18S rRNA. The results are represented as heatmap using z-scores from three individual biological experiments. 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).

A


shControl
shIWS1
WT-R
MT-R
NCI-H522
VSV-GFP (16h)
Reovirus (48h)
Cell Survival (%)
100
75
50
25
0
0
0.01
0.1
1
10
100
MOI
0
0.005
0.05
0.5
5
50
MOI

B


DMSO
MK2206
NCI-H522
VSV-GFP (16h)
Reovirus (48h)
Cell Survival (%)
100
75
50
25
0
0
0.01
0.1
1
10
100
MOI
0
0.005
0.05
0.5
5
50
MOI

C

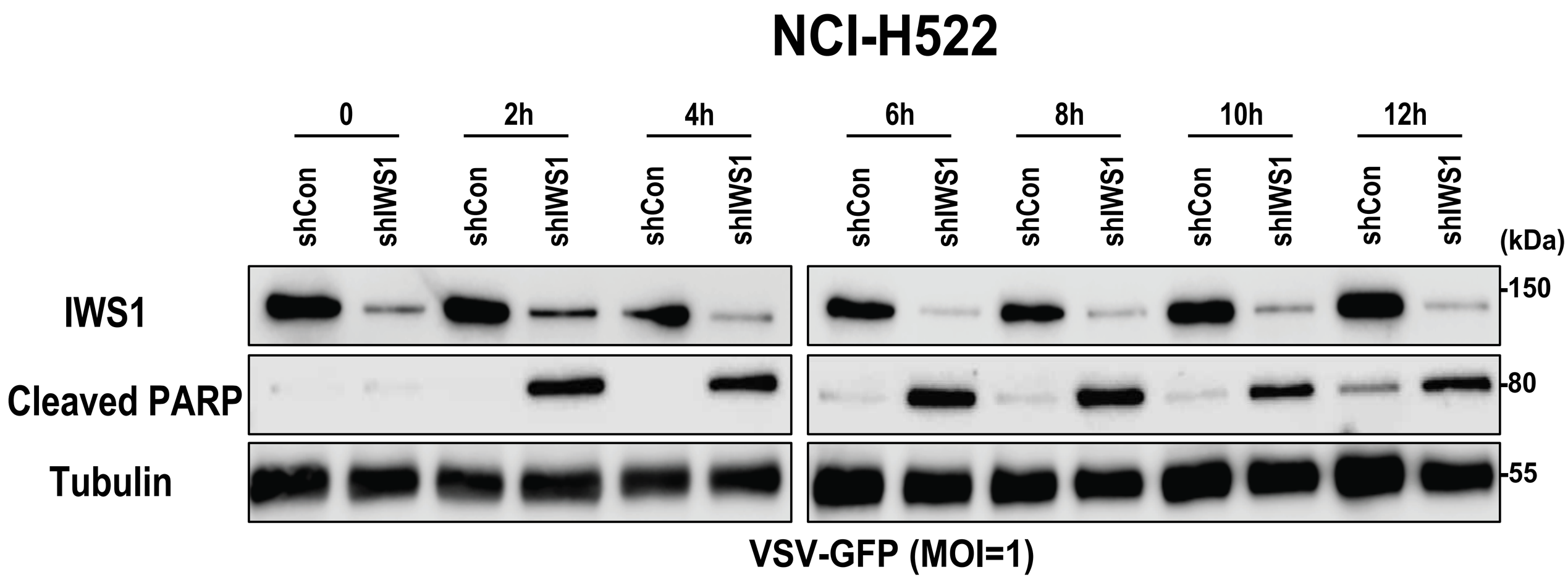

NCI-H522
0
2h
4h
6h
8h
10h
12h
shCon
shIWS1
(kDa)
IWS1
Cleaved PARP
Tubulin
150
80
55
VSV-GFP (MOI=1)

Figure 7. Inhibition of the AKT/p-IWS1 axis sensitizes lung adenocarcinoma cells to oncolytic viral killing.

A. *The loss of IWS1 phosphorylation sensitizes lung adenocarcinoma cells to oncolytic viral killing.* NCI-H522 cells transduced with the indicated constructs were exposed to increasing MOI of VSV-GFP or Reovirus for 16h and 48h, respectively. Cell proliferation was assessed by fluorescent detection of the reduction of resazurin to resorufin by viable cells and expressed as a percentage of uninfected cells. Mean values are shown ±SD (n=3) and data are representative of two independent experiments.

B. *The loss of AKT/p-IWS1 axis sensitizes lung adenocarcinoma cells to oncolytic viral killing.* NCI-H522 cells treated with DMSO or 5μM of the pan-AKT inhibitor MK2206, were exposed to increasing MOI of VSV-GFP or Reovirus for 16h and 48h, respectively. Cell proliferation was assessed by fluorescent detection of the reduction of resazurin to resorufin by viable cells and expressed as a percentage of uninfected cells. Mean values are shown ±SD (n=3) and data are representative of two independent experiments.

C. *The loss of IWS1 phosphorylation sensitizes lung adenocarcinoma cells to oncolytic viral killing via Caspase-mediated death.* NCI-H522 shControl and shIWS1 cells were infected for the indicated time point intervals with VSV-GFP at a MOI=1. Western blot lysates from these time points were probed with the indicated antibodies, showing a robust cleavage of PARP, a sensitive marker of Caspase mediated apoptosis, in shIWS1 cells even in early time points of infections.

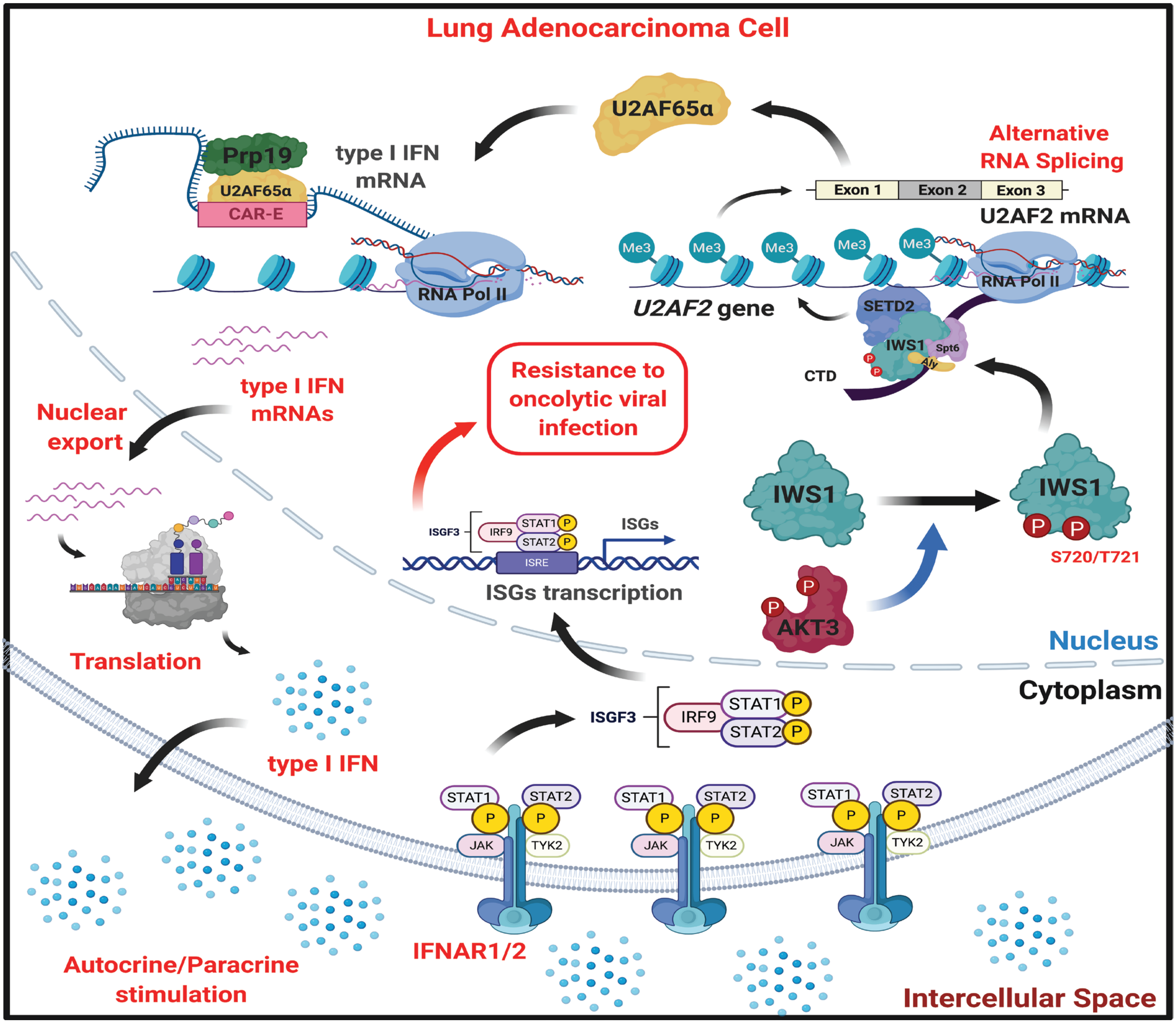

Lung Adenocarcinoma Cell
U2AF65α
Prp19
U2AF65α
CAR-E
type I IFN
mRNA
RNA Pol II
Alternative
RNA Splicing
Exon 1
Exon 2
Exon 3
U2AF2 mRNA
Me3
U2AF2 gene
SETD2
IWS1
Spt6
Aly
CTD
RNA Pol II
Resistance to
oncolytic viral
infection
type I IFN
mRNAs
Nuclear
export
IWS1
IWS1
S720/T721
AKT3
ISGF3
IRF9
STAT1
STAT2
ISRE
ISGs
ISGs transcription
Nucleus
Translation
Cytoplasm
ISGF3
IRF9
STAT1
STAT2
type I IFN
STAT1
STAT2
JAK
TYK2
IFNAR1/2
Autocrine/Paracrine
stimulation
Intercellular Space

Figure 8. Model of the effect of IWS1 phosphorylation at S720/721, on type I IFN expression and response to oncolytic viral infection.

Model of the effect of IWS1 phosphorylation at S720/721, on type I IFN expression and response to oncolytic viral infection. The phosphorylation of IWS1 at S720/T721 by AKT kinase controls the epigenetic regulation of *U2AF2* alternative RNA splicing, leading to inclusion of exon 2 in the mature *U2AF2* mRNA. Subsequently, the U2AF65α isoform binds CAR-E of type I IFNs and recruits Prp19, promoting their nucleocytoplasmic export and expression. During oncolytic viral infection, type I IFNs are properly exported, expressed and by acting in an autocrine and paracrine fashion, stimulate the IFNAR1/2 receptor. The latter leads to p-STAT1 activation and assembly of the ISGF3 on ISGs promoters. This confers resistance of lung adenocarcinoma cells to oncolytic viral infection.

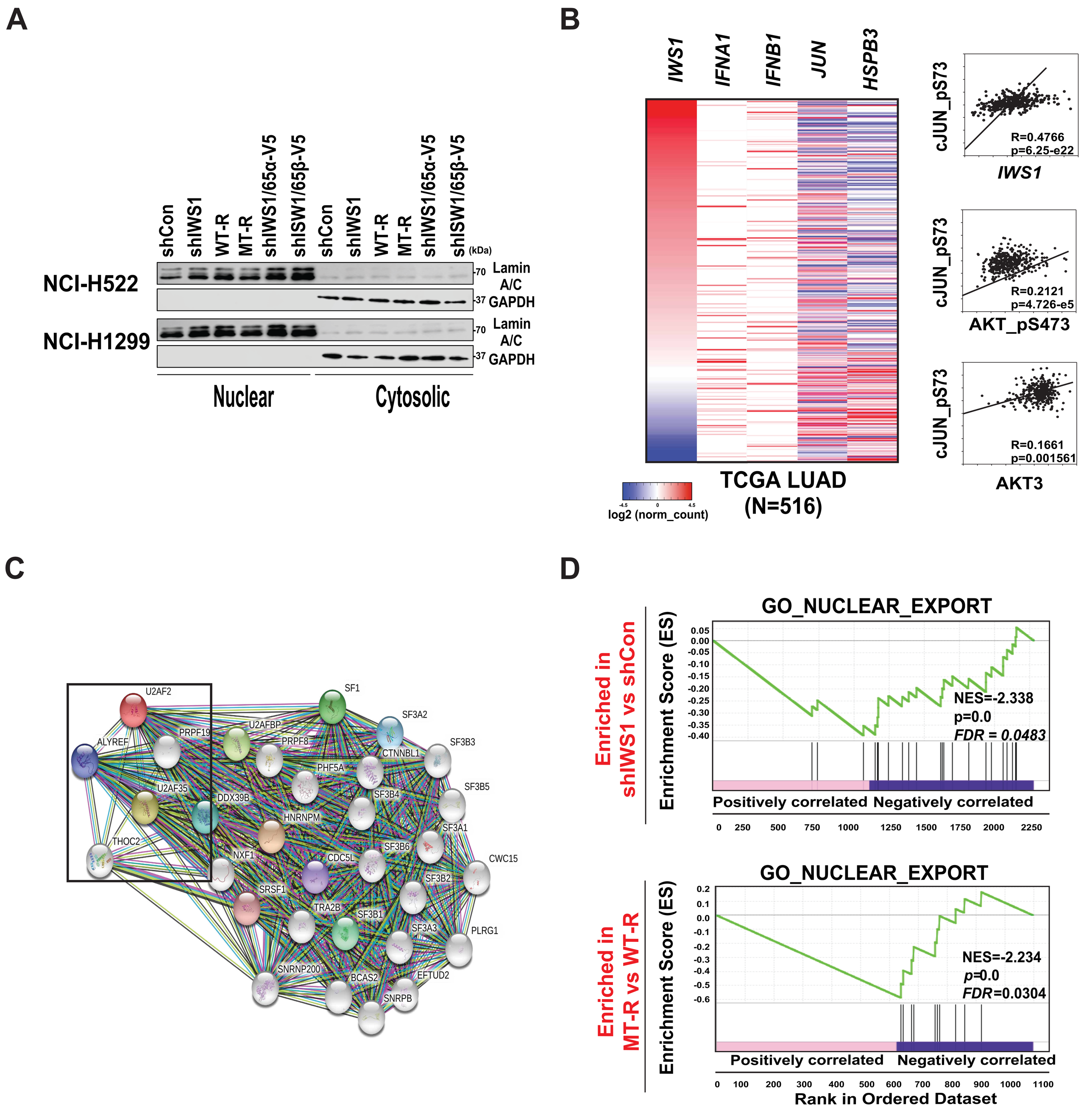

A
shCon
shIWS1
WT-R
MT-R
shIWS1/65α-V5
shISW1/65β-V5
(kDa)
NCI-H522
NCI-H1299
70
37
Lamin A/C
GAPDH
Nuclear
Cytosolic
B
IWS1
IFNA1
IFNB1
JUN
HSPB3
TCGA LUAD
(N=516)
log2 (norm_count)
-4.5
0
4.5
cJUN_pS73
IWS1
R=0.4766
p=6.25-e22
AKT_pS473
R=0.2121
p=4.726-e5
AKT3
R=0.1661
p=0.001561
C
U2AF2
SF1
SF3A2
U2AFBP
PRPF19
ALYREF
PRPF8
SF3B3
CTNNBL1
PHF5A
U2AF35
SF3B5
DDX39B
SF3B4
HNRNPM
SF3A1
THOC2
SF3B6
NXF1
CDC5L
CWC15
SF3B2
SRSF1
TRA2B
SF3B1
SF3A3
PLRG1
SNRNP200
BCAS2
EFTUD2
SNRPB
D
GO_NUCLEAR_EXPORT
Enriched in shIWS1 vs shCon
Enrichment Score (ES)
NES=-2.338
p=0.0
FDR = 0.0483
Positively correlated
Negatively correlated
GO_NUCLEAR_EXPORT
Enriched in MT-R vs WT-R
Enrichment Score (ES)
NES=-2.234
p=0.0
FDR=0.0304
Positively correlated
Negatively correlated
Rank in Ordered Dataset

Figure S1 (relative to Figure 1). IWS1 phosphorylation regulates the nucleocytoplasmic transport of mRNAs transcribed from a set of intronless genes, via *U2AF2* alternative RNA splicing.

A. The cytosolic and nuclear compartment of the indicated NCI-H522 (upper two lines) and NCI-H1299 cells (lower two lines) were probed with the indicated antibodies, to validate the fractionation.

B. (Left panel) Heat Maps showing the correlation of IWS1 RNA levels with intronless genes in the TCGA LUAD database. The RNA-seq expression values from each individual patient were expressed as $\log_2$ normalized counts and they were used to calculate the correlation coefficients. (Right panel) Correlation coefficient graphs of Reverse Phase Protein Assay (RPPA) levels of p-c-Jun (S73) in TCGA LUAD with the RNA levels of IWS1 (top), RRPA levels p-AKT (S473) (middle) and RNA levels of AKT3 (lower).

C. The Protein-Protein Interaction (PPI) network analysis of U2AF65-Prp19 using the STRING database. U2AF65 and Prp19 interact with components of TREX complex and Ref/Aly controlling the export of naturally intronless genes (red box)

D. Gene Set Enrichment Analysis (GSEA) of genes differentially expressed in shIWS1 versus shControl (left panels) and in shIWS1/MT-R versus shIWS1/WT-R (right panels) NCI-H522 cells demonstrates significant enrichment in genes involved in Nuclear Export. (NES–normalized enrichment score, FDR–false discovery ratio)

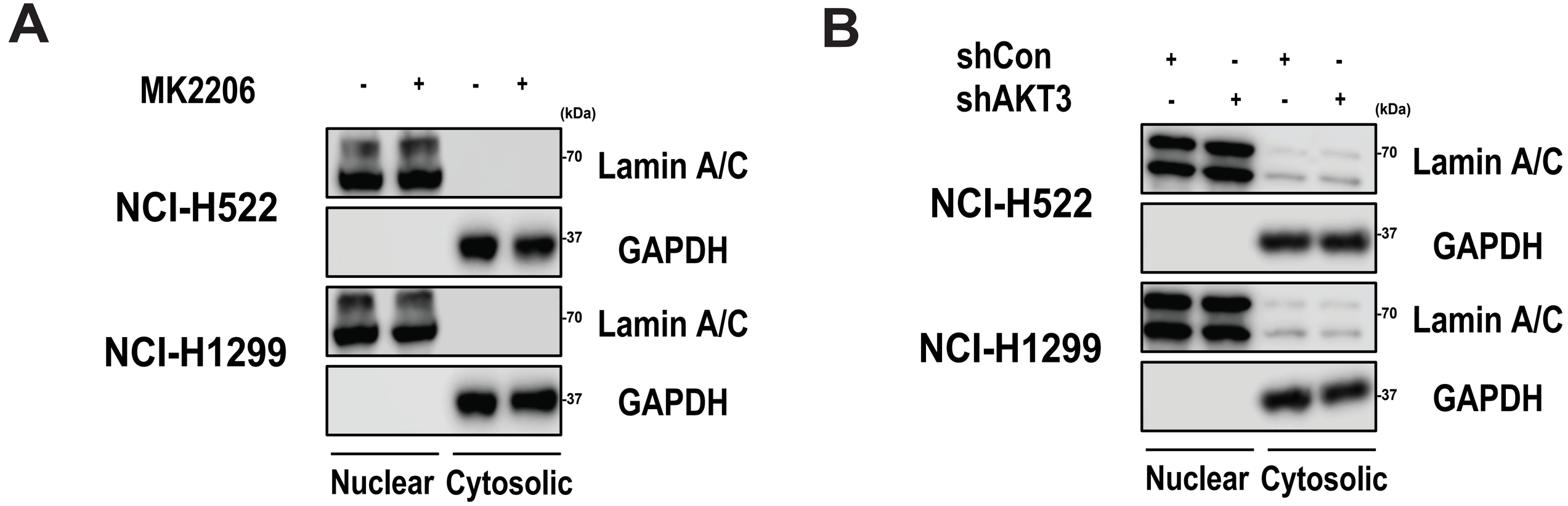

A
MK2206
- + - +
(kDa)
NCI-H522
-70
Lamin A/C
-37
GAPDH
NCI-H1299
-70
Lamin A/C
-37
GAPDH
Nuclear Cytosolic
B
shCon
shAKT3
+ - + -
- + - +
(kDa)
NCI-H522
-70
Lamin A/C
-37
GAPDH
NCI-H1299
-70
Lamin A/C
-37
GAPDH
Nuclear Cytosolic

Figure S2 (relative to Figure 2). AKT3 kinase affects the nucleocytoplasmic export of RNAs transcribed from intronless genes, thought IWS1 phosphorylation.

A and B. The cytosolic and nuclear compartment of the indicated NCI-H522 (upper two lines) and NCI-H1299 cells (lower two lines) were probed with the indicated antibodies, to validate the fractionation.

A

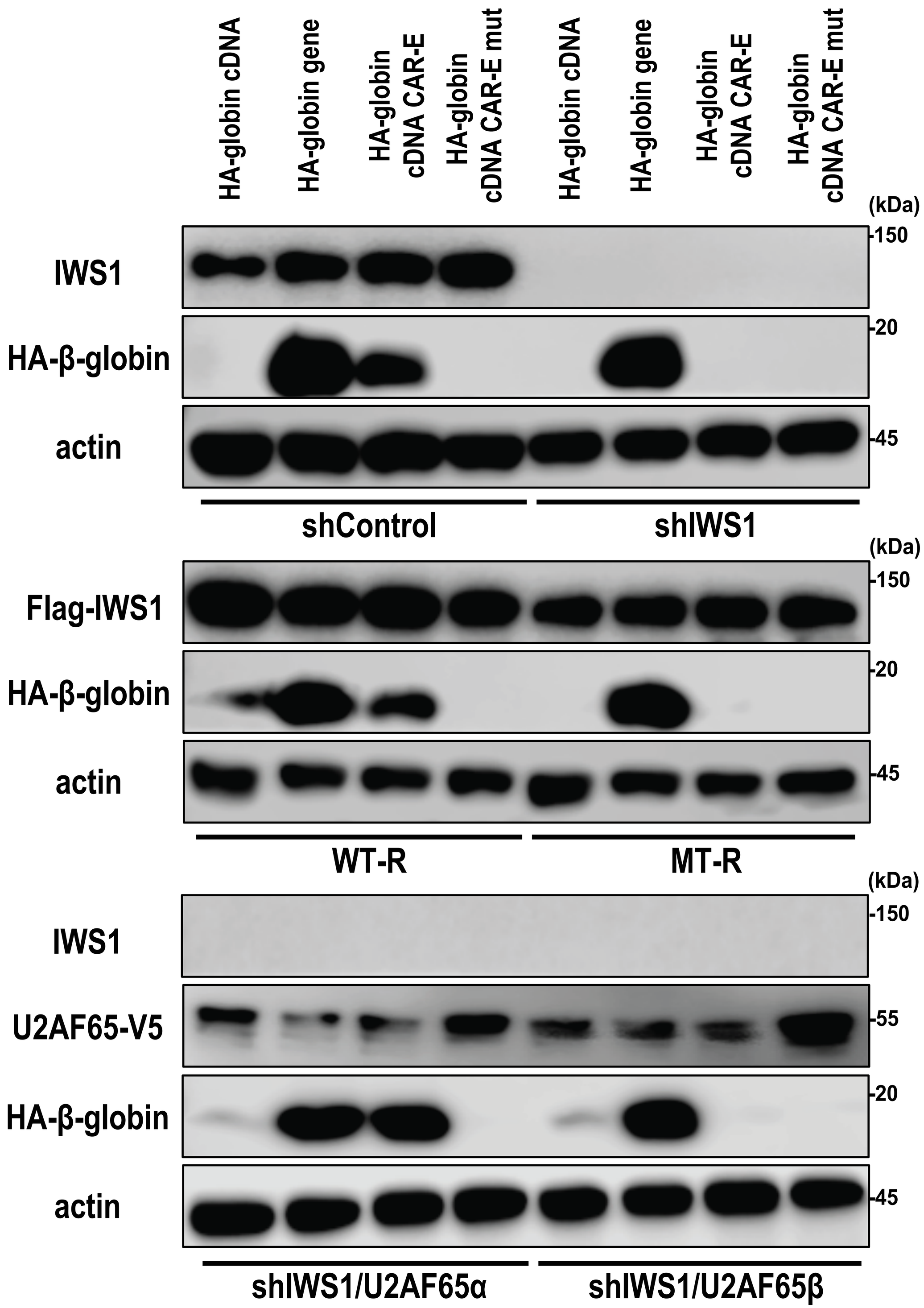

NCI-H1299
HA-globin cDNA
HA-globin gene
HA-globin cDNA CAR-E
HA-globin cDNA CAR-E mut
HA-globin cDNA
HA-globin gene
HA-globin cDNA CAR-E
HA-globin cDNA CAR-E mut
(kDa)
IWS1
150
HA-β-globin
20
actin
45
shControl
shIWS1
(kDa)
Flag-IWS1
150
HA-β-globin
20
actin
45
WT-R
MT-R
(kDa)
IWS1
150
U2AF65-V5
55
HA-β-globin
20
actin
45
shIWS1/U2AF65α
shIWS1/U2AF65β

Figure S3 (relative to Figure 3). IWS1 phosphorylation is required for the proper function of the CAR-E elements.

A. *IWS1 phosphorylation is required for CAR-E function.* NCI-H1299 cells transduced with the indicated constructs, were transfected transiently with pCMV HA-β-globin cDNA, HA-β-globin wt, HA-β-globin CAR-E and HA-β-globin CAR-E_{mut}. After 48h were harvested, and their lysates were probed with the indicated antibodies.

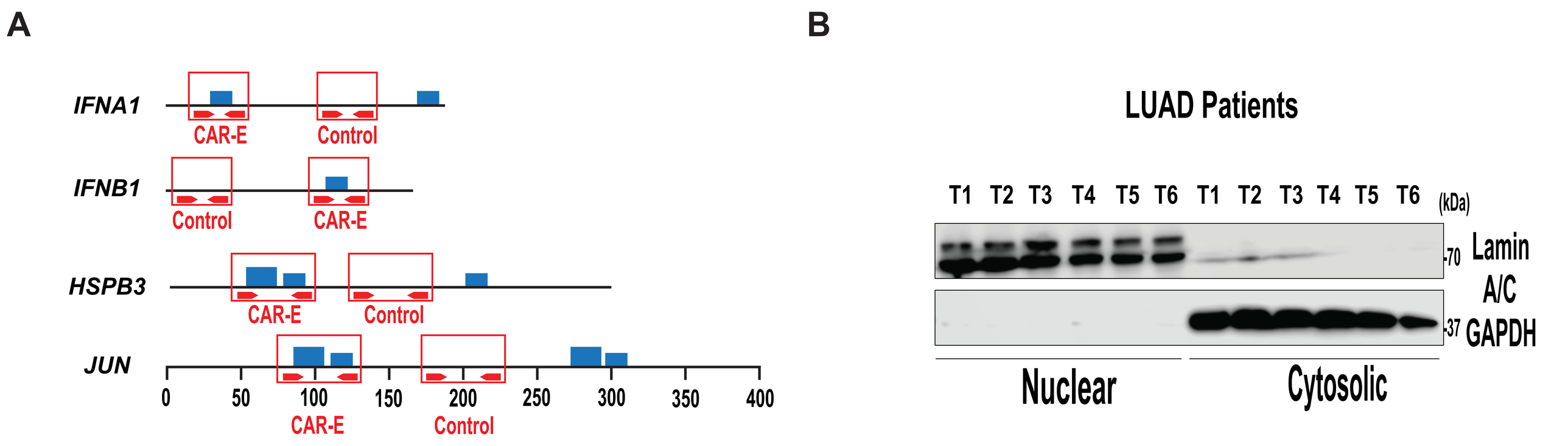

A
IFNA1
CAR-E
Control
IFNB1
Control
CAR-E
HSPB3
CAR-E
Control
JUN
0
50
100
150
200
250
300
350
400
CAR-E
Control
B
LUAD Patients
T1 T2 T3 T4 T5 T6 T1 T2 T3 T4 T5 T6
(kDa)
70
Lamin A/C
37
GAPDH
Nuclear
Cytosolic

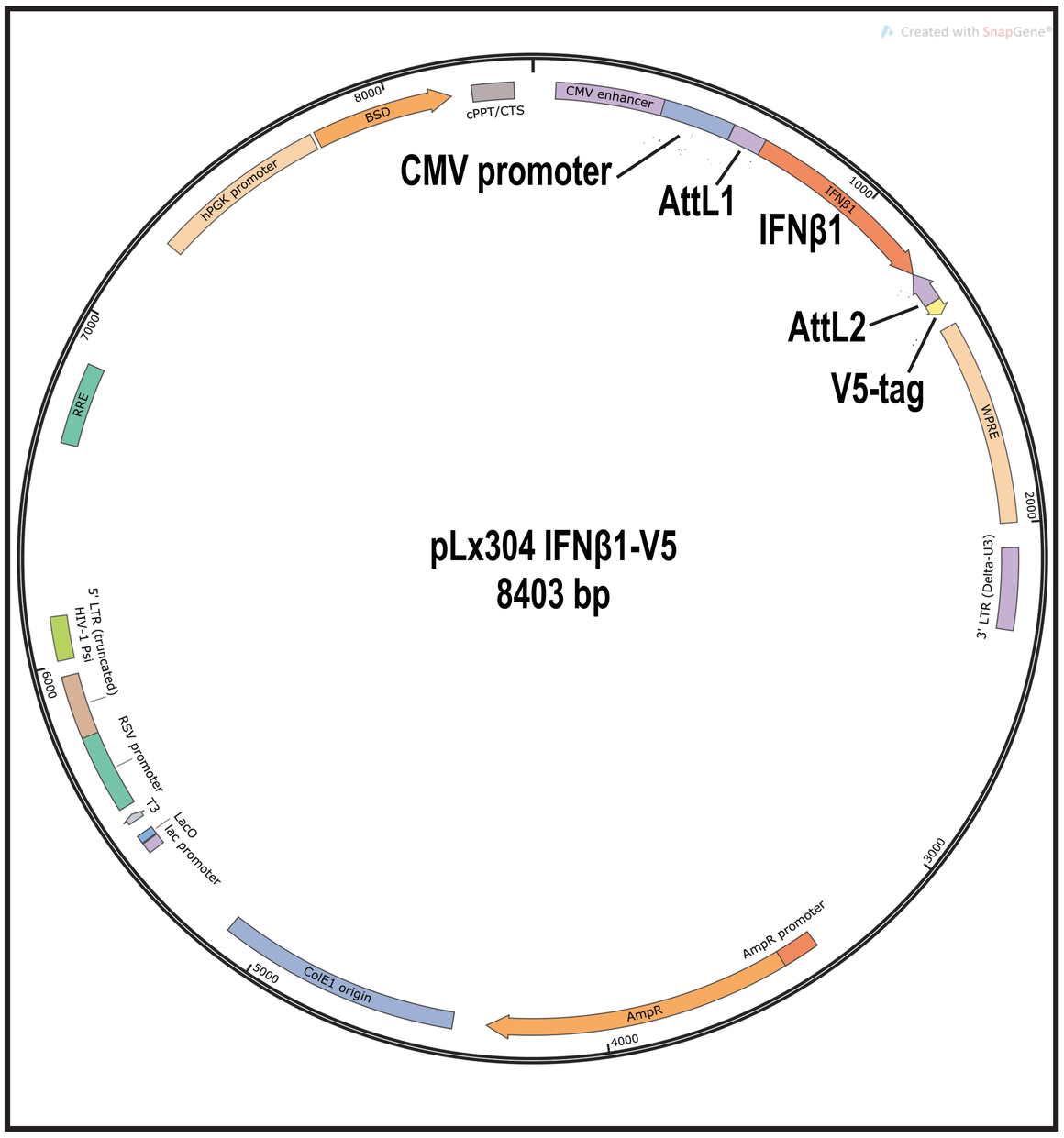
Figure S4 (relative to Figure 4). The splicing of the *U2AF2* mRNA downstream of IWS1 phosphorylation, regulates the Prp19 recruitment on the CAR-E elements.

A. The 10-nt consensus element (CAR-E) identified in the CARs from *IFNα1, IFNβ1, c-Jun or HSPB3* is shown, as described previously (Lei et al., 2013[13]). The locations of the CAR-Es in each CAR are indicated by the boxes. The numbering is relative to the first nucleotide of each CAR sequence. In red boxes with the red arrows shows the primers used to amplify the CAR-E elements (CAR-E) or the control binding regions (Control) in the indicated genes.

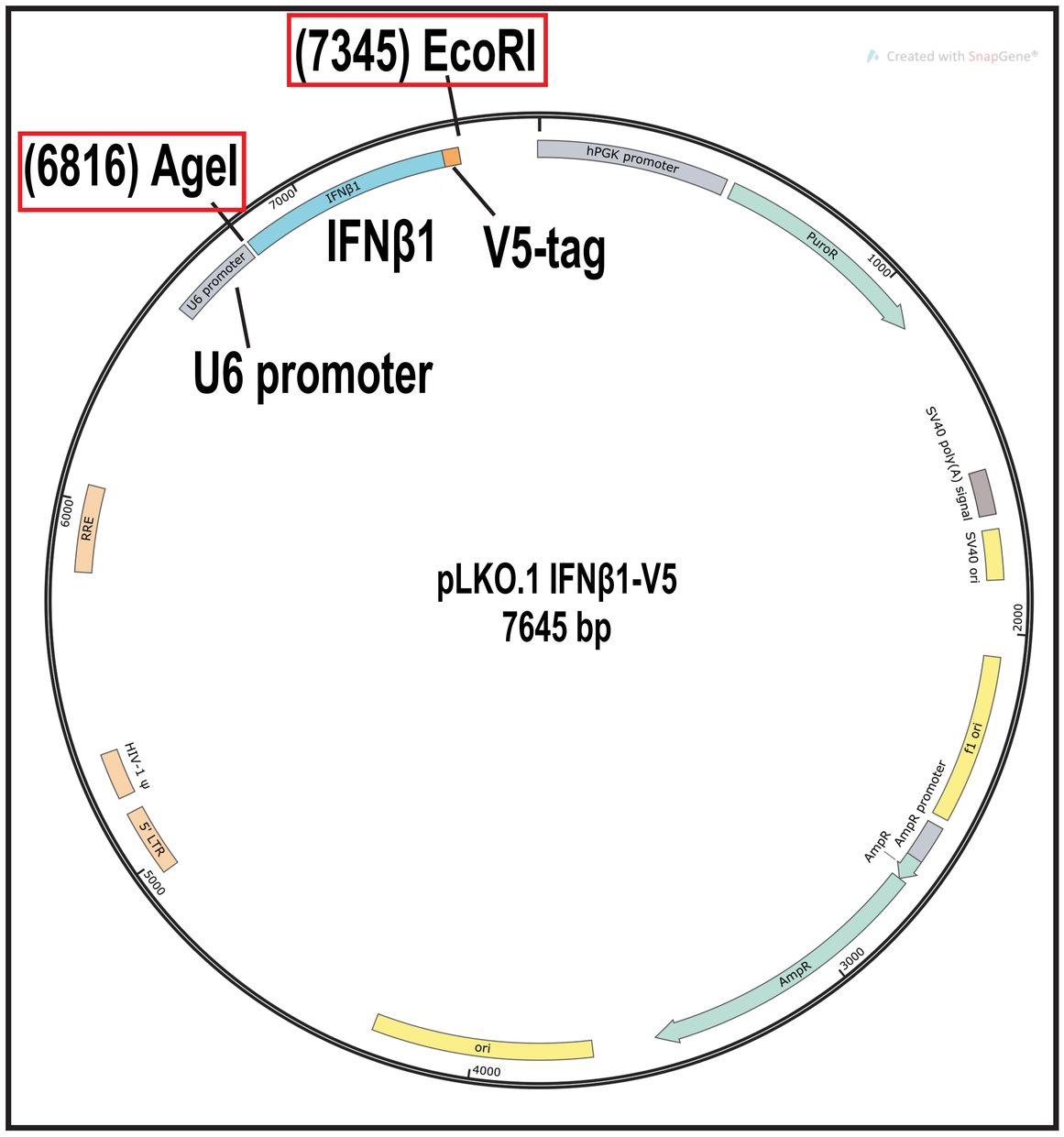
B. The cytosolic and nuclear compartment of the indicated low and high-IWS1 LUAD group were probed with the indicated antibodies, to validate the fractionation.

A

CMV_promoter (RNA Pol II_driven)



U6_promoter (RNA Pol III_driven)



B

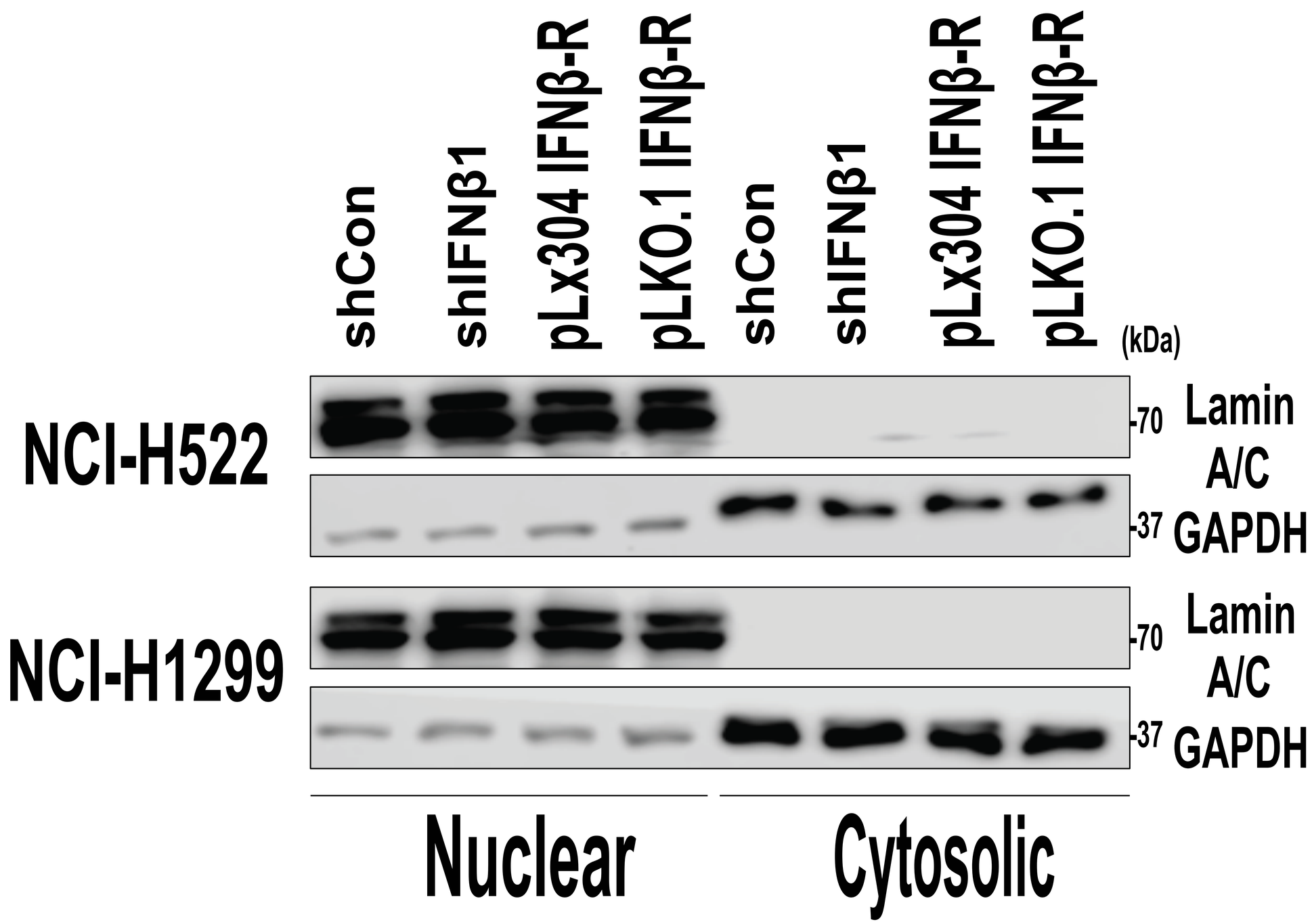
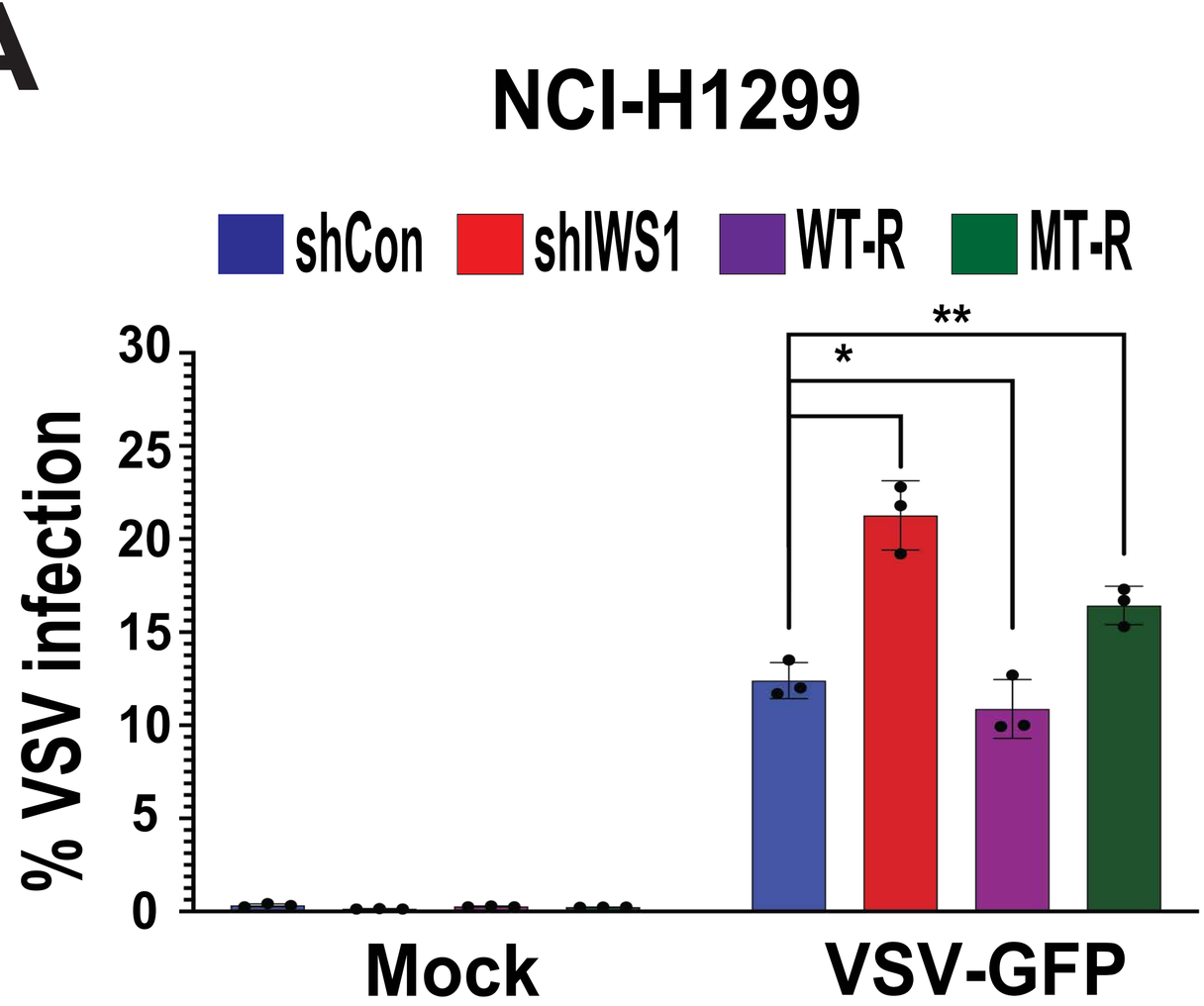


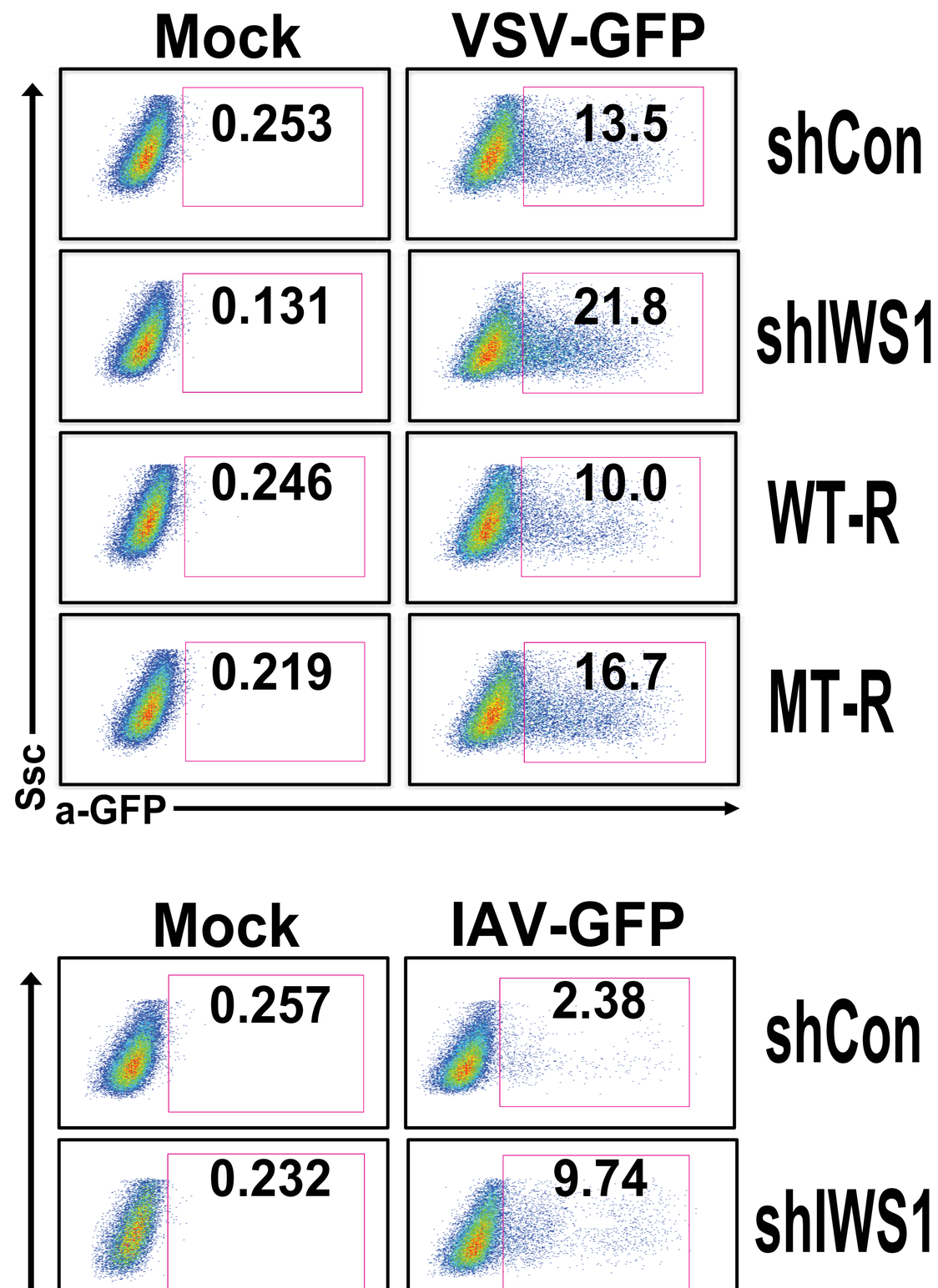
Figure S5 (relative to Figure 5). RNA-Pol II affects the splicing-independent nuclear export of intronless genes.

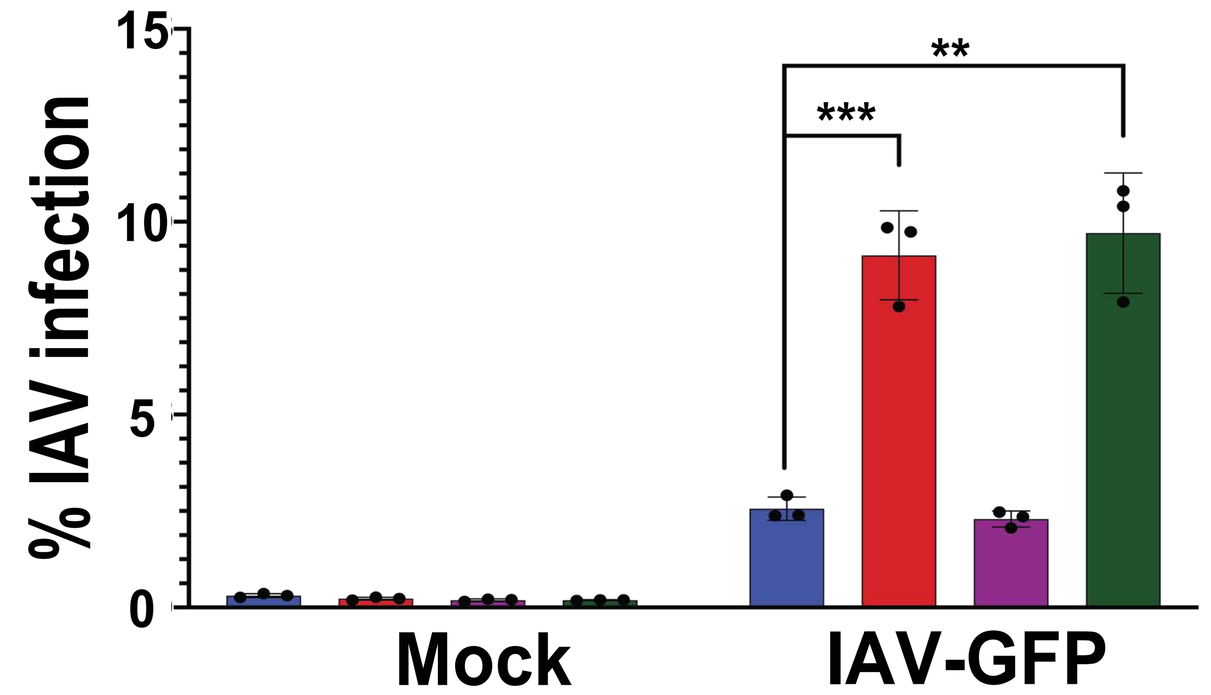
A. Schematic representation of CMV pLx304 IFNβ1-V5 (RNA Pol II-driven) and U6 pLKO.1 IFNβ1-V5 (RNA Pol III-driven) used in Fig. 5A and 5B is shown. The red boxes in pLx304 indicate the Gateway LR sites and in pLKO.1 map the restriction enzyme sites used for proper IFNβ1-V5 cloning into pLKO.1 (AgeI-EcoRI sites)

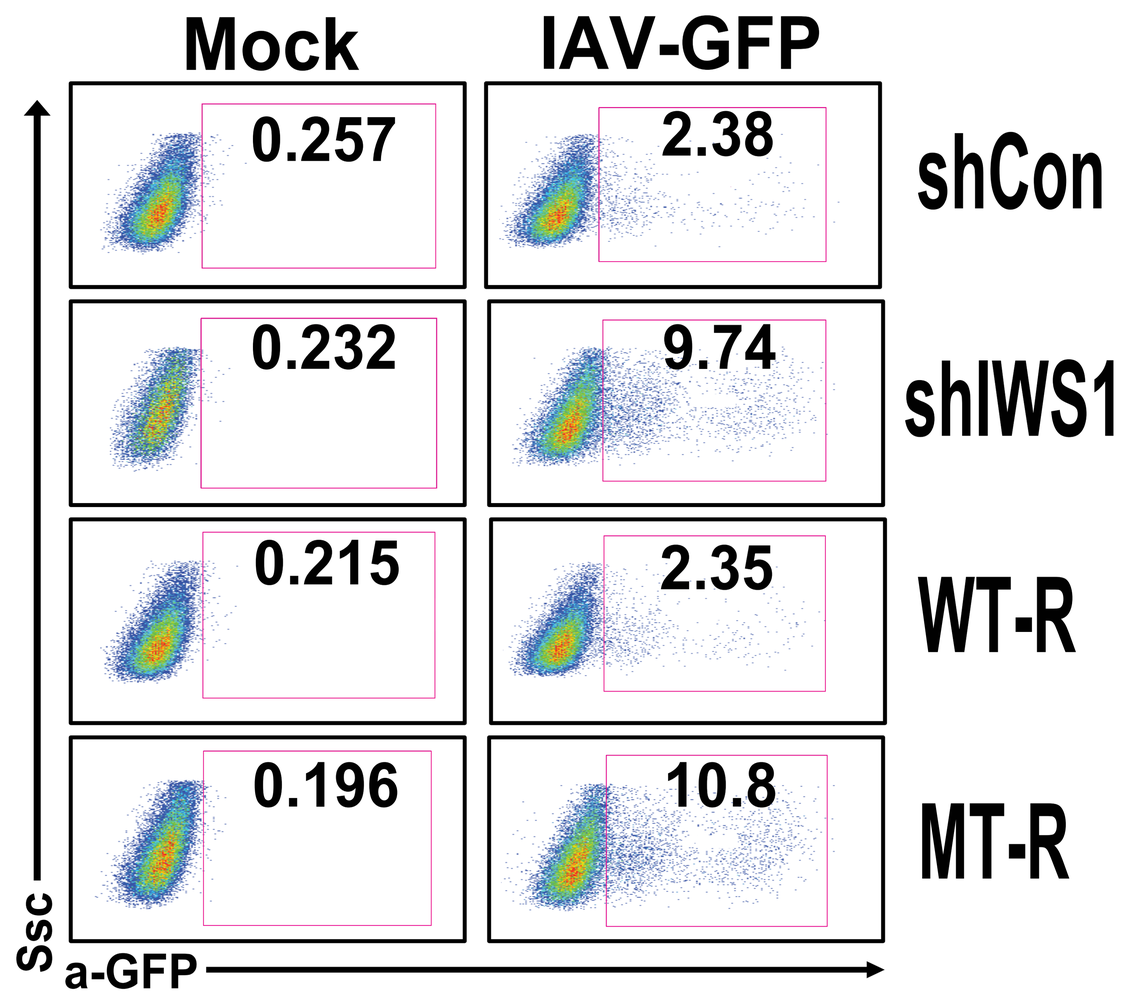
B. The cytosolic and nuclear compartment of the indicated NCI-H522 (upper two lines) and NCI-H1299 cells (lower two lines) were probed with the indicated antibodies, to validate the fractionation.

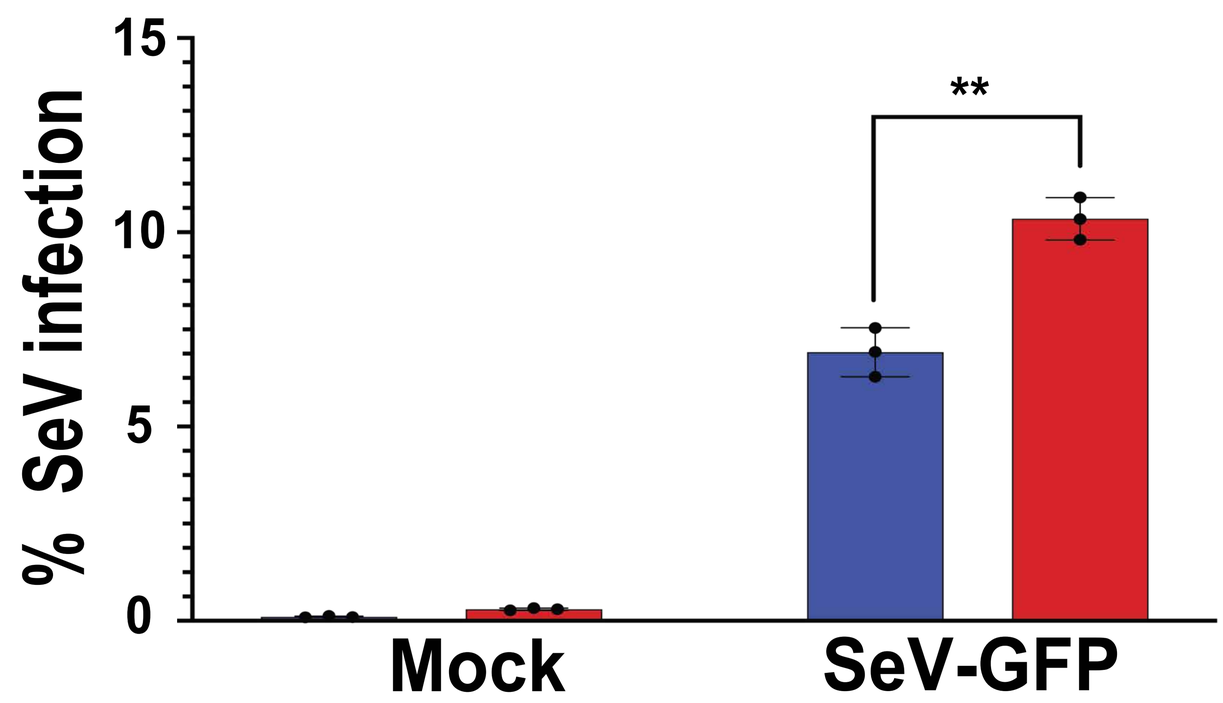
A

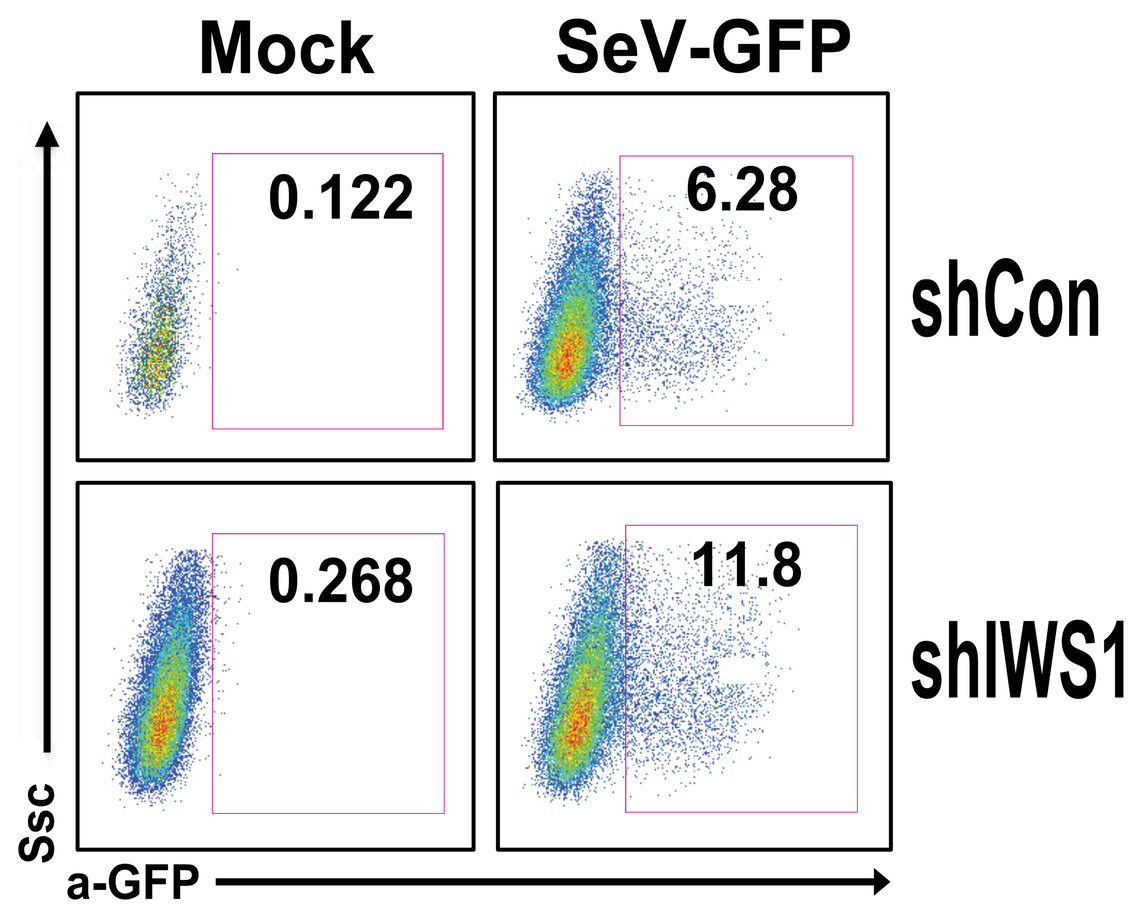

NCI-H1299
shCon
shIWS1
WT-R
MT-R
% VSV infection
30
25
20
15
10
5
0
*
**
Mock
VSV-GFP


Mock
VSV-GFP
0.253
13.5
shCon
0.131
21.8
shIWS1
0.246
10.0
WT-R
0.219
16.7
MT-R
Ssc
a-GFP


% IAV infection
15
10
5
0
***
**
Mock
IAV-GFP


Mock
IAV-GFP
0.257
2.38
shCon
0.232
9.74
shIWS1
0.215
2.35
WT-R
0.196
10.8
MT-R
Ssc
a-GFP


% SeV infection
15
10
5
0
**
Mock
SeV-GFP


Mock
SeV-GFP
0.122
6.28
shCon
0.268
11.8
shIWS1
Ssc
a-GFP

B

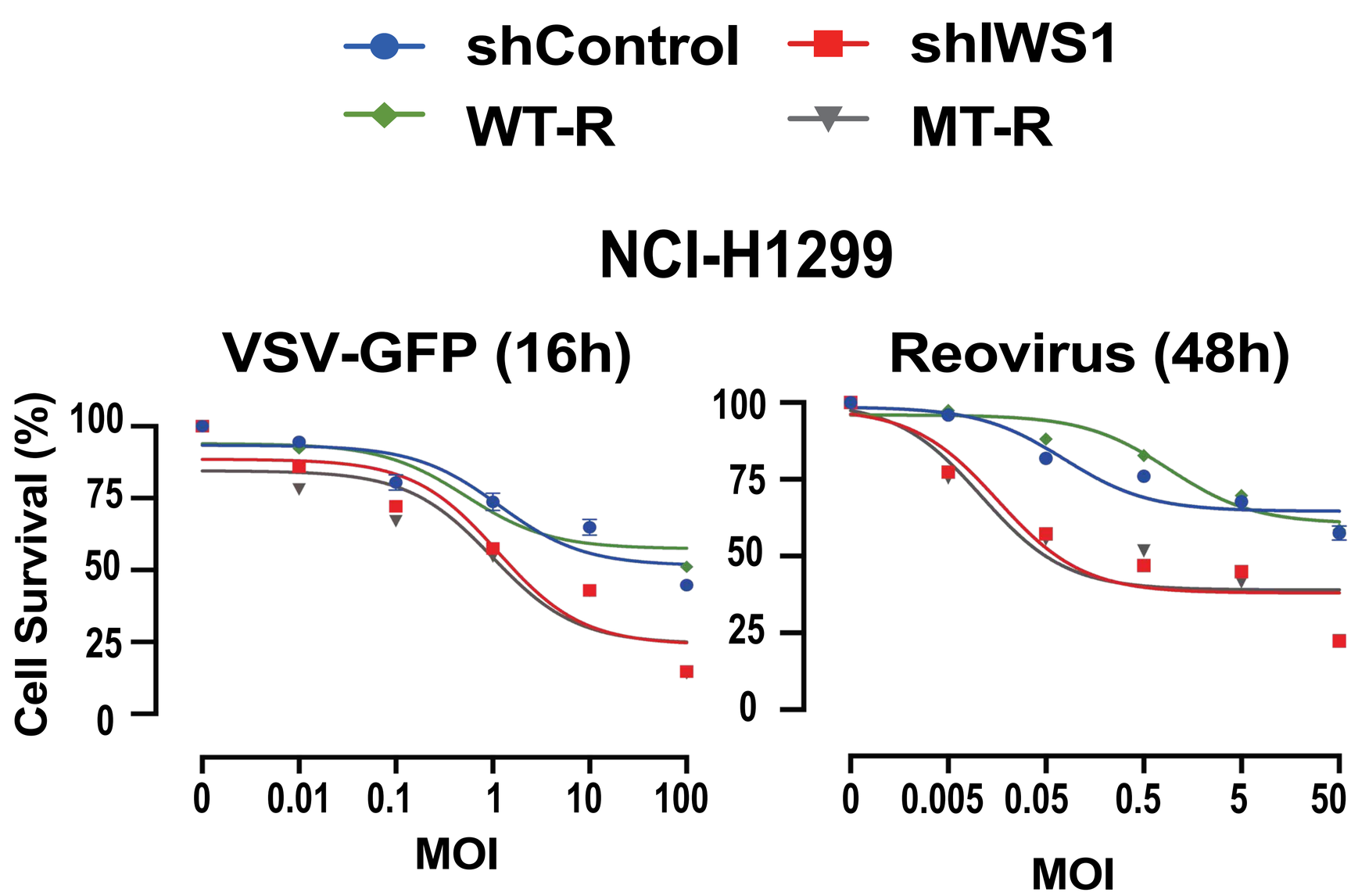
IFNβ1 (1000U/mL)
Unstim.
5
10
15
20
30
40
IFNβ1
Tubulin
-20
-55
NCI-H522

Figure S6 (relative to Figure 6). The loss of IWS1 phosphorylation enhances viral replication, through the aberrant p-STAT1 signaling and transcription of ISGs.

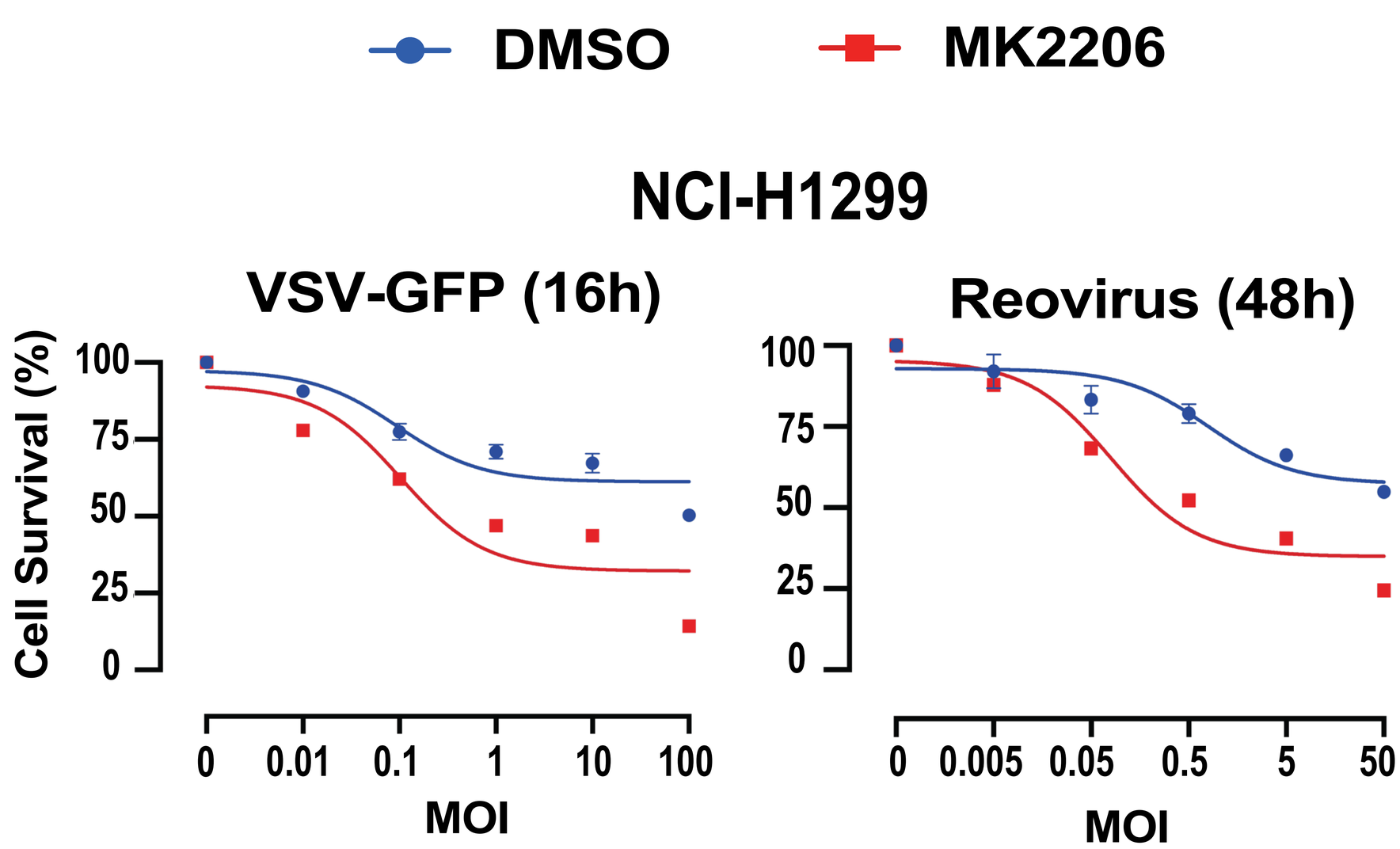
A. *The loss of IWS1 phosphorylation enhances viral replication.* The indicated NCI-H1299 cells were infected with VSV-GFP, SeV-GFP or Influenza-GFP at an MOI of 0.25 for 24h hours (VSV-GFP and SeV-GFP) and MOI of 1 for 24 hours (IAV-GFP), respectively. Cells were then fixed and were examined for GFP positivity to measure the percentage of cells infected using flow cytometry. Results shown are representative of at least three independent experiments, each performed with triplicate samples. Bars show the % infection of each viral strain ±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).

B. NCI-H522 cells were stimulated with 1000u/mL human IFNβ1 for time point intervals and their lysates were probed with the indicated antibodies. The results confirm the endocytosis of exogenous IFNβ1 in these cells.

A


shControl
shIWS1
WT-R
MT-R
NCI-H1299
VSV-GFP (16h)
Reovirus (48h)
Cell Survival (%)
100
75
50
25
0
0
0.01
0.1
1
10
100
MOI
0
0.005
0.05
0.5
5
50
MOI

B


DMSO
MK2206
NCI-H1299
VSV-GFP (16h)
Reovirus (48h)
Cell Survival (%)
100
75
50
25
0
0
0.01
0.1
1
10
100
MOI
0
0.005
0.05
0.5
5
50
MOI

C

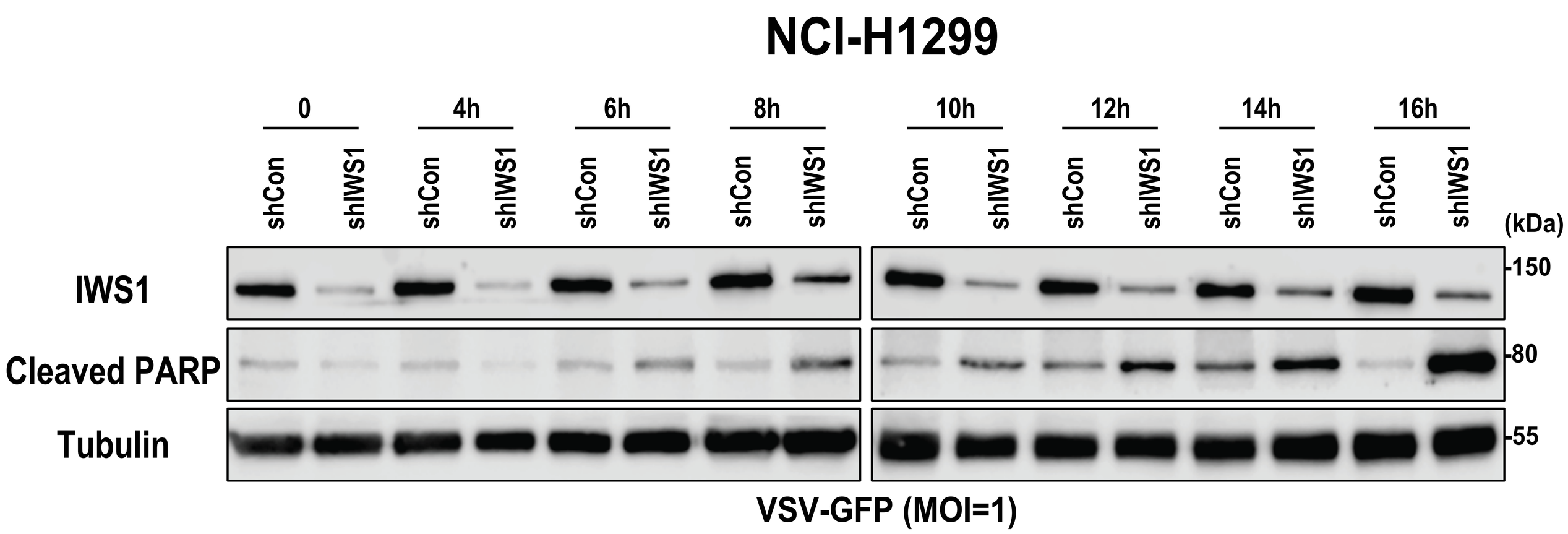

NCI-H1299
0
4h
6h
8h
10h
12h
14h
16h
shCon
shIWS1
(kDa)
IWS1
Cleaved PARP
Tubulin
150
80
55
VSV-GFP (MOI=1)

Figure S7 (Relative to Figure 7). Inhibition of the AKT/p-IWS1 axis sensitizes lung adenocarcinoma cells to oncolytic viral killing.

A. *The loss of IWS1 phosphorylation sensitizes lung adenocarcinoma cells to oncolytic viral killing.* NCI-H1299 cells transduced with the indicated constructs were exposed to increasing MOI of VSV-GFP or Reovirus for 16h and 48h, respectively. Cell proliferation was assessed by fluorescent detection of the reduction of resazurin to resorufin by viable cells and expressed as a percentage of uninfected cells. Mean values are shown ±SD (n=3), data are representative of two independent experiments.

B. *The loss of AKT/p-IWS1 axis sensitizes lung adenocarcinoma cells to oncolytic viral killing.* NCI-H1299 cells treated with DMSO or 5μM of the pan-AKT inhibitor MK2206, were exposed to increasing MOI of VSV-GFP or Reovirus for 16h and 48h, respectively. Cell proliferation was assessed by fluorescent detection of the reduction of resazurin to resorufin by viable cells and expressed as a percentage of uninfected cells. Mean values are shown ±SD (n=3), data are representative of two independent experiments.

C. *The loss of IWS1 phosphorylation sensitizes lung adenocarcinoma cells to oncolytic viral killing via Caspase-mediated death.* NCI-H1299 shControl and shIWS1 cells were infected for the indicated time point intervals with VSV-GFP at a MOI=1. Western blot lysates from these time points were probed with the indicated antibodies, showing a robust cleavage of PARP, a sensitive marker of Caspase mediated apoptosis, in shIWS1 cells even in early time points of infections.