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ROR2 interacts with JAK1 and JAK2 and potentiates the IFN γ -induced JAK/STAT signaling pathway in colorectal adenocarcinoma

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Abstract: 173 words

Abstract

The Wnt receptor ROR2 is a developmentally critical pseudokinase aberrantly overexpressed in aggressive cancers, where it correlates with poor prognosis and metastasis. However, the molecular mechanisms underlying these pro-tumorigenic effects remain poorly understood. In this study, we profiled the ROR2 interactome in a murine colorectal carcinoma model using co-immunoprecipitation coupled to mass spectrometry, and identified JAK1 and JAK2 as ROR2-binding partners. Functionally, we demonstrated that ROR2 overexpression potentiates the IFN γ -activated JAK/STAT signaling, leading to the upregulation of the immunosuppressive effectors PD-L1 and IDO1. Using computational structural analyses, we identified conserved JAK-binding motifs within the intracellular C-terminal region of ROR2 and demonstrated its functional requirement for IFN γ -JAK/STAT signaling enhancement. Finally, we established the clinical relevance of this ROR2-mediated IFN γ -JAK/STAT axis in human colorectal adenocarcinoma, and showed that high ROR2 expression positively correlates with the IFN γ signature, JAK/STAT signaling, and expression of downstream immunosuppressive effectors. Collectively, our findings uncover a previously unrecognized role for ROR2 as a modulator of a key immune regulatory pathway that drives immune evasion, highlighting ROR2 as a potential therapeutic target.

Introduction

Wnt signaling is an evolutionarily conserved pathway that governs fundamental cellular processes and plays a critical role in embryonic development and adult tissue homeostasis. Dysregulation of the pathway, due to mutations or aberrant activation of its components, is a hallmark of many malignancies and represents a major target for therapeutic intervention [1-3]. The mammalian Wnt ligands bind to Frizzled (Fzd) receptors, initiating intracellular responses commonly classified into canonical (β -catenin-dependent) and non-canonical (β -catenin-independent) signaling pathways. In the canonical Wnt signaling cascade, ligands engage the Fzd together with the low-density lipoprotein receptor-related protein 5/6 (LRP5/6) co-receptors, leading to cytoplasmic accumulation and nuclear translocation of β -catenin and subsequent activation of TCF/LEF-mediated transcriptional program that regulates cell proliferation, stem cell maintenance, cell fate determination and tissue regeneration [3-5]. Non-canonical Wnt ligands, in contrast, activate responses independently of β -catenin activity, primarily through the Planar Cell Polarity (PCP) and Ca^{2+} pathways to regulate cell migration, polarity, cytoskeletal organization and Ca^{2+} signaling [6-8]. To effectively propagate Wnt responses intracellularly and achieve signaling diversity and specificity, Wnt receptors often rely on additional transmembrane co-receptors, including the receptor orphan tyrosine kinase-like receptor 2 (ROR2).

ROR2 is a single-pass transmembrane receptor tyrosine kinase (RTK) highly expressed in various tissues during embryogenesis, where it plays an essential role in the morphogenesis of the skeletal and cardiovascular systems [9-12]. While ROR2 is largely silenced or expressed at low levels in most healthy adult tissues, it is frequently aberrantly overexpressed in several human malignancies [13]. High ROR2 expression is commonly associated with poor survival and increased metastatic risk across diverse solid tumors, including pancreatic ductal adenocarcinoma [14-16], clear cell renal carcinoma [17], cervical cancer [18], laryngeal squamous cell carcinoma [19], and breast cancer [20-23]. Mechanistically, ROR2 confers a pro-tumorigenic advantage to cancer cells by driving proliferation, invasion and metastasis [6-8, 20, 22, 24-27], primarily through (i) the activation of non-canonical Wnt/PCP pathway, where activated RhoA/ROCK or JNK/AP-1 drives cytoskeletal remodeling, invasion and motility [6-8, 19, 20,

24, 25, 28-31], (ii) the modulation of focal adhesion dynamics via FAK- and Src-related programs [25, 27], and (iii) the promotion of cell survival and proliferation via the PI3K/AKT/mTOR pathway [22, 26, 27].

In colorectal carcinoma (CRC), however, the role of ROR2 appears more complex and context- and tumor stage-dependent. While ROR2 mRNA is detectable at low levels in normal colon tissue, its expression in tumor tissue is often substantially altered and strongly influenced by epigenetic status, hypoxia and EMT-inducing transcription factors [32]. On the one hand, some studies suggest a tumor-suppressive function for ROR2 in CRC, in which its re-expression can suppress tumor growth by inhibiting β -catenin and AKT signaling pathways [32-34]. On the other hand, other studies show that elevated ROR2 levels in metastatic and late-stage tumors promote migration and invasion, and strongly correlate with advanced disease and unfavorable prognosis [8, 32, 35-37]. Despite its clear clinical relevance in CRC, the signal transduction mechanisms used by ROR2 remain poorly understood. ROR2 is classified as a pseudokinase due to evolutionary substitutions in key residues within its tyrosine kinase domain, rendering it catalytically inactive. Like other receptor pseudokinases, e.g., HER3, PTK7, ROR2 is thought to act primarily as a molecular scaffold or allosteric modulator, recruiting effector proteins to the cell membrane to assemble signaling complexes and transmit intracellular signals. Yet, the precise identity of these effectors and the structural basis of their recruitment, particularly in the context of advanced CRC, remain to be fully elucidated.

IFN γ is a soluble cytokine with a pivotal function in cancer immune surveillance, orchestrating both innate and adaptive immune responses [38, 39]. It is secreted mainly by activated T cells and natural killer cells as an antiparallel homodimer of two non-covalently associated 17kDa polypeptide subunits [40, 41]. The IFN γ receptor consists of two protein chains, the ligand-binding Interferon gamma receptor 1 (IFNGR1) and the signal-transducing Interferon gamma receptor 2 (IFNGR2), which together form a signaling-competent tetramer upon ligand binding. Because IFNGR1 and IFNGR2 lack intrinsic kinase activity, they constitutively associate with Janus Kinase 1 (JAK1) and JAK2, respectively, to transmit intracellular signaling [42]. JAK1 and JAK2 interact with their cognate receptor monomers

through their N-terminal FERM (four-point-one, ezrin, radixin, moesin) and SH2 (Src homology 2) domains, which recognize conserved membrane-proximal “Box1” and “Box2” motifs on the receptor intracellular tails [43]. Box1 is a proline-rich sequence that serves as the primary binding site for the FERM domain, whereas Box2 is a hydrophobic segment located downstream of Box1 to stabilize the receptor-JAK complex [44]. Although the crystal structures of the IFNGR1-JAK1 and IFNGR2-JAK2 complexes have not yet been resolved, recent studies on JAK1 interactions with the related receptors Interferon lambda receptor 1 (IFNLR1) and Interleukin-10 receptor subunit alpha (IL10RA) have provided high-resolution insights into Box1/Box2-mediated docking and JAK activation [44, 45]. JAK activation occurs as a sequential event: without ligands, JAK1 adopts a compact “closed” conformation in which the C-terminal kinase domain folds back onto the N-terminal FERM-SH2 module, occluding the kinase active site [44]. Upon cytokine binding, receptor dimerization brings the intracellularly associated JAK1 and JAK2 into proximity, enabling auto- or trans-phosphorylation of their activation loops. This unlocks JAK1 and JAK2 from their autoinhibitory state and initiates downstream signaling through phosphorylation of tyrosine residues on the intracellular domain of the receptor subunits, which then serve as docking sites for signal transducer and activator of transcription 1 (STAT1) and STAT3. Once docked, STAT1 and/or STAT3 are phosphorylated by activated JAK1/2, leading to their dimerization and nuclear translocation, where they drive a broad transcriptional program composed of numerous target genes called Interferon Stimulated Genes (ISGs). These ISGs mediate diverse, and sometimes opposing, effects in cancer cells, acting as both positive and negative regulators of inflammatory signaling [46-50]. Indeed, while IFN γ triggers antiproliferative [51], anti-angiogenic [52], pro-apoptotic effects [53, 54] and antigen-presentation programs [55] in neoplastic cells, it also drives immune-evasive mechanisms through upregulation of programmed cell death ligand 1 (PD-L1) and indoleamine 2,3-Dioxygenase 1 (IDO1), both of which attenuate T-cell function [56-58]. Thus, the net impact of IFN γ on tumor progression is highly context-dependent, shaped by the composition of cell types and the intensity of the signal within the tumor microenvironment [38, 39].

In this study, using a murine colorectal carcinoma cell line, we mapped the ROR2 interactome and identified JAK1 and JAK2 as its binding partners, and our subsequent investigation positioned ROR2 as a previously unrecognized component of the IFN γ -induced JAK/STAT signaling. We showed that ROR2 overexpression enhanced the IFN γ -driven JAK/STAT signaling and upregulated key downstream ISGs, including the immunosuppressive effectors PD-L1 and IDO1, and that this modulation required the Serine/Threonine/Proline-rich (STP) domain and C-terminal tail of ROR2 that contain BOX1 and BOX2 motifs. Finally, analysis of colorectal adenocarcinoma samples from The Cancer Genome Atlas Program (TCGA) revealed that high ROR2 expression positively correlates with the IFN γ response and JAK/STAT signaling signatures, and expression of PD-L1 and IDO1, suggesting an immunosuppressive role for ROR2 overexpression in CRC.

Results

Identification of ROR2's interactome in murine colon adenocarcinoma cells

ROR2 exhibits a complex dual role in colorectal adenocarcinoma (COAD), with reports of both up- and down-regulation compared to normal colon tissues [8, 32-37]. However, elevated ROR2 expression is consistently associated with poor clinicopathologic features and outcomes. In the cohort of COAD patients, tumor *Ror2* mRNA levels were increased with advanced TNM (Tumor/Nodes/Metastasis) stage, in which ROR2 expression is significantly higher in stage II-IV as compared to those at stage I (Figure 1A). Consistently, patients with ROR2-high tumors had markedly shorter disease-free survival than those with ROR2-low tumors, highlighting the association of elevated ROR2 expression with an increased risk of postoperative relapse (Figure 1B).

These clinical findings motivated the choice of a widely used colon adenocarcinoma cell line, CT26, derived from BALB/c mice, as a model to study the molecular mechanisms underlying the pro-tumorigenic functions of ROR2 in CRC. To do so, we overexpressed ROR2 in CT26 cells, in which endogenous ROR2 expression is relatively low, by transducing a C-terminally Flag-tagged ROR2 (ROR2-Flag) via lentiviral transduction. We first confirmed the efficacy of the overexpressed ROR2 in CT26 by determining its cellular localization and signaling transducing ability, and compared them to the cells transduced with an empty vector (EV). As shown, the overexpressed ROR2 proteins were successfully located at the cell membrane (Figure 2A), and they were also functionally active to transduce Wnt signaling activities as evidenced by the increases in phosphorylation of non-canonical Wnt effectors, JNK and PKC α (Figure 2B), as well as the enhancement in Wnt3a-induced β -catenin-dependent transcription activity, validated using the TOPFlash luciferase reporter assay (Figure 2C). These results confirmed that the overexpressed ROR2-Flag is functional and capable of modulating both canonical and non-canonical Wnt signaling as reported previously [32, 33].

To map the ROR2 interactome and thereby investigate its signal transduction mechanism, we performed co-immunoprecipitation (co-IP) for Flag-tagged ROR2 using anti-Flag or anti-HA (negative

control) magnetic beads followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Figure 2D). Analysis of two independent replicates identified high-confidence ROR2-interacting proteins based on the following criteria: (i) abundance ratios equal or above those of known ROR2-binding proteins (≥ 2.3 ; pull-down from protein extracts of CT26 expressing ROR2-Flag using anti-Flag versus those using anti-HA non-specific coated beads), (ii) a peptide spectrum match (PSM) ≥ 2 , and (iii) interactants identified in both independent experiments. Our identification yielded 86 confident candidate proteins (Table S1). Scatter plot analysis (Log2 Abundance ratio versus PSM; Figure 2E) showed that previously reported ROR2-binding proteins, Map3k7 (also called TAK1) [59] and Filamin A (FlnA) [60], were also identified in our pull-down, validating the reliability of the protocol. Gene Ontology (GO) enrichment analysis on the 86 identified interactants using the Reactome database via the Database for Annotation, Visualization and Integrated Discovery (DAVID) annotation tool revealed that, beyond expected associations with proteins participating in non-canonical β -catenin-independent Wnt signaling that regulates cell adhesion, migration, polarity and endocytosis via intersection with signaling mediated by Rho GTPases and Eph/ephrin, ROR2 interactome was also significantly enriched for proteins involved in immune system processes and cytokine signaling (Figure 2F). Notably, a critical mediator of cytokine signaling, JAK1, was identified among other signaling regulators. We then examined the interaction between ROR2 and JAK1 by performing co-IP and immunoblotting with independent sets of CT26 protein lysates. As shown in Figure 2G, endogenous JAK1 protein was precipitated in the pull-down of ROR2-Flag, confirming their interaction. The interaction between ROR2 and JAK1 was further validated in the reciprocal co-IP assays described below.

ROR2 interacts with JAK1 and JAK2

JAK1 is a critical mediator of JAK/STAT signaling, involved in multiple cytokine pathways, including IFN γ . IFN γ is a pleiotropic cytokine of the tumor microenvironment that shapes both innate and adaptive immune responses. Given that ROR2 harbors an intracellular domain capable of engaging

signaling kinases, we investigated whether ROR2 modulates IFN γ signaling through interactions with JAK1 and JAK2, the two JAK family members involved in IFN γ signaling.

To validate this hypothesis, we first confirmed their interaction by performing reciprocal co-IP assays with protein extracts from HEK293T cells transiently co-transfected with constructs expressing ROR2-Flag and JAK1 using anti-Flag or anti-JAK1 antibody (Figure 3A). As shown, pulling down ROR2-Flag protein precipitated JAK1 (Figure 3A, top); *vice versa*, precipitating JAK1 protein also pulled down ROR2-Flag (Figure 3A, bottom). Transducing JAK1-dependent signaling activated by IFN γ requires the coordination of JAK2 that is associated with IFNGR2; thus, we also assessed the interaction between ROR2 and JAK2 by conducting similar reciprocal co-IP assays in which ROR2-Flag and C-terminally HA-tagged JAK2 were transiently co-expressed in HEK293T cells and pulled down using anti-Flag or anti-HA antibody (Figure 3B). Interestingly, ROR2-Flag precipitation not only co-immunoprecipitated JAK1 but also pulled down JAK2-HA (Figure 3B, top); in agreement, ROR2 was also detected in the pulled-down protein complex of JAK2-HA (Figure 3B, bottom).

Furthermore, to determine if ROR2-JAK1 and -JAK2 associations are direct physical protein-protein interactions rather than indirect recruitment within a large protein complex, we performed a Bioluminescence Resonance Energy Transfer (BRET)-based assays in live HEK293T cells. We engineered energy donor constructs that express C-terminally NanoLuc Luciferase-fused ROR2 (NL-ROR2) and had constructs expressing Interferon Gamma Receptor 1 (NL-IFNGR1) or 2 (NL-IFNGR2) as positive controls; we also generated energy acceptor constructs which encode HaloTag-tagged JAK1 (HT-JAK1) or JAK2 (HT-JAK2) (Figure 3C). By co-expressing the energy donor and acceptor constructs in the presence of the HaloTag ligand, we were able to detect the emitted light from which the bioluminescent donor protein transferred energy to the acceptor protein if these two proteins were in close proximity. As shown in Figure 3D, cells co-expressing NL-IFNGR1 (positive control) or NL-ROR2 with HT-JAK1 showed specific energy transfer signals significantly higher than those from the negative control cells (NL-empty). Similarly, significant BRET signals were also detected from cells co-expressing NL-IFNGR2

(positive control) or NL-ROR2 with HT-JAK2 (Figure 3E). These NanoBRET results highly suggest that ROR2-JAK1 and ROR2-JAK2 interactions are the close-proximity binding of proteins.

ROR2 enhances the IFN γ -activated JAK/STAT signaling

To determine the functional consequences of the ROR2-JAK1/JAK2 interaction, we investigated the impact of ROR2 overexpression on the IFN γ -induced activation of JAK/STAT signaling. We first examined STAT1 activation in CT26 cells stably expressing Flag-tagged ROR2. As shown in Figure 4A, immunoblotting revealed that ROR2 overexpression significantly enhanced the IFN γ -induced STAT1 phosphorylation. Importantly, this signaling amplification was completely abrogated by the JAK1/JAK2 inhibitor, Ruxolitinib (Rux; Figure 4A), indicating that ROR2-mediated enhancement in STAT1 activation depends on the kinase activity of JAK1/2. This result was further corroborated by luciferase reporter assays, where ROR2 overexpression potentiated the IFN γ -induced transcriptional activities of STAT1/STAT3/STAT5, and this activation was also dampened by JAK1/2 inhibition (Figure 4B). Notably, the addition of Wnt3A or Wnt5A, a Wnt ligand that is able to bind to ROR2 to activate canonical or non-canonical Wnt signaling, respectively, did not interfere with IFN γ -activated and ROR2-enhanced JAK/STAT transcriptional activity (Figure S1), suggesting that the ROR2-mediated regulation on IFN γ -JAK/STAT signaling is independent of Wnt ligands.

Upon IFN γ stimulation, the IFNGR1/2 complex, particularly the subunit IFNGR1, undergoes rapid ligand-induced internalization from the cell membrane, and this process is crucial for activating downstream signaling [61]. Given ROR2 being a membrane receptor, we wondered whether ROR2 overexpression would modulate the cell surface expression or stability of IFNGR complex, thereby enhancing the downstream activity. As expected, IFN γ treatment significantly reduced the cell surface levels of IFNGR1 and, to a lesser extent, those of IFNGR2 (Figure S2A; IFN γ vs V); however, ROR2 overexpression did not alter their cell surface expression as compared to the control (Figure S2A; ROR2 vs EV), suggesting that ROR2-enhanced JAK/STAT signaling activity is not due to the modulation of IFNGR1/2 cell surface expression.

We further investigated whether ROR2 was closely associated with the IFNGR1 or IFNGR2 and acted as a co-receptor in the IFNGR complex to potentiate the IFN γ -activated downstream signaling. To examine this possibility, we first performed the co-IP assays in which ROR2-Flag was co-expressed with IFNGR1 or IFNGR2 in HEK293T cells. As shown, we did not observe the interaction between ROR2-Flag and IFNGR2 (Figure S2B); however, the reciprocal co-IPs using anti-Flag or anti-IFNGR1 antibody revealed a clear association between ROR2 and IFNGR1 (Figure S2C), implicating that other than JAK1 and JAK2, ROR2 is also associated with IFNGR complex via interaction with IFNGR1, even without IFN γ stimulation.

Since IFNGR1 is the ligand-binding subunit of IFNGR complex and therefore required for initiation of IFN γ signaling [62], we investigated whether ROR2 could functionally compensate for IFNGR2, the signal transducing subunit of the complex. To do so, we knocked down IFNGR2 expression in cells stably expressing ROR2 by introducing two independent shRNAs against IFNGR2, and then determined IFN γ -induced JAK/STAT downstream activity by performing luciferase reporter assays. As shown, significant reduction by shRNAs in *Ifngr2* expression confirmed their knockdown efficiency (Figure S2D), and these IFNGR2 knockdowns were sufficient to completely abrogate IFN γ -induced STAT transcriptional activity upon ROR2 overexpression (Figure S2E). Taken together, these results indicate that ROR2-enhanced IFN γ signaling requires both IFNGR1 and IFNGR2 subunits, and that despite an association with IFNGR1, ROR2 could not functionally substitute for IFNGR2 in mediating IFN γ -induced signaling. Although IFNGR2 does not show an interaction with ROR2, its presence in the complex remains essential for transduction of JAK/STAT signaling.

In addition to the reporter assays, the impact of ROR2 overexpression on JAK/STAT transcriptional activity was also assessed by measuring the expression levels of the downstream target ISGs by RT-qPCR analysis. As shown in Figure 4C, ROR2 potentiated the IFN γ -induced transcription of several ISGs in CT26 cells, including *Cd274* (encoding PD-L1), *Ido1*, *Cxcl10* and *Isg15*. This ROR2-mediated upregulation of ISGs was strictly dependent on JAK1/JAK2 activity as Ruxolitinib treatment abolished the effect. IFN γ is a pleiotropic cytokine capable of driving both anti- and pro-tumorigenic programs.

Given that high ROR2 expression in COAD correlates with poor prognosis (Figure 1B), we focused on downstream targets involved in cancer immune modulation. We found that ROR2 overexpression enhanced the protein levels of the immunosuppressive checkpoint protein PD-L1 and IDO1, both involved in immune evasion (Figure 4D). Furthermore, flow cytometry analysis further demonstrated that the ROR2-upregulated PD-L1 protein was present at cell surface of CT26 cells (Figure 4E, left). Interestingly, while the cell surface expression of Major Histocompatibility Complex Class I (MHC-I) proteins was also upregulated by ROR2, this elevation was seemingly independent of JAK activation as JAK inhibition did not abolish ROR2-enhanced MHC-I expression (Figure 4E, right). Taken together, these data implicate that ROR2 is able to modulate the expression of immune checkpoint proteins and enhance antigen presentation machinery, thereby remodeling the tumor microenvironment.

To confirm that the amplified IFN γ -JAK/STAT signaling in CT26 cells overexpressing ROR2 was directly attributed to the increased level of ROR2, we introduced two independent shRNAs against *Ror2* by lentiviral transduction to knock down the overexpressed ROR2 in the CT26 cells. The knockdown efficiency of shRNA was confirmed by immunoblotting against ROR2-Flag (Figure 5A). As expected, the knockdown of ROR2 reduced the protein induction of PD-L1 and IDO1 (Figure 5A) and compromised the ROR2-potentiated ISG transcription (Figure 5B). These results confirm that ROR2 is a driver to enhance IFN γ -activated downstream signaling response.

Identification of putative JAK-binding motifs within ROR2

We have shown that ROR2 functionally potentiates the IFN γ -JAK/STAT signaling, and we next sought to identify the structural basis of ROR2-JAK1/JAK2 interaction. Classical cytokine receptors recruit JAKs via conserved membrane-proximal domains, known as Box1 and Box2 motifs [43, 44]. To identify analogous motifs within ROR2, we first better defined the amino acid consensus sequence of the Box1 and Box2 motifs, and for this we applied the AlphaFold-based workflow [63] by modeling the interactions between JAK1/JAK2 and their canonical receptors to derive high-confidence characteristic sequence signatures of these motifs (Methods and Figure 6A, left). This analysis allowed us to refine

the definitions of Box1 and Box2 motifs beyond those reported by the existing literatures, including the Protein Data Bank (PDB) structures: 6E2P/6E2Q [64], 5IXI [45] and 7T6F [44]. Using the structure-based sequence alignment, we classified the Box1 motifs into two distinct categories (Figure 6A, “Box1” grey box at right): (i) Type 1 motifs: resembling those observed in PDB 6E2P/6E2Q, with the consensus regular expression, (P|PS)xPx{2–5}[FLI], where x denotes any amino acid, and (ii) Type 2 motifs: similar to those in PDB 5IXI, following the consensus, [LM]PxxL. While these signatures capture the majority of known receptors, several validated JAK-binding partners display related but non-identical sequence patterns, suggesting additional sequence diversity. As for Box2, no simple regular motif sequences could be defined. Instead, we identified a rule-based pattern characterized by a specific combination of hydrophobic and acidic residues, within a 5-residue window (Figure 6A, “Box2” grey box at right): either (i) 2 hydrophobic residues (F, L, I, P, V, M, W) and 3 acidic residues (D or E), or (ii) ≥ 3 hydrophobic residues and ≥ 1 acidic residue. As with Box1, this rule likely represents an incomplete definition as Box2 motifs within several validated JAK-binding receptors deviate from this pattern, yet it captures the majority of known Box2 sequences (Figure 6A, right).

According to the defined motif signatures of Box1 and Box2 described above, we screened the cytoplasmic region of ROR2 protein to identify putative JAK1/2-binding motifs reassembling Box1 and Box2. Interestingly, three consensus sequences (amino acid 795-802, 813-819 and 826-834) for Box1 and two consensus sequences (amino acid 920-927 and 932-942) for Box2 motifs were found in the cytoplasmic tail of ROR2 (Figure 6B). Phylogenetic analysis using ProViz, a visualization tool to investigate evolutionary functional regions of a protein [65], revealed that these predicted binding motifs are highly conserved across ROR2 orthologs (Figure 6B).

Lastly, to structurally validate these putative evolutionarily conserved binding sites, we modeled the ROR2-JAK1/JAK2 interaction using AlphaFold3 (Figure 6C). The predictions of ROR2-JAK1/2 bindings (in dark blue) were compared with reference crystal structures of a Box1 from the erythropoietin receptor in complex with JAK2 FERM-SH2 (PDB: 6E2P/6E2Q; in red) and a Box2 from Interferon lambda receptor 1 in complex with JAK1 (PDB: 7T6F; in red), and this analysis revealed that

the predicted Box1 and Box2 motifs in ROR2 adopt conformations that are structurally compatible with canonical JAK1 (Box1, ipTM=0.6; Box2, ipTM=0.38) and JAK2 (Box1, ipTM=0.37; Box2, ipTM=0.46) (Figure 6C). To sum up, these computational structure analyses (i) refined the consensus Box1 and Box2 motifs from known JAK1/2 canonical receptors, (ii) revealed that the cytoplasmic tail of ROR2 contains those conserved Box1 and Box2 consensus motifs, and (iii) demonstrated architectural compatibility of binding interface between Box1/Box2 of ROR2 and FERM-SH2 of JAK. These predictions suggest that the conserved Box1 and Box2 motifs are potentially essential for ROR2 to recruit JAK1/2 and thereby to activate downstream JAK/STAT signaling.

The STP domain and C-terminal tail of ROR2 are crucial for IFN γ -JAK/STAT signaling enhancement

ROR2, structurally belonging to the RTK superfamily [66], is a single-pass transmembrane receptor, composed by a conserved extracellular region consisting of Immunoglobulin-like (IG), Frizzled-like cysteine-rich domain (CRD) and Kringle domain (KRD), and an intracellular region comprising a tyrosine (pseudo)kinase domain (TKD) followed by a proline-rich domain (PRD) flanked by two serine-threonine-rich domains (ST1 and ST2), together called as Serine-Threonine-Proline-rich (STP) domain [67, 68] (Figure 7A). Our computational analysis identified three putative Box1 motifs located within the STP domain and two putative Box2 motifs located within the C-terminal tail of ROR2 (Figure 7A). Since the STP domain is a well-established interaction hub for ROR2-binding partners, including Dishevelled 2 (Dvl2), CK1 ϵ , p120-catenin, FlnA, Src, TGF- β activated kinase 1 (TAK1), and Neurotrophin receptor-interacting MAGE homologue (NRAGE) [59, 60, 69-72], and its C-terminal region contains several predicted Box1 and Box2 motifs, we hypothesized that this C-terminal region is critical for the ROR2-mediated potentiation on IFN γ -induced JAK/STAT signaling activity. To test this hypothesis, we generated a mutant form of ROR2 protein lacking the STP domain and the C-terminal tail (ROR2- Δ C; Figure 7A-B) and assessed its impact on IFN γ -activated transcriptional activities of STAT1/3/5 by performing luciferase reporter assays. As shown in Figure 7C, the results of this assay revealed that

while full-length ROR2 (ROR2-FL) significantly increased IFN γ -induced STAT1/3/5 transcriptional activities, this elevation was completely abolished in cells expressing ROR2- Δ C. Consistent with this, the upregulation of ISG expression detected in cells expressing ROR2-FL was absent with those expressing ROR2- Δ C (Figure 7D). These results demonstrated that the STP domain and C-terminal tail of ROR2, harboring the predicted Box1 and Box2 motifs, are required for the functional regulation of IFN γ -JAK/STAT signaling. Surprisingly, despite this loss of function in signaling transduction, NanoBRET assays for ROR2- Δ C with JAK1 or JAK2 protein showed that ROR2- Δ C retained the ability to physically interact with both JAK1 and JAK2 (Figure S3). This result suggests that the TKD alone is sufficient for ROR2 to mediate binding to JAK kinases. However, this TKD-mediated interaction appears to be non-effective to transduce JAK/STAT signaling as the absence of the STP domain and C-terminal tail abolished the downstream signaling amplification. Thus, we proposed that while the TKD of ROR2 protein may provide a primary docking or scaffolding interface for JAK1/2 binding, the STP domain and C-terminal tail that contain conserved Box1 and Box2 motifs are essential to properly orient or activate the JAK/STAT complex for efficient signal transduction.

Correlation of ROR2 expression with IFN γ -JAK/STAT signaling in COAD

To establish the translational significance of the ROR2-potentiated IFN γ -JAK/STAT signaling axis uncovered in murine colorectal adenocarcinoma cells, we analyzed the correlation with human COAD samples from TCGA. Consistent with our mechanistic findings, we observed a robust positive correlation between ROR2 transcript levels and the IFN γ response signature in human COAD (Figure 8A; $R=0.408$, $p=5.79 \times 10^{-13}$). Given that JAK1 and JAK2 are critical regulators in signaling pathways that are activated by a broad spectrum of cytokines beyond IFN γ , including IL-6, type I interferons, and growth factors [73-78], we further investigated the correlation between ROR2 expression and the signaling activity of the global JAK/STAT pathway. As shown, high ROR2 expression displayed significant association with the expression of JAK/STAT signaling signature in human COAD (Figure 8B; $R=0.377$, $p=3.68 \times 10^{-11}$), suggesting that ROR2 might act as a generalized amplifier for JAK-mediated signaling in

the COAD. Given that high level of ROR2 expression is correlated with unfavorable clinical outcomes (Figure 1B), IFN γ response and activation of JAK/STAT signaling (Figure 8A-B), we wonder whether the transcripts encoding key drivers of immune evasion are also associated with ROR2 expression. As shown, ROR2 expression levels are positively correlated with the levels of transcripts encoded for the immune checkpoint protein PD-L1 (*CD274*) (Figure 8C) and the metabolic enzyme IDO1 (Figure 8D). Interestingly, the expression levels of transcripts encoding MHC-I components (HLA-A, HLA-B, HLA-C, and B2M), responsible for the antigen presentation machinery, were also positively associated with ROR2 expression (Figure S4); however, this correlation was not as significant as those with immunosuppressive effectors. Notably, the correlation with HLA-C ($R=0.275$), which plays a dominant role in providing inhibitory signals to Natural Killer cells via their Killer-Cell Immunoglobulin-like Receptors [79], was stronger than HLA-A ($R=0.179$) and HLA-B ($R=0.186$), which are the primary targets for Cytotoxic T lymphocyte recognition, supporting a model of ROR2 promoting immune evasion. This differential correlation aligns with our observation that high ROR2 expression is associated with poor prognosis (Figure 1), suggesting that the ROR2-mediated amplification of IFN γ -JAK/STAT signaling predominantly favors an immunosuppressive program (high PD-L1/IDO1) over increased immunogenicity (moderate MHC-I), thereby fostering immune evasion.

Discussion

ROR2 is a developmentally critical transmembrane tyrosine kinase receptor that is largely silenced in adult tissues but frequently overexpressed in aggressive cancers, including COAD. While higher ROR2 expression is linked to poor prognosis and metastatic phenotypes through regulation of proliferation, migration, and invasion [8, 32, 35-37, 80], the molecular mechanisms underlying its pro-tumorigenic function remain incompletely understood. In this study, we uncovered a previously unrecognized function of ROR2 as a scaffolding receptor for JAK1 and JAK2, thereby regulating the IFN γ -induced JAK/STAT signaling to promote an immunosuppressive tumor microenvironment.

Given that ROR2 is classified as a pseudokinase lacking robust intrinsic catalytic activity, we investigated the ROR2 signaling network by mapping its interactome in COAD cells through co-IP coupled to LC-MS/MS. In addition to previously reported ROR2-binding partners, such as Filamin A and TAK1, we identified JAK1 as a new ROR2-interacting protein. By performing reciprocal co-IP and live-cell NanoBRET assays, we verified that ROR2 interacts not only with JAK1 but also with JAK2. We then demonstrated that the ROR2-JAK1/2 interaction is functionally critical, as ROR2 overexpression amplified the IFN γ -induced STAT1/3/5 transcriptional activities and upregulated the expression of their downstream target ISGs. Analysis of COAD samples from TCGA revealed a strong positive correlation between ROR2 expression and IFN γ -JAK/STAT signature, including immunosuppressive PD-L1 and IDO1, confirming that the IFN γ signaling amplification by ROR2 overexpression observed *in vitro* is clinically relevant in COAD patients.

To gain structural insight into the ROR2-JAK1/2 interaction, we refined the consensus definitions of JAK1/JAK2-binding motifs using AlphaFold3 and identified putative Box1 and Box2 motifs within the STP domain and C-terminal tail of ROR2. Despite the well-established receptor preferences for JAK1 or JAK2, our predictions revealed no detectable differences in the sequence signatures of motifs involved in the JAK1/2 interactions. Instead, both JAK proteins were predicted to share two recurrent Box1 motif types. Our inability to distinguish JAK1- from JAK2-specific Box1 and Box2 signatures may stem from variations within the corresponding binding regions of JAK1 and JAK2 themselves, or from more

subtle sequence determinants that cannot be resolved without experimentally mapping binding affinities to individual motifs. While the intracellular ROR2 tyrosine (pseudo)kinase domain (TKD) alone was sufficient for the interaction with JAK1/JAK2, this binding was insufficient to transduce JAK/STAT signaling, as deletion of the C-terminal STP domain and C-terminal tail completely abolished the ROR2-mediated enhancement of IFN γ -JAK/STAT signaling. This suggests that the STP domain and C-terminal tail of ROR2 is essential for the trans-activation or productive orientation of the JAK/STAT complex. Our findings are consistent with previous reports demonstrating the critical role of the STP domain in mediating ROR2 interactions with CK1 ϵ [70, 81], Filamin A [60, 82, 83], GSK3 β [84, 85], PKC [6, 83, 86], EphrinB2 [87], DVL2 [87], Dlxin-1 [88], and likely Wtip [89].

Wnt5a-ROR2 signaling has been previously reported to modulate JAK/STAT expression and activation, as well as chemokine/cytokine expression. It has been shown that ROR2 overexpression increases STAT3 phosphorylation through activation of Src and Fyn [90]. Moreover, *Ror2* knockdown in mouse bone-marrow mesenchymal stem cells reduces STAT3 expression [91]. In mouse tendon stem/progenitor cells (TSPCs), *Ror2* knockdown diminishes Wnt5a-induced phosphorylation of JAK2 and STAT3 required for promoting TSPC senescence, demonstrating the regulatory role of Wnt5a-ROR2 signaling in JAK/STAT activation. Interestingly, IFN γ treatment is able to restore JAK/STAT activation that was dampened by Wnt5a knockdown, further highlighting the cross-regulation between Wnt5a-ROR2 and IFN γ -JAK/STAT signaling [92]. Furthermore, a colitis mouse model in which conditional deletion of *Wnt5a* or *Ror2* attenuated dextran sodium sulfate (DSS)-induced colitis demonstrates that the Wnt5a-ROR2 axis is essential for the production of inflammatory cytokines, in turn promoting IFN γ -induced JAK1/STAT1 signaling in the bone-marrow-derived dendritic cells [93]. Beyond this, Wnt5a-ROR2-driven chemokine or cytokine expression has also been reported (i) in bone marrow-derived mesenchymal stem cells, in which Wnt5a-ROR2 signaling enhances the expression and secretion of Cxcl16 [94], (ii) in human umbilical vein endothelial cells, where *Ror2* knockdown inhibited the TNF- α -induced release of inflammatory cytokines, IL-1 β , IL-6 and MCP-1 [95], (iii) in mouse lung adenocarcinoma cells, where ROR2 downregulation reduces lipopolysaccharide (LPS)-

induced secretion of pro-inflammatory cytokine, TNF α , IL-1 β and IL-8, through inhibiting JNK and ERK signaling pathways [96], and (iv) in human bronchial epithelial cells where ROR2 knockdown reduces NF- κ B activation and cytokine production [97]. Our discovery unifies these studies and provides a mechanistic explanation for these reports by demonstrating a physical coupling between ROR2 and the JAK/STAT machinery, which further potentiates cytokine-activated immune signaling in a Wnt ligand-independent manner.

The biological outcome of the ROR2-dependent enhancement of JAK/STAT signaling is likely to be context-dependent. While in the colitis model, ROR2 in dendritic cells promotes a pro-inflammatory Th1 response [93], in our COAD model, ROR2 may drive immune evasion by regulating the expression of immunosuppressive targets, PD-L1 and IDO1. Correlation analysis of a COAD cohort from TCGA, revealing a positive association between ROR2 expression and IFN γ targets, further strengthens the immunosuppressive role of ROR2 in COAD. Although ROR2 expression in COAD was also positively correlated with expression of MHC-I components, the upregulation of which usually increases immunogenicity, this association was relatively weaker, and the elevated MHC-I expression may be a direct consequence of a cytokine-driven transcriptional program, particularly involving IFN γ . Thus, based on our analyses and *in vitro* data, we propose a model in which high ROR2 expression potentiates the IFN γ -activated JAK/STAT signaling, which in turn drives an immunosuppressive state within the tumor microenvironment and thereby contributes to poor prognosis in COAD.

In conclusion, our findings identify ROR2 as a previously unrecognized modulator of the IFN γ -JAK/STAT axis, through physical interaction with JAK1 and JAK2, thereby potentiating IFN γ -driven signaling. As JAK1 and JAK2 are signaling mediators shared among multiple cytokine receptors, ROR2's close association with these kinases may have broader functional significances beyond IFN γ . It will be important to determine whether ROR2 can also modulate signaling downstream of other cytokine receptors engaging JAK1 and/or JAK2, thereby integrating non-canonical ROR2 inputs into a wider cytokine regulatory network. Moreover, given that IFN γ is a double-edged sword in cancer with both anti-tumor and pro-tumorigenic/immune-evasive effects, ROR2-dependent amplification of this

pathway is likely to have context-dependent consequences that vary across tumor types and microenvironmental states. In some settings, enhanced IFN γ signaling via ROR2 may strengthen antigen presentation and cytotoxic immunity; in others, it could favor chronic inflammation, immune checkpoint upregulation, or T-cell dysfunction. Thus, ROR2 expression in tumors may not only serve as a biomarker for signaling activation, but also represent a context-specific therapeutic target at the interface of tumor-intrinsic plasticity and tumor-extrinsic immune regulation. Future studies across diverse tumor contexts will be required to define how this ROR2-JAK interface shapes the balance between anti-tumor immunity and immune evasion.

Materials and methods

Dataset collection and analysis of survival curve, TNM stage, and TCGA correlation

The microarray datasets GSE39582 and GSE17536 from the NCBI Gene Expression Omnibus (GEO) were used for TNM stage and survival curve, respectively. Additionally, gene expression data from RNA-Seq STAR counts for colorectal adenocarcinoma (COAD), along with clinicopathologic characteristics, were obtained from The Cancer Genome Atlas (TCGA) via the GDC portal (<https://portal.gdc.cancer.gov/>). The count data was then converted to log₂ (TPM+1) data. Kaplan–Meier survival analyses were performed using the survival and survminer R packages, with continuous variables dichotomized at the median. P values were calculated using the log-rank test. Correlation plots and bar charts were generated using the ggplot2 R package. Spearman correlation analysis was used to assess associations between different variables, and the Wilcoxon test was applied to compare ROR2 expression across different stages.

Cell culture

Cell lines CT26 and B16, originated, respectively, from mouse BALB/C colon adenocarcinoma and C57BL/6 melanoma, were purchased from the American Type Culture Collection (ATCC), cultured and maintained in IMDM medium (21980032, Gibco) supplemented with 10% FBS (10270-106, Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin (15140-122, Gibco), and a mix of amino acids containing 0.24 mM L-asparagine, 0.55 mM L-arginine and 1.5 mM L-glutamine at 37°C under 5% CO₂. For mitogen treatment, cells were treated with vehicle (PBS) or carrier-free 100 ng/ml Wnt3A (1324-WN-CF, R&D Systems) for 6 hours (h) to activate canonical Wnt signaling, or with 50 ng/ml recombinant mouse IFN γ (PMC4033, Thermo Fisher Scientific) to activate IFN γ signaling for 6 h prior to harvesting for reporter assay and RT-qPCR analysis, or 24 h for protein purification. To inhibit JAK activity, vehicle (DMSO) or 1 µM Ruxolitinib (S1378, Selleck Chemicals) were added to their culture medium containing IFN γ .

Lentiviral transduction assay for ROR2 expression

HEK293T cells (Thermo Fisher Scientific) were transfected with a TurboFect Transfection reagent (R0531, Thermo Fisher Scientific) with a plasmid mix containing pLKO.1 lentiviral expressing plasmid, the viral packaging plasmids MD2 and PAX2. 12 h post-transfection, HEK293T culture medium was replaced with a virus-producing medium (VPM) composed of IMDM culture medium. 36 h post-transfection, virus containing VPM from HEK293T cells was collected, filtered and mixed with Polybrene (Sigma-Aldrich) and additional 10% FBS and then applied to CT26 cells. Viral infection was performed by centrifugation of the CT26 or B16 cells with the virus-containing mix at 1100 g for 1 h at 35°C. Three days after infection, cells were selected with addition of 10 µg/ml blasticidin or 1 µg/ml of puromycin. Lentiviral plasmids used for lentiviral transduction were the empty vector, pLKO.1-MCS-Blasticidin (derived from pLKO.1-NLS-iCre-mRFP [98], where NLS-iCre was replaced by multiple cloning sites [99]) and pLKO.1-MCS-Blasticidin containing the insertion of C-terminally Flag-tagged full-length ROR2 (ROR2-Flag or ROR2-FL) or C-terminal truncated ROR2 (ROR2-ΔC) in MCS. ROR2-ΔC insertion was amplified from pEF1a-mRor2delta745 (22640, Addgene). pLKO.1-scramble, *Ror2* or *Infgr2* shRNA-puromycin plasmids were from mouse TRC1.0 shRNA library (Sigma-Aldrich).

Immunofluorescence microscopy

CT26 cells were cultured on Nunc Lab-Tek II Chamber slides (Thermo Fisher Scientific) coated with 10 µg/ml fibronectin (Merck Millipore), then fixed in 4% PFA for 10 minutes (min) at 37°C and permeabilized in 0.5% Triton-X100 for 20 min. Fixed cells were incubated in immunofluorescence blocking buffer (0.3% Triton-X100, 1% bovine serum albumin, 1% gelatin, 5% normal goat serum, 5% normal donkey serum in PBS) at room temperature (RT) for 1 h, and then with primary antibody Anti-DYKDDDDK tag (FLAG) at 1:100 dilution (2368, Cell Signaling Technology) at 4°C overnight. Cells were washed and incubated with Alexa-Fluor conjugated secondary antibodies (Jackson ImmunoResearch) at RT for 1 h before mounted with medium containing DAPI (Roth). Images of immunofluorescence

staining were acquired with a Cell Observer Spinning Disk (COSD) confocal microscope and analyzed using Zen3.4 software (Zeiss).

Luciferase reporter assay

B16 cells carrying an empty vector or expressing ROR2-Flag were plated and co-transfected with a TOPFlash (for TCF/LEF transcriptional activity) or pGRR5 (for STAT transcriptional activity) luciferase reporter together with a Renilla plasmid using TurboFect Transfection reagent. 48 h post-transfection, cells were treated with 100 ng/ml Wnt3A or 50ng/ml IFN γ for 6 h prior to analysis. Luciferase activity was detected using Dual-Luciferase reporter assay system (E1910, Promega) and by a GLOMAX luminometer (Promega) based on manufacturer's instructions.

Flow cytometry analysis

CT26 cells were cultured and treated prior to being detached with 2 mM EDTA in PBS for 5 min. Collected cells were suspended in PBS containing 2% FBS and 1 mM EDTA, and incubated with antibodies before analysis. Stained cells were then analyzed using a FACS LSR Fortessa (BD Biosciences). Viability was measured by staining with Fixable Viability Dye eFluor 780 (65-0865, eBioscience). Antibodies used for flow cytometry are PE-conjugated anti-PD-L1 (1:200, 124308, Biolegend), FITC-conjugated H2Kd (1:200, 116606, Biolegend), APC-conjugated anti-IFNGR1 (1:200, ab275700, Abcam) and APC-conjugated anti-IFNGR2 (1:200, 130-105-672, Miltenyi Biotec).

Co-immunoprecipitation coupled to liquid chromatography and mass spectrometry

CT26 cells were lysed in a non-denaturing lysis buffer containing 50 mM Tris-HCl, 100 mM NaCl, 0.1 mM EDTA, 0.5 mM MgCl₂, 1% NP-40, 10% glycerol, protease inhibitors (78430, Thermo Fisher Scientific) and phosphatase inhibitors (78440, Thermo Fisher Scientific). Lysates were cleared by centrifugation at 17000 g for 20 min and subjected to co-immunoprecipitation by incubation with covalently conjugated magnetic beads coupled to anti-FLAG (M8823, Merck Millipore) or anti-HA

(SAE0197, Merck Millipore) antibodies at 4°C overnight with gentle mixing. Beads were washed in lysis buffer and the immunoprecipitated proteins were eluted with 1x LDS sample buffer (NP0007, Thermo Fisher Scientific).

Co-immunoprecipitated proteins were separated in a 10% acrylamide gel at 120 V for 15 min, and protein bands were visualized and in-gel digested with trypsin. Peptides were extracted, dried, dissolved in solvent A (2% acetonitrile in 0.1% TFA), directly loaded onto reversed-phase pre-column (Acclaim PepMap 100, Thermo Fisher Scientific) and eluted in backflush mode. Peptide separation was performed using a reversed-phase analytical column (Acclaim PepMap RSLC, 0.075 x 250 mm, Thermo Fisher Scientific) with a linear gradient of 4%-27.5% solvent B (98% acetonitrile in 0.1% formic acid) for 40 min, 27.5%-50% solvent B for 20 min, 50%-95% solvent B for 10 min and holding at 95% for the last 10 min at a constant flow rate of 300 nl/min on an Vanquish Neo system. The peptides were analyzed by an Orbitrap Fusion Lumos tribrid mass spectrometer (Thermo Fisher Scientific). The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Fusion Lumos coupled online to the nano-LC. Intact peptides were detected in the Orbitrap at a resolution of 120,000. Peptides were selected for MS/MS using HCD setting at 30, and ion fragments were detected in the linear ion trap (LUMOS). A data-dependent procedure that alternated between one MS scan followed by MS/MS scans was applied for 3 seconds for ions above a threshold ion count of 2×10^4 in the MS survey scan with 40.0s dynamic exclusion. The electrospray voltage applied was 2.1 kV. MS1 spectrum was obtained with an AGC target of 4×10^5 ions and a maximum injection time of 50ms, and MS2 spectra were acquired with an AGC target of 5×10^4 ions and a maximum injection time set to dynamic. For MS scans, the m/z scan range was 375 to 1800. The resulting MS/MS data was processed using Sequest HT search engine within Proteome Discoverer 2.5 SP1 against a *Mus Musculus* protein database obtained from Uniprot. Trypsin was specified as cleavage enzyme allowing up to 2 missed cleavages, 4 modifications per peptide and up to 5 charges. Mass error was set to 10 ppm for precursor ions and 0.1 Da for-fragment ions (LUMOS) or 10 ppm (Exploris240). Oxidation on Met (+15.995 Da), conversion of Gln (-17.027 Da) or Glu (- 18.011 Da) to pyro-Glu at the peptide N-term were considered as variable

modifications. False discovery rate (FDR) was assessed using Percolator, and thresholds for protein, peptide and modification site were specified at 1%. For abundance comparison, abundance ratios were calculated by Label Free Quantification (LFQ) of the precursor intensities within Proteome Discoverer 2.5 SP1.

ROR2-interacting proteins were selected based on the following criteria: (i) abundance ratio ≥ 2.3 (anti-Flag co-IP vs anti-HA coIP with protein extracts of CT26 expressing ROR2-Flag, (ii) PSM ≥ 2 , and (iii) common interactants identified in two independent experiments. The identified 86 putative ROR2-interacting proteins are listed in Table S1. Gene ontology analysis was performed using the DAVID annotation tool.

Co-immunoprecipitation and immunoblotting

HEK293T cells were co-transfected with empty plasmid (control) or plasmids carrying Flag-tagged ROR2 with plasmid containing JAK1 (pEF-BOS-Jak1; kind gift from S. Constantinescu), HA-tagged JAK2 (pMX-IRES-GFP-Jak2; kind gift from L. Dumoutier), IFNGR1 and IFNGR2 (pEF-BOS-Ifngr1 or -Ifngr2; kind gift from S. Constantinescu) using TurboFect Transfection reagent. 48 h after transfection, protein lysates were harvested, cleared and subjected to co-immunoprecipitation by incubation of Dynabeads Protein G (10003D, Thermo Fisher Scientific) with Anti-FLAG (2368, Cell Signaling Technology), anti-HA (3724, Cell Signaling Technology), anti-JAK1 (3344, Cell Signaling Technology) or anti-IFNGR1 (PA5-27841, Thermo Fisher Scientific) antibodies at 4°C overnight with gentle mixing. The pulled down proteins were eluted in 1x LDS sample buffer containing 0.1 mM DTT at 85°C for 6 min, and then subjected and analyzed by immunoblotting analysis.

To extract total proteins, cells were incubated with M-per buffer (78503, Thermo Fisher Scientific) containing protease and phosphatase inhibitors on ice for 45 min, and then cleared and denatured in LDS+DTT sample buffer at 85°C for 6 mins. For immunoblotting, protein lysates were then loaded on a 4-12% Bolt Bis-Tris gel (NW04125BOX, Thermo Fisher Scientific) and transferred onto a nitrocellulose membrane with the iBlot 3 Western Blot Transfer System (Thermo Fisher Scientific). Transferred

membranes were blocked with 5% BSA in TBST buffer at RT for 1 h, and then incubated with primary antibodies at 4°C overnight. After incubation, blots were washed and then incubated with the HRP-conjugated secondary antibodies (Jackson ImmunoResearch) at RT for 1 h. The blots were developed using chemiluminescence detection reagent (SuperSignal West substrates, Thermo Fisher Scientific), and signals were then detected using the Fusion SL imaging system (Vilber).

Primary antibodies used for immunoblotting: FLAG (DYKDDDDK) tag (2368), p-JNK^{T183/Y185} (4668), JNK (9258), p-PKC^{S660} (9371), PKCα (2056), p-STAT1^{Y701} (7649), STAT1 (9172), PD-L1 (60475), IDO1 (51851), JAK1 (3332), HA-tag (3724) and α-Tubulin (2144) from Cell Signaling Technology, and IFNGR1 (PA5-27841) and IFNGR2 (MA5-26271) from Thermo Fisher Scientific.

NanoBRET assays

NanoBRET assay was performed based on manufacturer's instructions (Promega). In brief, full-length ROR2 and ROR2-ΔC were cloned into a pNL1 NanoLuc vector (N1361, Promega) with NanoLuc (NL) tagged at its intracellular C-terminus; JAK1 and JAK2 were cloned into a pHTN HaloTag® CMV-neo vector (G7721, Promega) with HaloTag (HT) fused at their N-terminus. HEK293T cells were transiently co-transfected with NL-ROR2 and HT-JAK1 or -JAK2 with the TransIT-LT1 Transfection Reagent (Mirus, Madison, WI, USA) in a 96-well plate. Fluorescent NanoBRET HaloTag 618 Ligand was added (N1662, Promega) 4 h after transfection. 24 h post-transfection, the NanoBRET™ NanoLuc Substrate was added and bioluminescence was analyzed using a GLOMAX Discover multiplate reader (Promega) at 37 °C using the 450BP (donor) and 600LP (acceptor) built-in filters.

Reverse Transcription and quantitative real-time PCR (RT-qPCR)

Total RNAs from cultured CT26 cells were extracted with TRIzol reagent (15596026, Thermo Fisher Scientific), purified with the Direct-zol RNA MiniPrep kit (R2052, Zymo Research) and reverse transcribed with ProtoScript(r) II First Strand cDNA Synthesis Kit (E6560S, New England Biolabs). Quantitative PCR was performed with indicated primers in the SYBR Green (Bio-Rad) on the

Thermoblock 96 Silver Block Real-Time PCR system (Bio-Rad), and cycle numbers were recorded by CFX Manager v3.1 software. The relative expression levels were normalized to PCR amplification of the housekeeping gene *Rps16*. Primers used for qPCR analysis are listed in Table S2.

Analyses for motif identification and structural modeling

To predict Box1 and Box2 motif, interaction partners of human JAK1 and JAK2 were retrieved from BioGrid and IntAct. For each interactor, only cytoplasmic regions shorter than 1200 amino acids were retained for modeling. Protein-protein interactions between JAK1 and JAK2 and their respective partners were predicted using ColabFold [100] with default batch settings (predictions are available on Zenodo: 10.5281/zenodo.17791933). High-confidence motif-like segments were identified using MiniPAE, applying the adaptive confidence threshold described previously [63]. The predicted Box1 and Box2 motif signatures derived from the predictions were used to screen ROR2 sequence for putative analogous motifs. ProViz [65] was used to highlight the conservation of such motifs across evolution, further supporting the potential binding role of the predicted motifs.

Predictions for the interaction between mouse ROR2 and JAK1 or JAK2 were generated using the AlphaFold3 server with default parameters. For comparison to known structures, models showing the closest structural resemblance to the relevant reference PDB entry were selected and displayed. All structural models were inspected and rendered in PyMOL (Version 3.1, Schrödinger LLC). All JAK1 and JAK2 interaction predictions generated in this study are available on Zenodo at 10.5281/zenodo.17791933.

Data Availability

All data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Author Contributions

J.d.L. designed and performed the majority of the experiments, analyzed data, prepared figures and wrote the manuscript. M.V. and T.M. developed and performed computational and AlphaFold analyses; Z.X. conducted TCGA analyses and generated the graphs.; J.Z. co-supervised the study; W.-H.L. supervised the study, analyzed proteomic data and edited the manuscript. All authors provided intellectual input and approved the final manuscript.

Declaration of interests

The authors declare no competing interests.

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Figure legends

Figure 1: ROR2 expression increases with advancing TNM stage and poor prognosis of colorectal adenocarcinoma. (A) Scatter plot showing tumor ROR2 mRNA expression across pathological TNM stages I-IV with each dot representing an individual patient and horizontal bars indicating group medians. ROR2 levels are significantly higher in stage II-IV tumors as compared with stage I, indicating an increase in ROR2 expression with advanced disease. (B) ROR2 expression is correlated with poor prognosis of colorectal adenocarcinoma. Kaplan-Meier curves depict disease-free survival (DFS) probability in patients stratified by tumor ROR2 mRNA expression (high vs. low, median split; $n=72$ per group). Patients with ROR2-high tumors (red) experienced significantly shorter DFS (days) compared with those with ROR2-low tumors (grey). Shaded areas indicate 95% confidence intervals, and the table below shows the number of patients at risk at each time point.

Figure 2: Identification of ROR2-binding proteins in murine colon adenocarcinoma cells. (A) Immunofluorescence staining of murine colorectal cell line CT26 transduced with C-terminally Flag-tagged ROR2 (ROR2-Flag) or an empty vector (EV) stained for Flag (green) and DAPI (blue). As shown, ROR2 is localized at the cell membrane of CT26 cells. DAPI was used as a nuclear counterstain. (B) Immunoblotting for phosphorylated and total JNK and PKC α in CT26 carrying an EV or overexpressing ROR2. ROR2 overexpression shows an increase in phosphorylation of JNK and PKC α , indicating overexpressed ROR2 is functionally active for non-canonical Wnt signaling activation. Quantification of phosphorylated to total proteins is shown below. (C) Luciferase reporter assay for TCF/LEF transcriptional activity (TOPFlash) with B16 cells carrying an EV or overexpressing ROR2 upon treatment of vehicle or 100ng/ml Wnt3a for 6 h. ROR2 overexpression significantly enhanced Wnt3a-induced TCF/LEF transcriptional activity. Data are reported with bar representing the mean and the paired data sets are linked with lines; $n=4$ independent experiments. p -values were calculated using paired two-sided t -test on log2 transformed values; ***, $p<0.001$. (D) Workflow for identifying ROR2-binding partners. Flag-tagged ROR2 overexpressed in CT26 cells was pulled down by magnetic beads

covalently coupled to anti-Flag or anti-HA (negative control) antibody. Following wash and elution, immunoprecipitated protein complexes by ROR2-Flag were enzymatically digested into peptides, separated by LC, and identified by MS/MS. FDR was assessed using Percolator, and thresholds were specified at 1%; abundance ratios were calculated by LFQ of the precursor intensities within Proteome Discoverer 2.5 SP1 (see methods and materials). **(E)** Volcano plot displaying the 86 identified ROR2-binding proteins from one of duplicate datasets (Exp-2) with Log2 Abundance Ratio (ROR2-Flag pull-down using anti-Flag vs anti-HA non-specific beads; x-axis) plotted against peptide spectrum match (PSM; y-axis), a score reflecting the similarity between the detected mass spectrum of a peptide and its theoretical spectrum. Higher PSM values indicate greater confidence in peptide identification. **(F)** Gene ontology analysis using the DAVID annotation tool shows the biological processes enriched among the 86 ROR2-binding partners. n indicates the numbers of the identified proteins. **(G)** Co-immunoprecipitation for ROR2-Flag in CT26 carrying empty vector (ROR2-) and ROR2-Flag (ROR2+) using magnetic beads coupled to anti-Flag antibody (Anti-Flag beads+) or non-specific HA antibody (anti-Flag beads-). Co-immunoprecipitated proteins were then detected by immunoblotting. As shown, JAK1 was pulled down by ROR2-Flag, confirming the interaction between ROR2 and JAK1. $n=2$.

Figure 3: ROR2 interacts with JAK1 and JAK2. **(A)** Co-immunoprecipitation for ROR2 (top) or JAK1 (bottom) in HEK293T cells co-transfected with Flag-tagged and JAK1 using anti-Flag (top) or anti-JAK1 (bottom) antibody. Reciprocal co-IPs confirmed the interaction between ROR2 and JAK1. Red arrows indicate specific bands for JAK1; a red asterisk denotes non-specific bands from the co-IPs. α -Tubulin serves as loading control for input samples. $n=3$. **(B)** Co-immunoprecipitation for ROR2 (top) or JAK2 (bottom) in HEK293T cells co-transfected with Flag-tagged ROR2 and HA-tagged JAK2 using anti-Flag (top) anti-HA (bottom) antibody. In addition to JAK1, JAK2 was also found to interact with ROR2. $n=3$. **(C)** Schematic of NanoBRET assay for detecting ROR2-JAK interaction. The assay measures bioluminescence resonance energy transfer (BRET) from a NanoLuc luciferase donor fused with ROR2 (NL-ROR2) to a fluorescent HaloTag acceptor fused with JAK1 or JAK2 (HT-JAK1/2). When donor and

acceptor proteins are in close proximity, energy transfer from bioluminescent donor protein to the acceptor protein occurs, and the emitted fluorescence in the presence of the HaloTag ligand (HL) is detected, indicating their interaction. **(D-E)** NanoBRET assay for ROR2 and JAK1 (D) or JAK2 (E) in HEK293T cells co-transfected with NL-ROR2 and HT-JAK1 (D) or HT-JAK2 (E). Cells co-transfected with NL-empty as the negative control, and those with NL-IFNGR1 (D) or NL-IFNGR2 (E) as the positive control. As compared to their negative controls, increased BRET signal was detected from cells with NL-ROR2 for HT-JAK1 (D) and HT-JAK2 (E), indicating the close-proximity interaction of ROR2 with JAK1 and JAK2. Data in (D) and (E) are reported as mean $\pm$ s.d.; $n=4$ (D) and 3 (E) independent experiments. p -values were calculated using unpaired two-sided t -test on log2 transformed values; * $p<0.05$; ** $p<0.01$.

Figure 4: ROR2 enhances IFN γ -induced JAK/STAT signaling and ISG expression. **(A)** Immunoblotting for phosphorylated and total STAT1 in CT26 cells carrying an empty vector (-) or overexpressing ROR2-Flag (+) upon treatment of vehicle or 50ng/ml IFN γ without or with 1 μ M Ruxolitinib (Rux) for 24 h. IFN γ -induced STAT1 phosphorylation was increased by ROR2 overexpression, but fully abrogated by JAK inhibition. **(B)** Luciferase reporter assay for STAT1/3/5 transcriptional activity in B16 cells carrying an empty vector (EV) or overexpressing ROR2 upon treatment of vehicle or 50ng/ml IFN γ without or with 1 μ M Ruxolitinib for 6 h. IFN γ -induced STAT transcriptional activity was enhanced by ROR2 overexpression, but this activation was completely abolished when JAK activity was inhibited. **(C)** RT-qPCR analysis for mRNAs of ISGs in control (EV) and ROR2-overexpressed (ROR2) CT26 cells treated with vehicle, IFN γ and Rux for 6 h. ROR2 overexpression increased the expression of *Cd274*, *Cxcl10*, *Isg15* and *Ido1*, and this upregulation was fully dampened by Ruxolitinib treatment. **(D)** Immunoblotting (bottom) and quantification (top) for PD-L1 (left) and IDO1 (right) protein expression in control (EV) and ROR2-overexpressed (ROR2) CT26 cells treated with vehicle, IFN γ and Rux for 24 h. IFN γ -induced PD-L1 and IDO1 protein expression was elevated by ROR2 overexpression, but fully abolished by JAK inhibition. **(E)** Flow cytometry analysis showing mean fluorescence intensity (MFI)

fold change for cell surface PD-L1 (left) and MHC-I (right) expression in control (EV) and ROR2-overexpressed (ROR2) CT26 cells treated with vehicle, IFN γ and Rux for 24 h. Cell surface presentation of PD-L1 and MHC-I proteins were increased by ROR2 overexpression, and this elevation was abrogated in PD-L1 but not MHC-I protein by Ruxolitinib treatment, suggesting the upregulated MHC-I expression by ROR2 is independent of JAK activation. Data in (B)-(E) are reported with the bar representing the mean of 3 independent experiments, and the paired data sets are linked with lines. p -values were calculated using paired two-sided t -test on log2-transformed values. * $p<0.05$; ** $p<0.01$. When $p>0.05$, the value is indicated.

Figure 5: ROR2 knockdown impairs IFN γ -induced JAK/STAT signaling and ISG expression. (A) Immunoblotting (left) and quantification (right) of PD-L1 and IDO1 protein expression in control (EV) and ROR2-overexpressing (ROR2) CT26 cells stably transduced with scramble or two independent shRNAs against *Ror2* (shRNA#1 and #2) upon the treatment of 50ng/ml IFN γ for 24 h. A box plot showing quantification results from 3 independent experiments. As shown, ROR2 knockdown reduced ROR2-enhanced expression of PD-L1 and IDO1 in the presence of IFN γ . (B) RT-qPCR analysis of *Ror2* and interferon-stimulated genes *Cd274*, *Ido1*, *Cxcl10*, *Isg15* and *Oas1* mRNAs in control (EV) and ROR2-overexpressing (ROR2) CT26 cells stably transduced with scramble or *Ror2* shRNAs stimulated with 50ng/ml IFN γ for 24 h. ROR2-enhanced expression of ISGs upon IFN γ stimulation was compromised by ROR2 knockdown. Data are presented as box plots with the horizontal line representing the mean of 3 independent experiments. p -values were calculated using paired two-sided t -test on log2-transformed values. * $p<0.05$; ** $p<0.01$. When $p>0.05$, the value is indicated.

Figure 6: Computational predictions of JAK-binding motifs within ROR2 protein. (A) Computational workflow for identifying conserved JAK1/2-binding motifs, Box1 and Box2, using AlphaFold software. Known human JAK1- and JAK2-interacting receptors from BioGRID and IntAct databases (137 partners for JAK1 and 242 partners for JAK2) were analyzed to identify potential binding motifs bound to the

FERM and SH2 domains of JAK1 and JAK2. AlphaFold structural predictions and MiniPAE scoring identified high-confidence JAK-binding motifs in 32 JAK1- and 27 JAK2-binding partners. Structure-based sequence alignment of 20 JAK1- and 17 JAK2-binding partners revealed two conserved motif types for Box1, Type I ($P \mid [PS]xPx\{2,5\}[FLI]$) and Type II ($[LM]PxxL$), and a rule-based pattern containing a combination of hydrophobic and acidic residues within a 5-residue for Box2, “2 [FLIPVMW] + 3 [DE]” or “3 [FLIPVMW] + 1 [DE]”. Underlined residues in the alignment indicate structurally conserved positions across models. The dark shades colors indicate the consensus residues structurally aligned in the JAK1/JAK2 interactants. **(B)** ROR2 contains three predicted motifs corresponding to Box1 and two predicted motifs for Box2, conserved across species. Sequence alignment of ROR2 orthologs generated using Proviz software shows conservation of Box1 and Box2 motifs within ROR2 proteins across vertebrate species. Red boxes highlight the predicted consensus sequences for Box1 (top) and Box2 (bottom); those marked with blue asterisks indicate the sequences used for AlphaFold3 structure prediction shown in (C). **(C)** AlphaFold3-predicted structures of JAK-binding motifs (in blue), referred to Box1 (left) or Box2 (right), of mouse ROR2 (mROR2) binding to the FERM-SH2 domain (grey surface) of mouse JAK1 (mJAK1) and JAK2 (mJAK2). Predictions are structurally aligned with reference PDB structures (in red, PDB 6E2P/6E2Q for Box1; PDB 7T6F for Box2). Consensus conserved residues are shown in darker colors, non-consensus residues are shown in lighter colors. Model quality metrics: ipTM (interface predicted template modeling score) indicates interface confidence, and pTM indicates overall model confidence (both range 0-1, higher values indicate better predictions).

Figure 7: The STP domain and C-terminal tail of ROR2 are required for the activation of IFN γ -activated JAK/STAT signaling. **(A)** Schematic representation of mouse ROR2 with its domain structure. Full-length ROR2 (ROR2-FL) contains an extracellular region composed of an immunoglobulin-like domain (IG, 55-145), a cysteine-rich domain (CRD, 169-303) and a Kringle domain (KRD, 316-394), and an intracellular region including a tyrosine (pseudo)kinase domain (TKD, 473-746), a proline-rich domain (PRD, 783-859) flanked by two serine-threonine-rich domains (ST1, 746-783; ST2, 859-883). The

predicted JAK1/2-binding motifs, Box1 and Box2 (as shown in Figure 6), are located within the STP (ST1-PRD-ST2) domain and C-terminal tail, respectively. A truncated ROR2 construct (ROR2-ΔC) was generated by deleting the C-terminal 200 amino acids that comprise the entire STP domain and C-terminal tail. **(B)** Immunoblotting for ROR2 in B16 cells transfected with an empty vector (EV), full-length ROR2 (ROR2-FL) or C-terminal truncated ROR2 (ROR2-ΔC). **(C)** Luciferase reporter assay for STAT1/3/5 transcriptional activity in B16 transfected with EV, ROR2-FL or ROR2-ΔC upon treatment of vehicle or 50ng/ml IFN γ for 6 h. While ROR2-FL significantly enhanced the IFN γ -induced STAT transcriptional activity, ROR2-ΔC failed to enhance it, demonstrating that the STP domain and C-terminal tail of ROR2 are required for its function in potentiating IFN γ -JAK/STAT signaling. **(D)** RT-qPCR analysis of ISGs in B16 cells transfected with EV, ROR2-FL or ROR2-ΔC upon treatment of vehicle or 50ng/ml IFN γ for 24 h. As shown, IFN γ -induced ISG expression was significantly upregulated by ROR2-FL; however, this upregulation was not detected in cell expressing ROR2-ΔC, indicating that the STP domain and C-terminal tail are critical for ROR2-mediated regulation of IFN γ responses. Data in (C) and (D) are reported with the bar representing the mean of 3 independent experiments, and the paired data sets are linked with lines. *p*-values were calculated using paired two-sided *t*-test on log₂-transformed values. * *p*<0.05; ** *p*<0.01; *** *p*<0.001. When *p*>0.05, the value is indicated.

Figure 8: ROR2 expression level positively correlates with IFN γ response, the activation of JAK/STAT signaling and the expression of immunosuppressive genes in human colorectal adenocarcinoma. (A-D) Scatter plots showing the correlation of ROR2 mRNA expression levels with the expression levels of IFN γ response signature genes (A), genes involved in the KEGG JAK-STAT signaling pathway (B), *CD274* (C) and *IDO1* mRNA (D) in human COAD samples available in the TCGA dataset. Higher ROR2 expression is significantly associated with the increased expression of IFN γ response (Pearson *R*=0.408, *p*=5.79x10⁻¹³) and JAK-STAT signaling (Pearson *R*=0.377, *p*=3.68x10⁻¹¹) signature genes, as well as with the expression levels of *CD274* (Pearson *R*=0.353, *p*=7.52x10⁻¹⁰) and *IDO1* (Pearson *R*=0.294, *p*=3.18x10⁻⁷).

In (A)-(D) panels, each dot represents an individual tumor sample, and orange lines depict linear regression fits with 95% confidence intervals (grey shading).

Supplementary figure legends

Figure S1: ROR2-mediated enhancement on IFN γ -JAK/STAT signaling is independent of Wnt ligands.

Luciferase reporter assay for STAT1/3/5 transcriptional activity in B16 cells carrying an empty vector (EV) or overexpressing ROR2 (ROR2) upon treatment of vehicle or 50ng/ml IFN γ without or with 100ng/ml Wnt3a or 300ng/ml Wnt5a for 6 h. As shown, ROR2 significantly enhanced the IFN γ -induced STAT transcriptional activity, but the addition of Wnt ligands did not interfere with this potentiation. Data are presented as mean of 2 independent experiments; *p*-values were calculated using paired two-sided *t*-test on log2 transformed values.

Figure S2: ROR2 does not alter IFNGR complex cell surface expression but IFNGR2 is required for IFN γ -JAK/STAT signaling. (A) Flow cytometry analysis for cell surface expression of IFNGR1 (left) and IFNGR2 (right) in vehicle- or IFN γ -treated CT26 cells transduced with EV or ROR2 in the absence or presence of 1 μ M Ruxolitinib (Rux) for 6 h. The cell surface expression of IFNGR1 was reduced upon IFN γ stimulation due to the internalization of IFNGR1-IFN γ complex. Note that the expression levels of IFNGR1 and IFNGR2 were not altered by ROR2 overexpression. MFI, mean fluorescence intensity. (B) Co-immunoprecipitation for ROR2 in HEK293T cells co-transfected with ROR2-Flag and IFNGR2 using anti-Flag antibody. IFNGR2 was not detected in pull-down of ROR2, suggesting no interaction between ROR2 and IFNGR2. (C) Reciprocal co-IPs for ROR2 (left) or IFNGR1 (right) in HEK293T cells co-transfected with ROR2-Flag and IFNGR1 using anti-Flag (left) or anti-IFNGR1 (right) antibody. Co-IP experiments show the interaction between ROR2 and IFNGR1. α -Tubulin in (B) and (C) serves as loading control for input samples. (D) RT-qPCR analysis for *Ifngr2* mRNA expression in B16 cells stably transduced with scramble or two independent shRNAs (shRNA#1 and shRNA#2) against *Ifngr2* showing the efficient knockdown of IFNGR2 expression. (E) Luciferase reporter assay for STAT1/3/5

transcriptional activity in ROR2-overexpressing B16 cells carrying scramble or *Ifngr2* shRNAs treated with vehicle or IFN γ for 6 h. Knockdown of IFNGR2 markedly impaired IFN γ -induced STAT1/3/5 transcriptional activity even upon ROR2 overexpression, demonstrating that the presence of IFNGR2 is essential for ROR2-enhanced IFN γ signaling response. Data in (A), (D) and (E) are reported as mean $\pm$ s.d.; $n=4$ (A), 3 (D), and 2 (E) independent experiments. p -values were calculated using paired two-sided t -test on log2 transformed values; *** $p<0.001$; ns, not significant.

Figure S3: C-terminal truncated ROR2 retains the ability of binding to JAK1 and JAK2. (A-B) NanoBRET assay for full-length (ROR2-FL) or truncated ROR2 (ROR2- Δ C) with JAK1 (A) or JAK2 (B) in HEK293T cells co-transfected with NL-ROR2-FL, NL-ROR2- Δ C or NL-IFNGR1 (A) or NL-IFNGR2 (B) together with HT-JAK1 (A) or HT-JAK2 (B). Cells with NL-empty as the negative control, and those with NL-IFNGR1 (A) or NL-IFNGR2 (B) as the positive control. BRET signals were increased in NL-ROR2-FL for both HT-JAK1 (A) and HT-JAK2 (B) as compared to their negative controls, and these signals remained detectable in cells expressing ROR2- Δ C, indicating the STP domain and C-terminal tail of ROR2 are dispensable for the ROR2-JAK1/2 interaction. Data are reported as mean $\pm$ s.d.; $n=4$ independent experiments. p -values were calculated using paired two-sided t -test on log2 transformed values; *, $p<0.05$.

Figure S4: ROR2 expression correlates with antigen-presentation genes in human COAD. (A-D) Scatter plots showing correlations of ROR2 mRNA expression levels with the expression levels of MHC-I genes, HLA-A (A), HLA-B (B), and HLA-C (C) and B2M (encoding β 2-microglobulin) in human COAD samples available in the TCGA dataset. ROR2 expression shows a modest positive correlation with the expression of MHC-I genes and B2M (Pearson $R=0.179$, 0.186 , 0.275 , and 0.282 , respectively). In all panels, each purple dot represents an individual tumor sample, and orange lines depict linear regression fits with 95% confidence intervals (gray shading).

Supplementary table legends

Table S1: List of identified ROR2-binding proteins by LC-MS/MS.

List of 86 ROR2-binding proteins identified by LC-MS/MS from two independent co-IP experiments (Exp-1 and Exp-2). Protein candidates are selected according to the following criteria: (i) Abundance ratios (AR; anti-Flag vs anti-HA pull down) ≥ 2.3 , (ii) Peptide spectrum match (PSM) ≥ 2 , and (iii) identification in both independent experiments.

Table S2: List of primers used for mRNA expression.

Sequences of primer sets (forward and reverse) used for RT-qPCR analysis are listed.

Figure 1

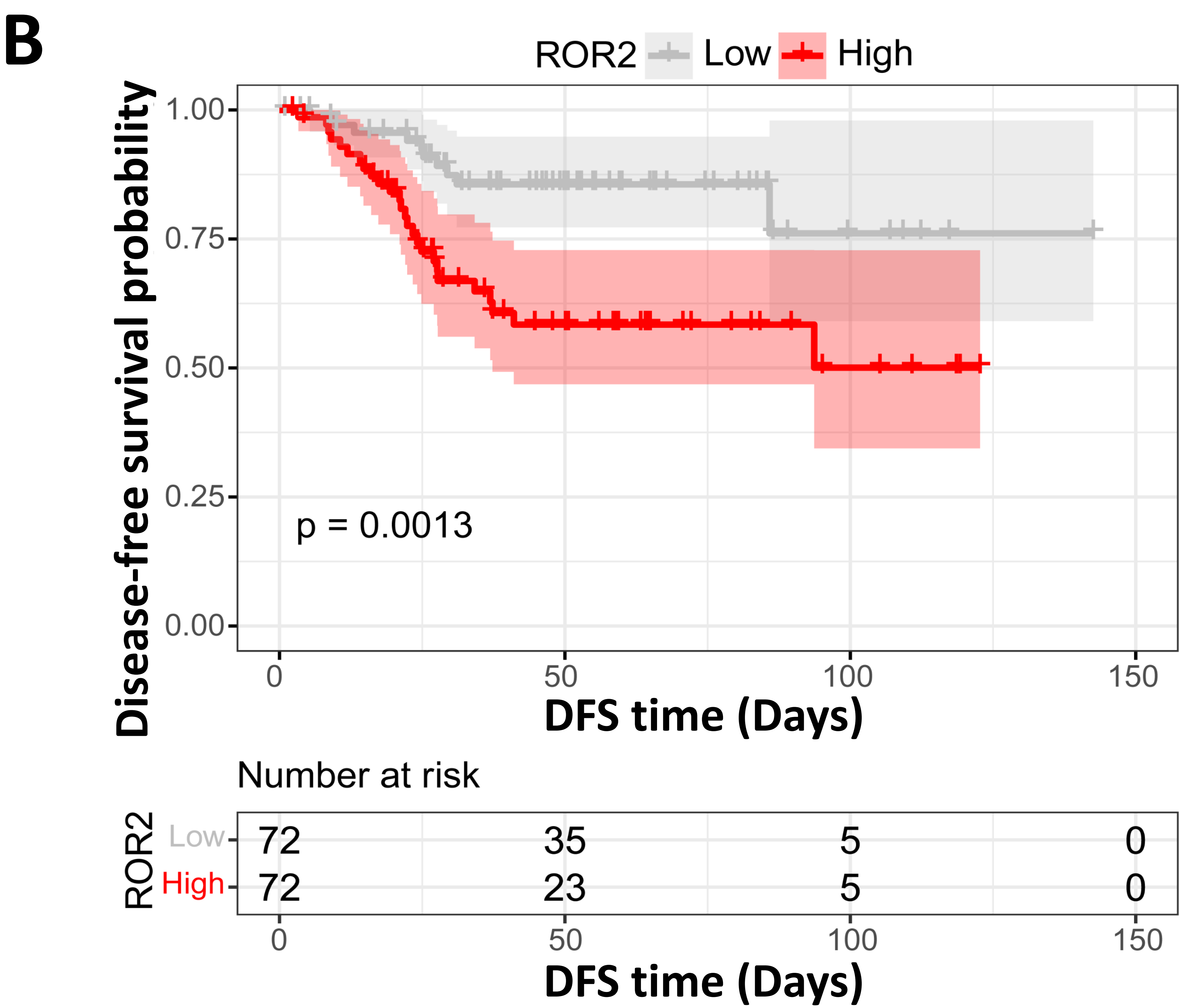
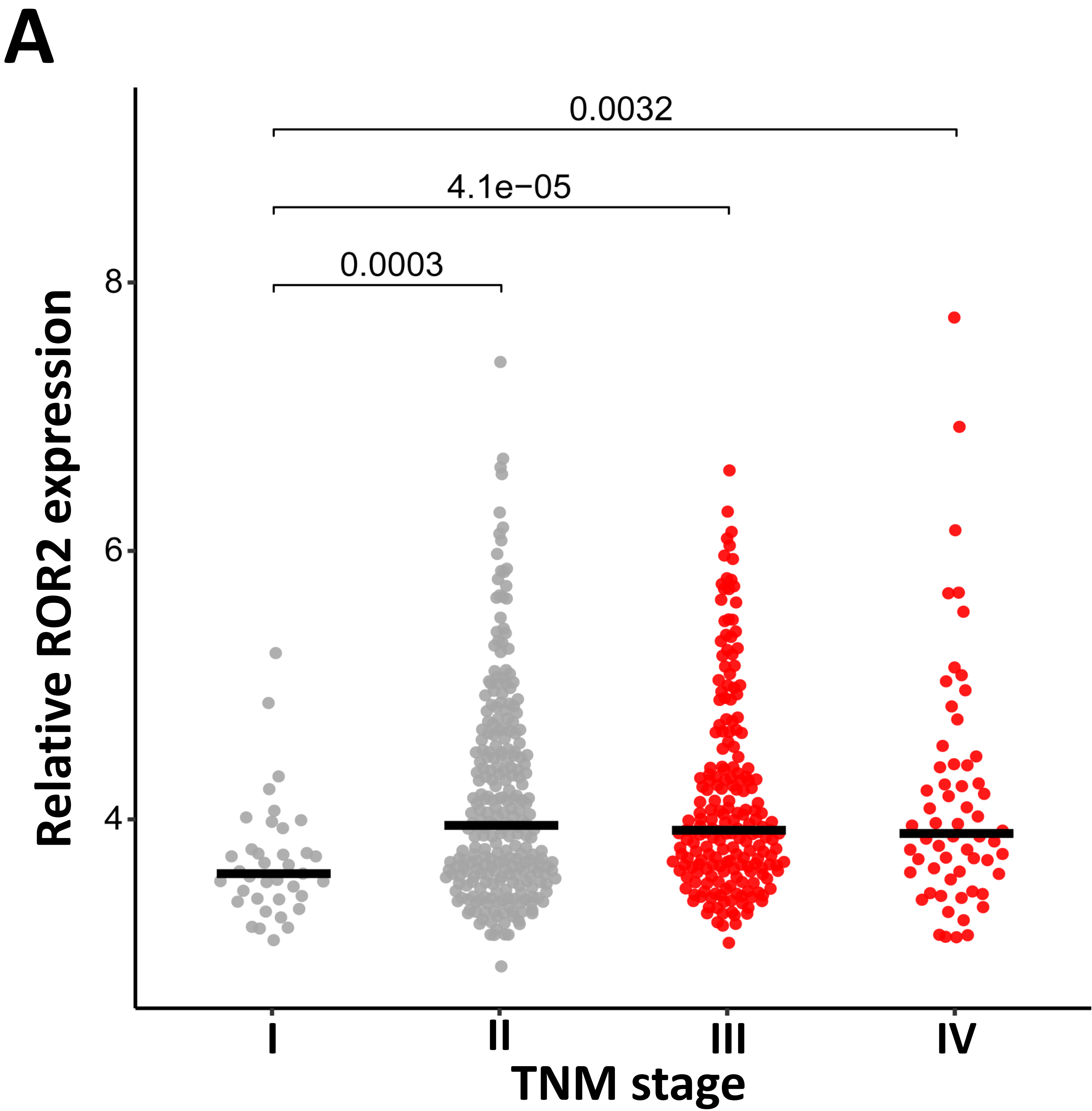


Figure 2

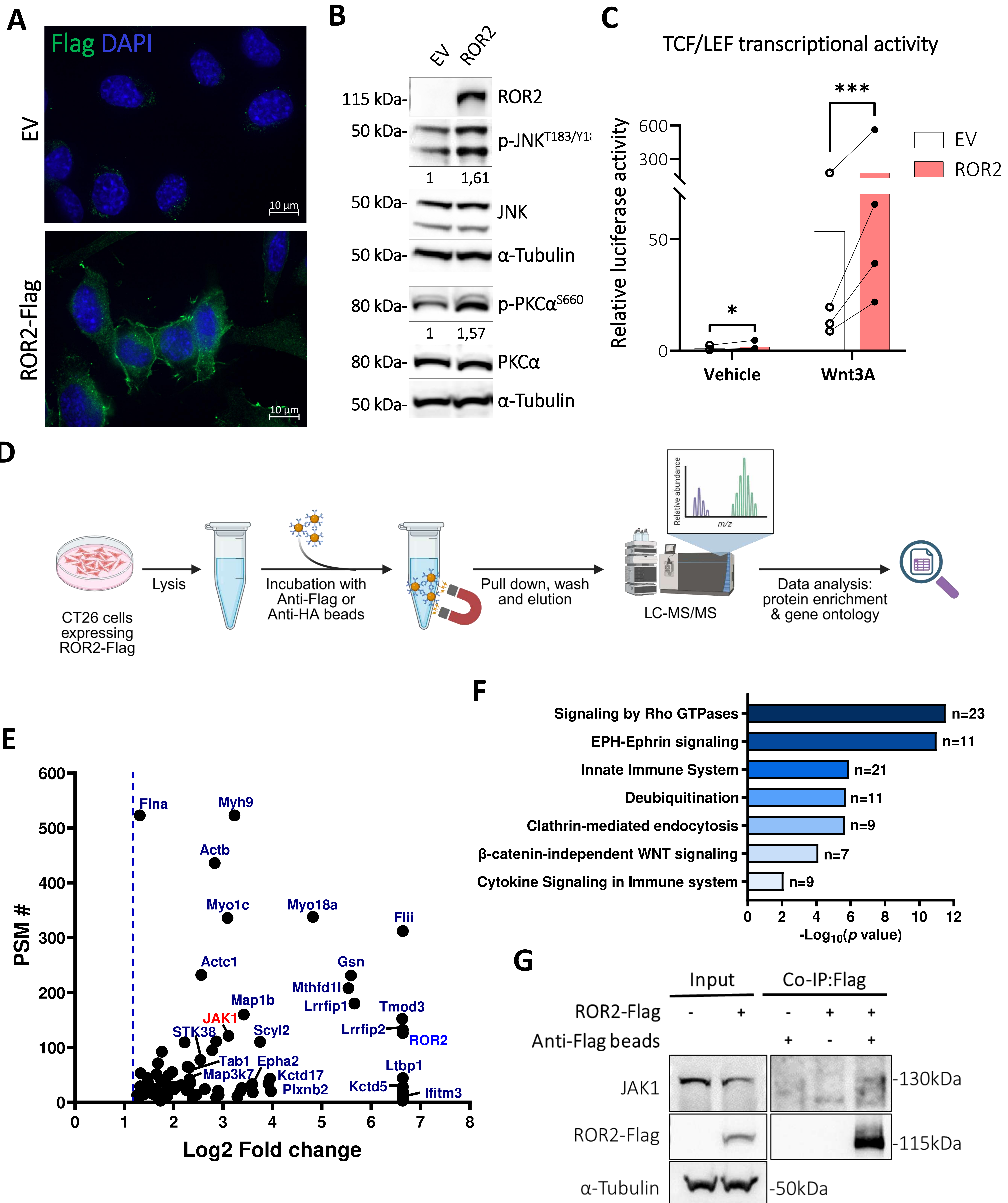


Figure 3

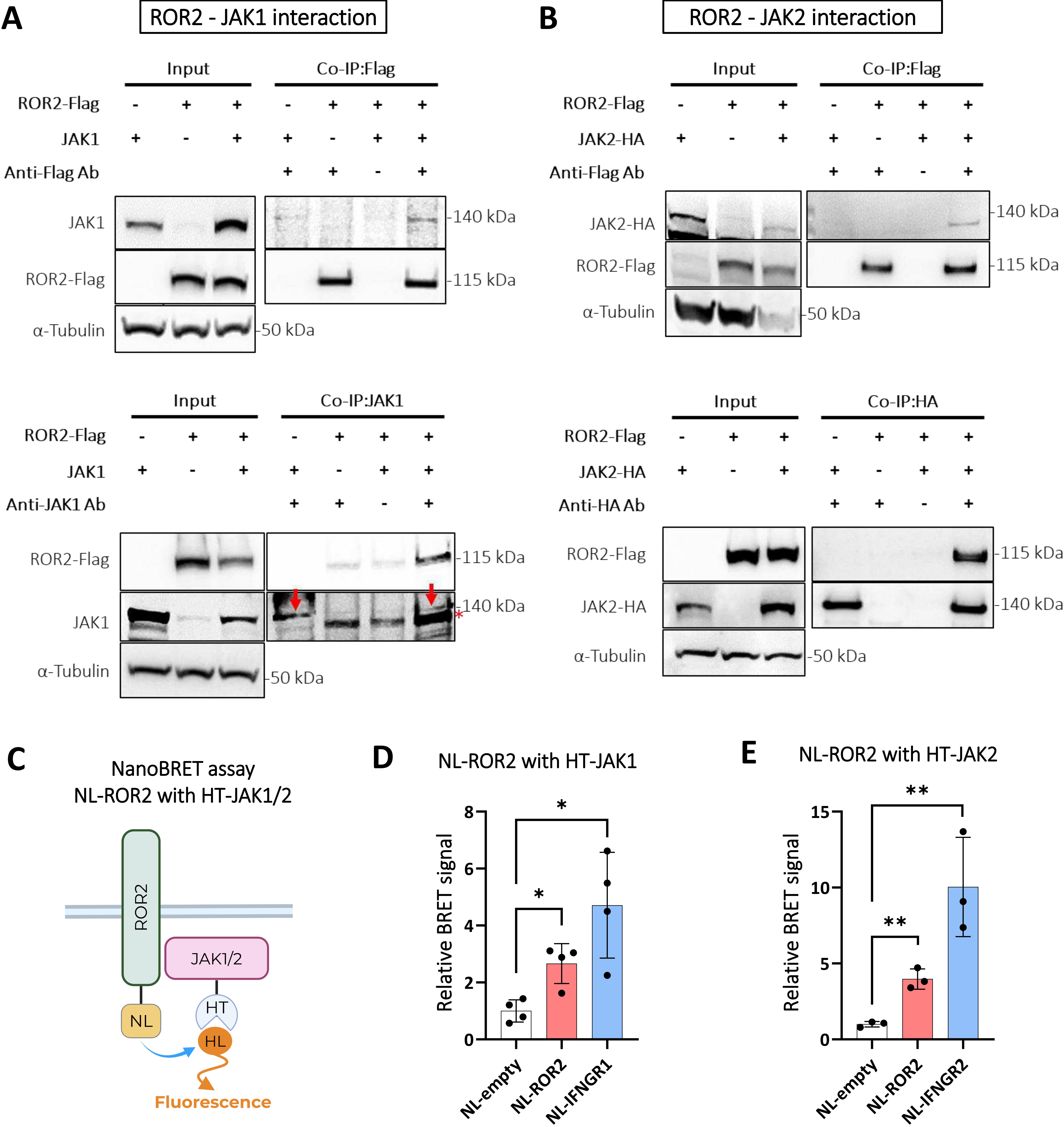


Figure 4

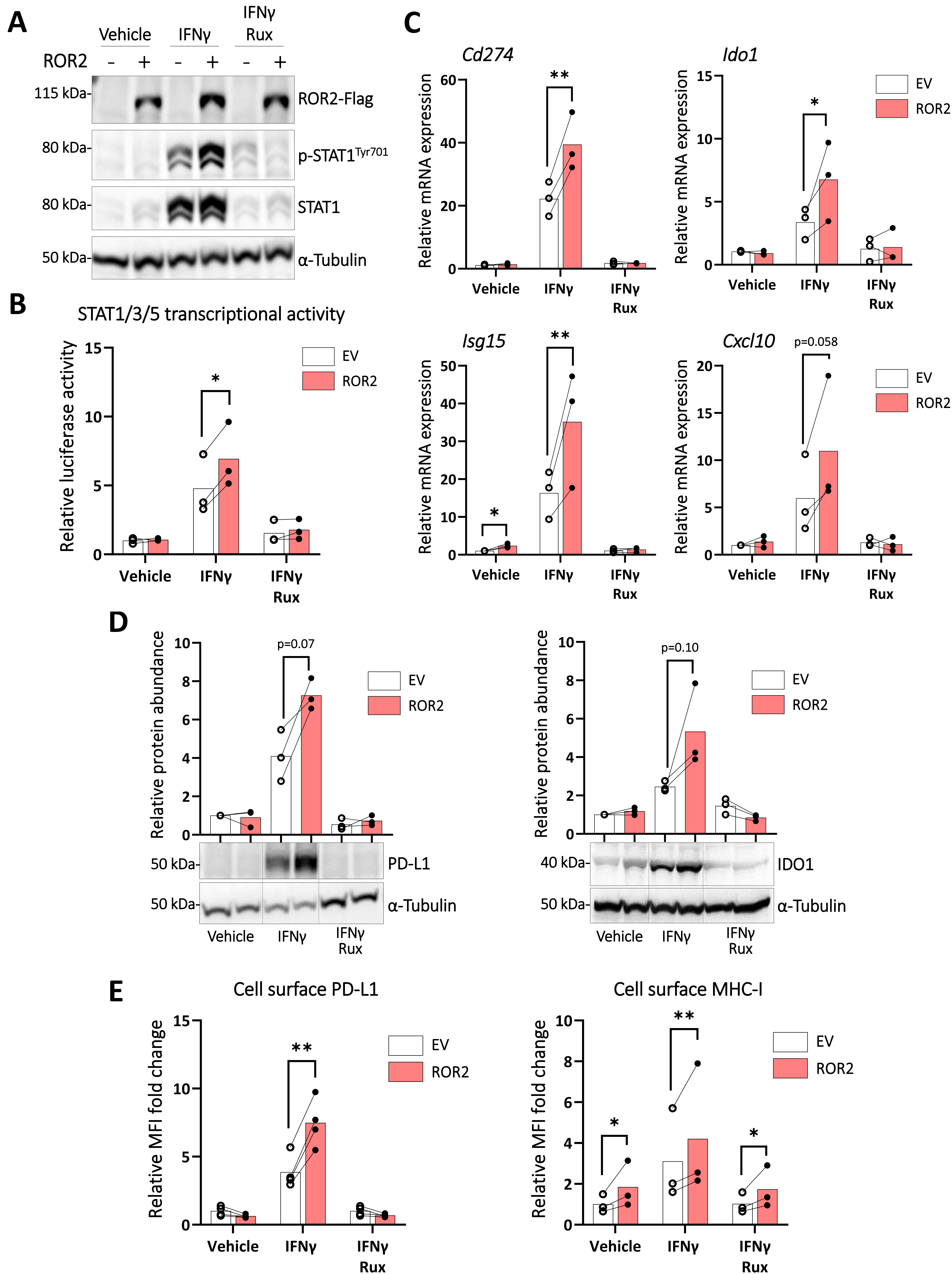


Figure 5

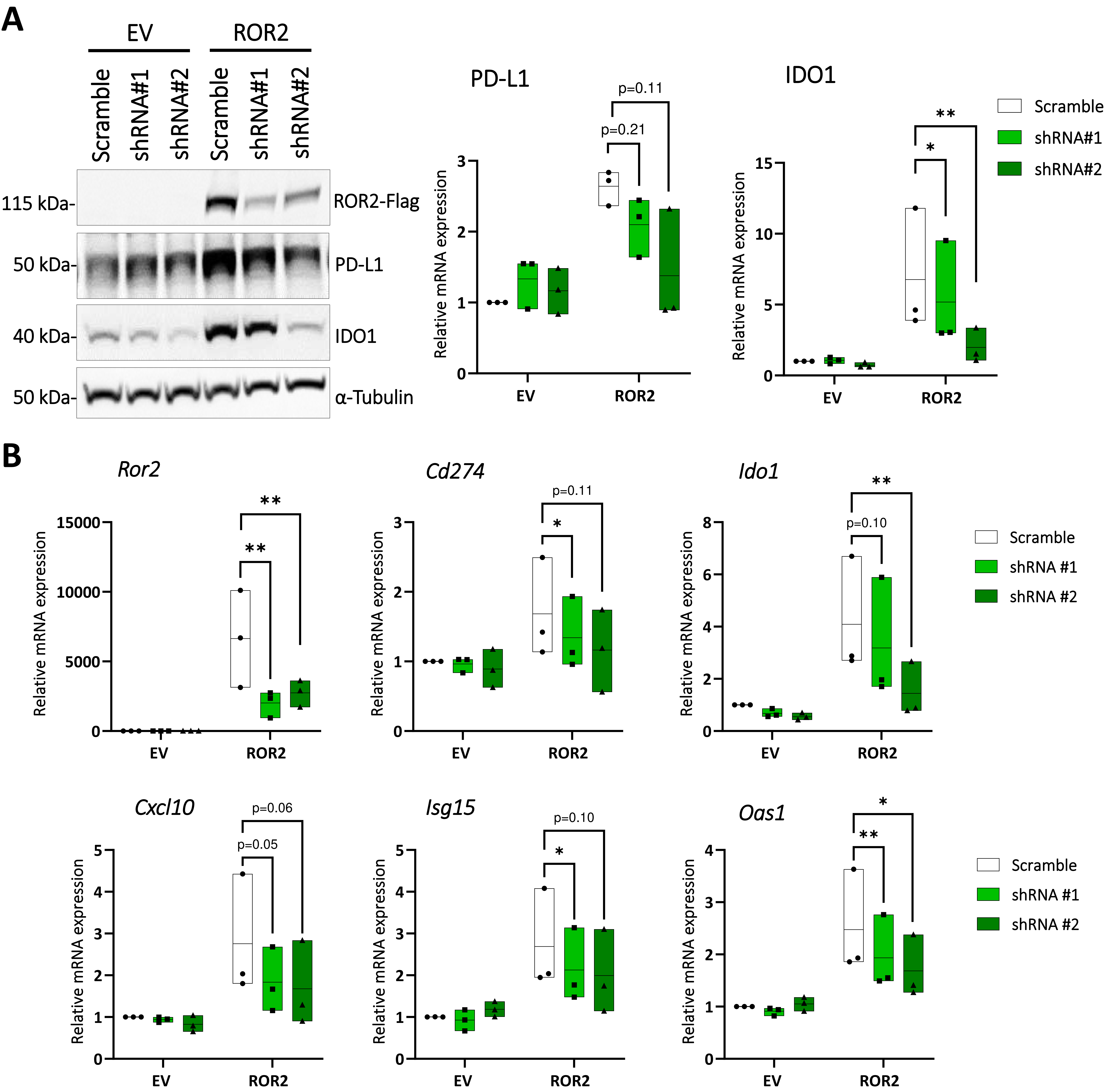
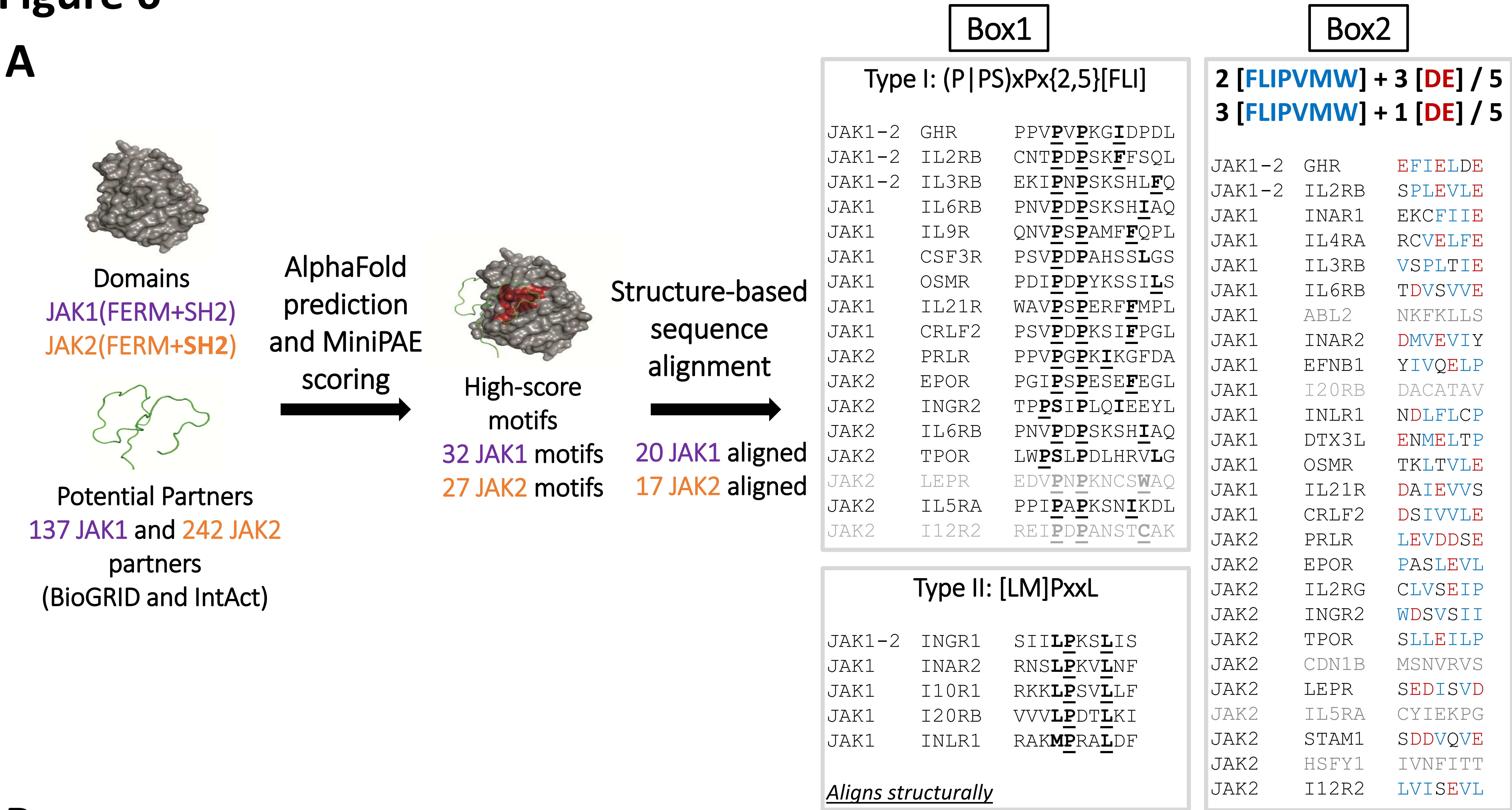
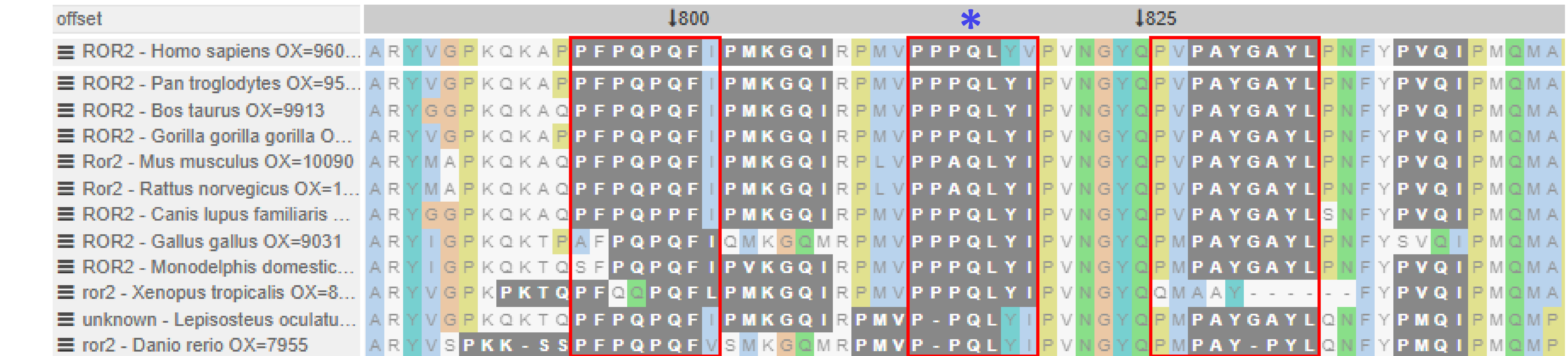


Figure 6

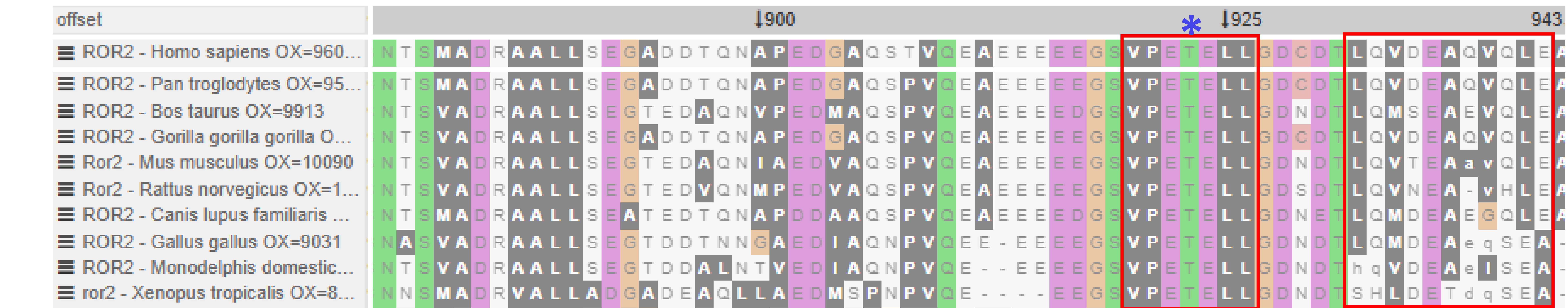
A



B Putative ROR2 Box1



Putative ROR2 Box2



C

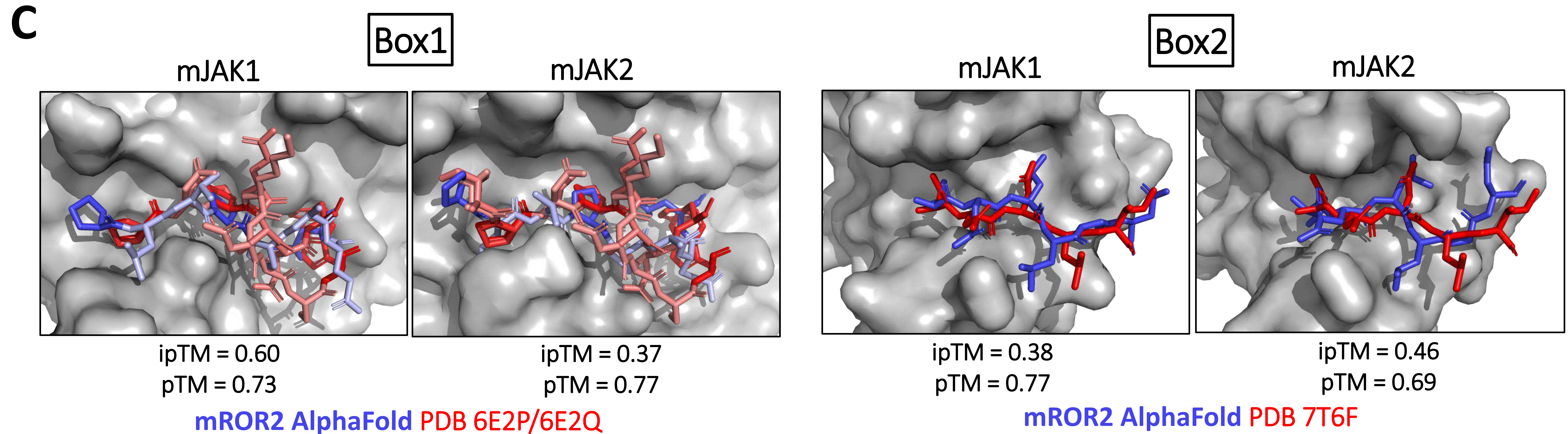


Figure 7

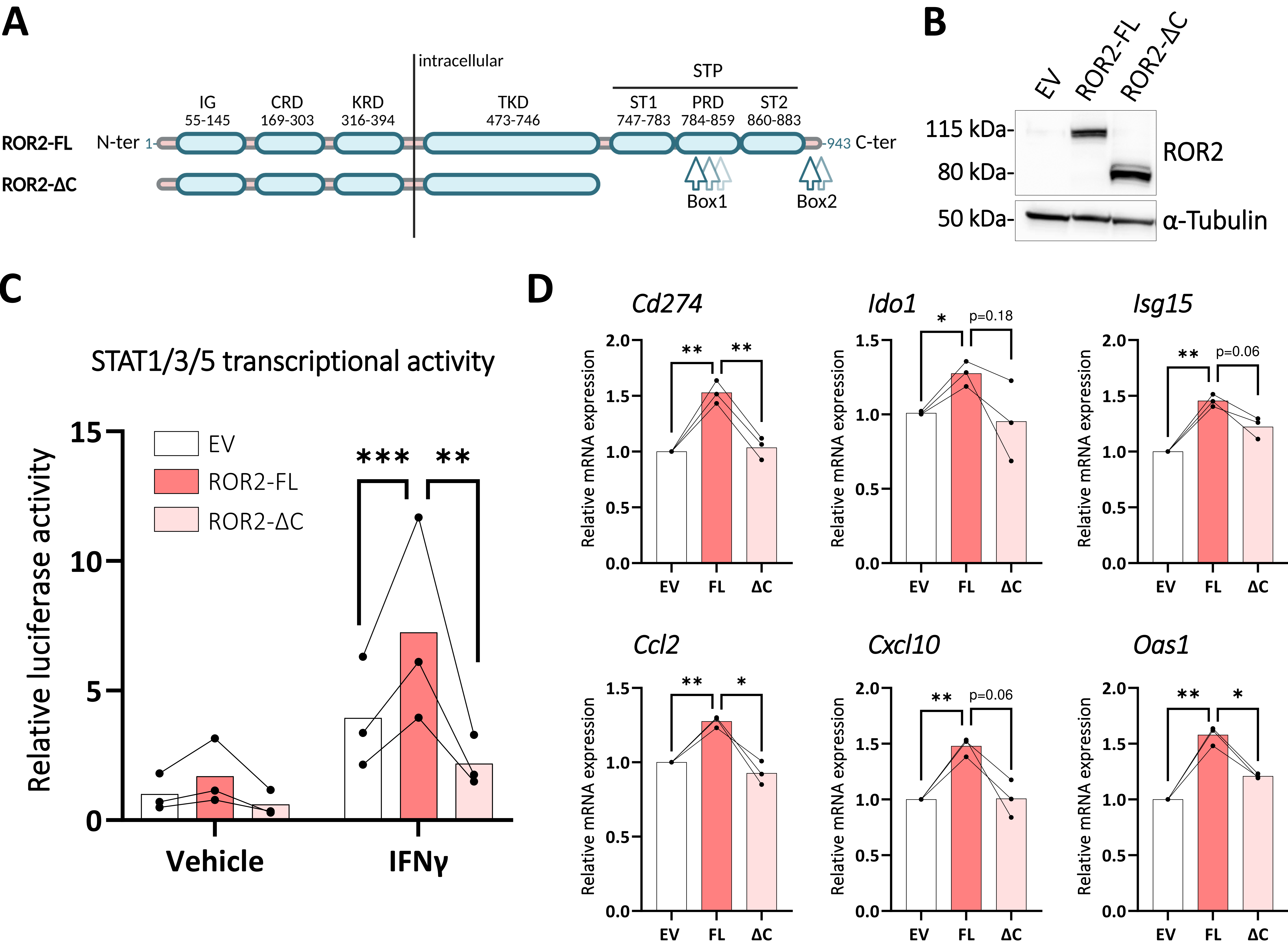
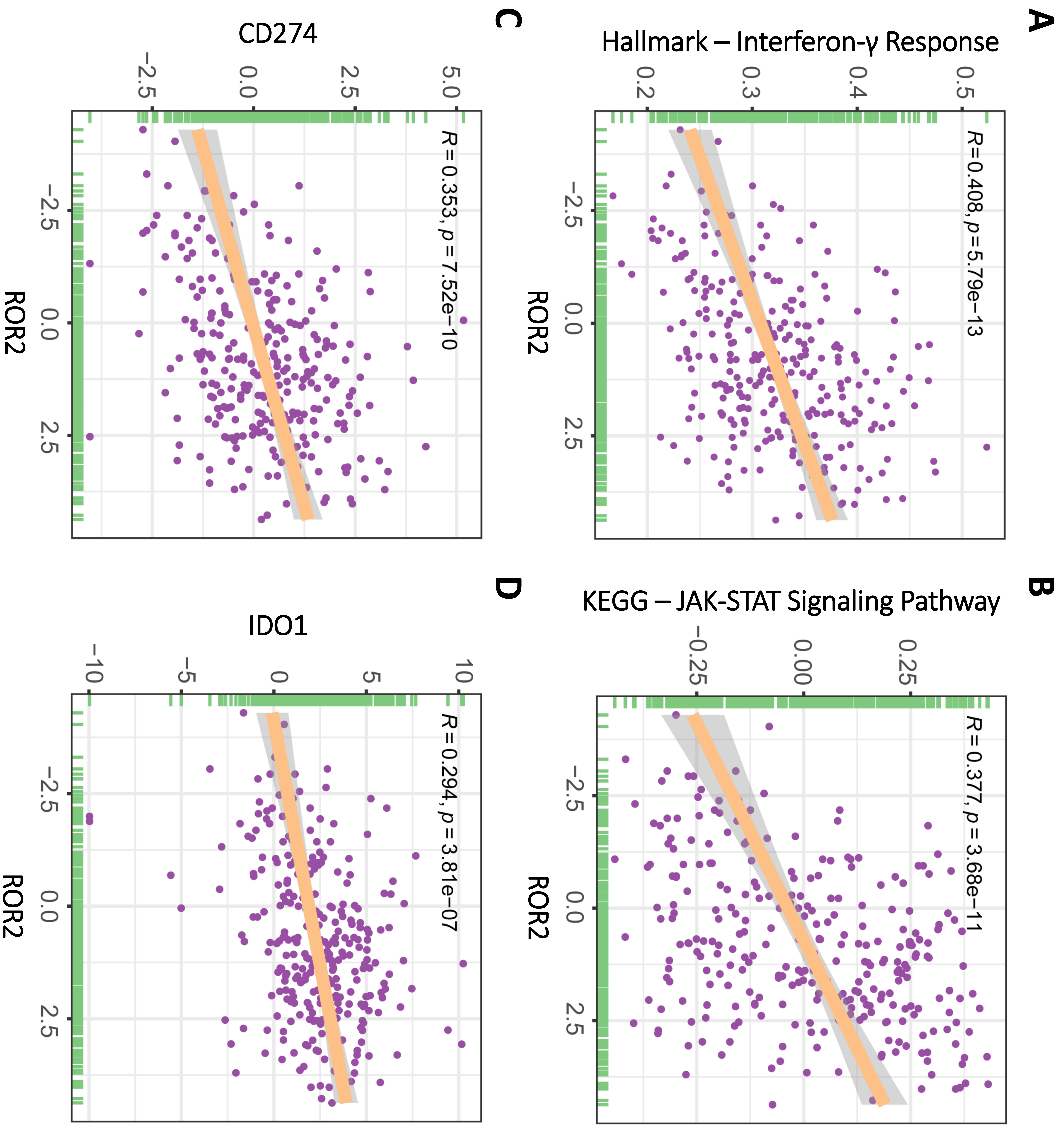


Figure 8



Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTable12.pdf](#)
- [SupplementaryFigureS1S4.pdf](#)