

**ROR1, a target of NKX2-1/TTF-1 lineage-survival oncogene, inhibits ASK1-mediated pro-apoptotic signaling in lung adenocarcinoma**

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## Summary

We previously identified receptor tyrosine kinase-like orphan receptor 1 (ROR1) as a transcriptional target of the NKX2-1/TTF-1 lineage-survival oncogene in lung adenocarcinoma. ROR1 consequently sustains a favorable balance between pro-survival PI3K-AKT and pro-apoptotic ASK1-p38MAPK signaling. In contrast to recent advances in understanding of how ROR1 sustains pro-survival signaling, the mechanism of ROR1 repression of pro-apoptotic signaling remains rather elusive. In the present study, we investigated the underlying mechanism of ROR1-mediated inhibition of the ASK1-p38MAPK signaling pathway. siROR1-mediated growth inhibition was partially but significantly alleviated by ASK1 co-knockdown in lung adenocarcinoma cell lines. Also, ASK1 phosphorylation at Thr845, which reflects its activated state, was clearly inhibited by ROR1 overexpression in both steady state and oxidative stress-elicited conditions in MSTO-211H cells. In addition, we found that ROR1 was physically associated with ASK1 at the C-terminal serine threonine-rich domain of ROR1. Furthermore, ROR1 kinase activity was shown to be required to repress the ASK1-p38 axis and oxidative stress-induced cell death. The present findings thus support our notion that ROR1 sustains lung adenocarcinoma survival, at least in part, through direct physical interaction with ASK1 and consequential repression of the pro-apoptotic ASK1-p38 axis in a ROR1 kinase activity-dependent manner.

**Keywords:** apoptosis, ASK1, lung cancer, ROR1, TTF-1

## Introduction

Lung cancer has long been the leading cause of cancer death and lung adenocarcinoma is the most prevalent among the four major histologic types. We previously reported that NKX2-1/TTF-1, an indispensable transcription factor for peripheral lung development and physiological functions, plays a role as a lineage-survival oncogene in lung adenocarcinoma.<sup>(1)</sup> In addition, we have shown that neuroendocrine lung cancer cells, such as small cell lung cancer, have dependence on lineage-survival signaling conferred by continued expression of ASH1, a transcription factor indispensable for pulmonary neuroendocrine cell development.<sup>(2-4)</sup> Together, those findings suggested that a “lineage-specific addiction” to signaling programmed for development of normal progenitor cells<sup>(5)</sup> is involved in the molecular pathogenesis of lung cancer.<sup>(6)</sup> The importance of NKX2-1/TTF-1 in lung adenocarcinoma development was further substantiated by subsequent identification of the NKX2-1/TTF-1 locus as a genomic region with frequent focal amplification.<sup>(7-9)</sup> Notably, we identified the receptor tyrosine kinase-like orphan receptor 1 (ROR1) as a direct transcriptional target of NKX2-1/TTF-1, which is crucial for maintaining a favorable balance between pro-survival PI3K-AKT and pro-apoptotic ASK1-p38MAPK signaling for lung adenocarcinoma survival.<sup>(10)</sup> Mechanistic insight into how ROR1 sustains EGFR-mediated PI3K-AKT signaling in both kinase-dependent and -independent manners has been well presented. In contrast, how ROR1 inhibits pro-apoptotic signaling remains elusive.

ASK1, a member of the MAPKKK family, is activated by various stimuli such as oxidative and ER stress, as well as by TNF- and Fas-elicited signals. ASK1 in turn activates the MAPKK 4 (MKK4)-JNK and MKK3/6-p38 pathways, leading to induction of cellular apoptosis.<sup>(11)</sup> ASK1 knockout mice were shown to be resistant to oxidative stress-induced cell death,<sup>(12)</sup> suggesting the functional importance of ASK1 for coping with cellular stress

induced by reactive oxygen species (ROS). To date, some proteins have been identified as negatively regulating ASK1 activity through their interactions, including TRX,<sup>(13)</sup> 14-3-3,<sup>(14)</sup> GSTM1-1,<sup>(15)</sup> Raf1,<sup>(16)</sup> CHA,<sup>(17)</sup> and CIB1.<sup>(18)</sup>

In this study, we attempted to gain insight into the mechanism of ROR1 repression of pro-apoptotic signaling to the ASK1-p38 axis. Our findings showed that ROR1 inhibits ASK1 activity in an ROR1-kinase dependent manner via physical interaction at the C-terminal serine threonine-rich domain of ROR1.

## **Materials and Methods**

### **Cell lines**

NCI-H1975 and NCI-H441 cells were purchased from the American Type Culture Collection, and PC-9 cells from RIKEN Cell Bank (Tsukuba, Japan). The SK-LC-5 cell line was a generous gift from the late Lloyd J. Old (Memorial Sloan-Kettering Cancer Center). All cell lines were authenticated by short tandem repeat DNA profiling and confirmed to be free from mycoplasma contamination. Summary of NKX2-1/TTF-1 and ROR1 expression is provided in Supplementary Table S1. Generations of wild-type or kinase-dead ROR1-expressing MSTO-211H cells were produced using a previously described technique.<sup>(10)</sup> All cells were maintained in RPMI-1640 with 10% fetal bovine serum.

### **Antibodies**

Recombinant MKK6 was obtained from Millipore (Watford, UK). Anti-ROR1, anti-ASK1, anti-phospho-ASK1 (T845), anti-phospho-ASK1 (S83), anti-MKK3/6, anti-phospho-MKK3/6 (S189/S207), anti-p38, anti-phospho-p38 (T180/Y182), anti-mouse IgG, and anti-rabbit IgG were purchased from Cell Signaling Technology (Danvers, MA), while anti-c-myc came from Santa Cruz Biotechnology (CA, USA), anti-GST and anti-MBP from MBL, anti-ROR1 and

anti-goat IgG from R&D systems, anti-JNK and anti-phospho-JNK (T183/Y185) from BD Bioscience (Bedford, MA) and anti- $\alpha$ -tubulin from Sigma.

### **RNA interference**

The following RNA oligomers were obtained from QIAGEN (Hilden, Germany) and Sigma-Aldrich (Dorset, UK), and used for RNA interference:

5'-CAGCAATGGATGGAATTTCAA-3', siROR1 #1;

5'-GGTATACATGAGTGGAATT-3', siASK1#1; and

5'-CACTATTGGATGTTCTCTA-3', siASK1 #2.

AllStars Negative Control siRNA (siControl) was also obtained from QIAGEN. Transfection of siRNAs (each at 20 nM) was performed using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Cells were harvested at 72 hours after transfection and subjected to western blotting (WB). Cell proliferation was determined 5 days after transfection by colorimetric assays with a Cell-Counting kit-8 (Dojindo Laboratories, Kumamoto, Japan).

### **Constructs**

Construction of expression vectors of full-length human ROR1 cDNA (pCMVpuro-ROR1) as well as its various derivatives was performed using a previously described method.<sup>(10)</sup>

Full-length human ASK1 cDNA was purchased from RIKEN and inserted into a pCMV-puro expression vector. The entire ASK1 open reading frame of the resultant construct (pCMVpuro-ASK1) was thoroughly sequenced. In addition, a myc-tagged derivative of pCMVpuro-ASK1 (pCMVpuro-ASK1-myc) was also constructed by *in vitro* mutagenesis using KOD-plus-DNA polymerase (TOYOBO, Osaka, Japan) and the following oligonucleotides:

ASK1-myc-F, 5'- TCCGAAGAGGACCTGTGACTGTTGCTCAATCTAATCTTC-3'; and  
ASK1-myc-R, 5'- GATCAGCTTCTGCTCAGTCTGTTTGTTCGAAAGTCAAT-3'.

### **Preparation of recombinant proteins**

GST-tagged ROR1 (intracellular domain) was expressed in Sf9 insect cells using a Gateway system (Invitrogen) and purified by glutathione-affinity chromatography, as previously described.<sup>(10)</sup> An *E. coli*-expressing protein of MBP-tagged kinase-dead MKK6 was purchased from Millipore.

### **WB and immunoprecipitation (IP)-WB analyses**

WB and IP-WB analyses were performed according to standard procedures using Immobilon-P filters (Millipore) and an Amersham ECL Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK). For analysis of physical interactions between ROR1 and ASK1, pCMVpuro-ASK1 was transfected with various ROR1 expression constructs, including wild-type,  $\Delta$ tyrosine kinase domain 1 ( $\Delta$ TK1),  $\Delta$ TK2,  $\Delta$ TK3,  $\Delta$ ST1,  $\Delta$ P, and  $\Delta$ ST2. Cells were harvested 24 hours after transfection with NP-40 lysis buffer containing 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 2 mM EDTA, 1% NP-40, 10% Glycerol, and 1 mM  $\text{Na}_3\text{VO}_4$ .

### **Analysis of responses to reactive oxygen species (ROS)-inducing conditions**

Wild-type and kinase-dead ROR1-expressing MSTO-211H cells were plated into 6-well plates. After 24 hours, the cells were harvested for WB analysis after treatment with or without 10 mM hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) for 15 minutes. Colorimetric assays were also performed to determine the influence on cell proliferation using a Cell-Counting kit-8 (Dojindo), following prolonged incubation in the presence of 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 24 hours.

Oxidative stress-generating ROS was also produced by treating cells with 100  $\mu$ M tert-butyl hydroperoxide (TBHP from Molecular Probes, Paisley, UK) for 120 minutes, after which the cells were harvested for WB analysis.

### ***In vitro* kinase assays**

293T cells were transiently transfected with pCMVpuro-ASK1-myc, then myc-tagged ASK1 was purified using a c-Myc tagged Protein MILD PURIFICATION KIT (Ver. 2; MBL, Nagoya, Japan). ASK1-myc and MBP-MKK6 cells were co-incubated at 37°C for 1 hour with GST or GST-ROR1-WT in phosphorylation buffer (25 mM Tris-HCl [pH 7.5], 5 mM MgCl<sub>2</sub>, 0.1 mM ATP), and subjected to SDS-PAGE, followed by WB analysis with a specific antibody.

### **Flow cytometric analysis**

Cell apoptosis was measured by flow cytometry after propidium iodide staining. Briefly, cells were treated with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 hours, then harvested by trypsinization. All cells were collected and fixed with 70% ethanol and 30% PBS at -20°C, followed by treatment with RNase and propidium iodide (Sigma-Aldrich), essentially as previously described.<sup>(4)</sup> Using a FACScalibur system, 1 x 10<sup>4</sup> cells were analyzed and sub-G1 cells were quantitated with Cell Quest Pro software (Becton, Dickinson and Company, Franklin Lakes, New Jersey).

## **Results**

### **ASK1-knockdown alleviates siROR1-elicited growth inhibition**

We examined the effects of co-treatment with siASK1 and siROR1 in the lung adenocarcinoma cell lines PC-9 and NCI-H1975, which readily express both ROR1 and

ASK1. Co-knockdown of ASK1 significantly repressed siROR1-induced phosphorylation of both MKK3/6 and p38 but not JNK in PC-9 and NCI-H1975 cells (Figs. 1a and 1b, respectively). Co-knockdown of ASK1 also significantly alleviated siROR1-induced growth inhibition in PC-9 cells (Fig. 1a), while similar but more modest effects were also observed in NCI-H1975 cells (Fig. 1b, as well as Figs. S1a and S1b with siASK1#2). These findings suggested that ASK1-mediated signaling to the MKK3/6-p38 axis is involved, at least in part, in siROR1-elicited growth inhibition in lung adenocarcinoma cells.

### **ROR1 is required for repression of ROS-induced ASK1 phosphorylation**

In order to investigate whether ROR1 is involved in regulation of the ASK1-p38 MAPK signaling pathway, we first compared steady state levels of phosphorylation of ASK1 and p38 in MSTO-211H cells, which had been stably transfected with either a wt-ROR1 expression construct or an empty vector. Significant decreases in auto-phosphorylation of ASK1 at Thr845 and p38 phosphorylation at T180 and Y182 were observed in ROR1-transfected MSTO-211H cells, suggesting ROR1-mediated negative regulation of the ASK1-p38 axis (Fig. 2a). Phosphorylation of ASK1 and p38 was also analyzed by WB analysis in the presence or absence of H<sub>2</sub>O<sub>2</sub>. Consequently, we observed marked repression of oxidative stress-induced, activation-reflecting phosphorylation of ASK1 (T845) and p38 (F180/Y182) in MSTO-211H cells overexpressing ROR1 (Fig. 2b). It was also noted that ROR1 overexpression significantly maintained ASK1 phosphorylation at serine 83, which is known to be phosphorylated by AKT and inhibits ASK1 function,<sup>(19)</sup> even in the presence of hydrogen peroxide. Similar results were obtained with another oxidative stress inducer, TBHP (Fig. 2c). An *in vitro* kinase assay was also performed using cellular ASK1 prepared from ASK1-transfected 293T cells as well as recombinant MBP-tagged kinase-deficient MKK6. While readily detectable auto-phosphorylation of ASK1 was found along with



phosphorylation of MKK6, which is a substrate of ASK1 and directly upstream of p38, co-incubation of ASK1 with recombinant GST-tagged ROR1 clearly diminished ASK1 auto-phosphorylation and MKK6 phosphorylation (Fig. 3). Taken together, these findings demonstrated that ROR1 negatively regulates pro-apoptotic signaling of the ASK1-MKK6-p38 axis.

### **ROR1 physically interacts with ASK1**

We next investigated whether ROR1 physically interacts with ASK1. 293T cells were co-transfected with ROR1 and ASK1, then subjected to IP-WB analysis. ASK1 was co-immunoprecipitated with an anti-ROR1 antibody, while an anti-ASK1 antibody was conversely co-immunoprecipitated with ROR1 (Fig. 4a). An association of transfected ROR1 and endogenous ASK1 was also observed in MSTO-211H cells transfected with ROR1 (Fig. 4b). Interactions between endogenous ROR1 and ASK1 proteins were further analyzed by IP-WB analysis using 4 different lung adenocarcinoma cell lines, which clearly demonstrated their physical associations (Fig. 4c for PC-9 and NCI-H1975, Fig. S2 for NCI-H441 and SK-LC-5). We also found a consistent association between ASK1 and ROR1 regardless of the presence of H<sub>2</sub>O<sub>2</sub> (Fig. 4d).

We then conducted detailed mapping of the ASK1 binding site of the ROR1 protein. Various deletion mutants of ROR1 were co-transfected with ASK1 into COS-7 cells (Fig. 5a), followed by IP-WB analysis of their interactions. We found that the interaction between ROR1 and ASK1 required the presence of the C-terminal serine/threonine-rich domain of ROR1 (Fig. 5b). These results demonstrated that ROR1 interacts with ASK1 at the C-terminus in both steady state and ROS-inducing conditions.

### **ROR1 kinase activity is required for inhibition of ASK1 phosphorylation**

Next, we examined whether the kinase activity of ROR1 is required for inhibition of ASK1 activity. MSTO-211H cells, which had been stably transfected with either a wild-type ROR1 (ROR1-WT) or a kinase-dead (ROR1-KD) mutant, were treated with H<sub>2</sub>O<sub>2</sub> and subjected to WB analysis. In contrast to ROR1-WT, ROR1-KD failed to significantly repress H<sub>2</sub>O<sub>2</sub>-induced ASK1 phosphorylation as well as consequential p38 phosphorylation (Fig. 6a). In addition, we noted that H<sub>2</sub>O<sub>2</sub>-induced cell growth inhibition was partly rescued by overexpression of ROR1-WT but not by that of ROR1-KD in MSTO-211H cells (Fig. 6b). Consistent with these findings, flow cytometric analysis revealed a significant reduction in sub-G1 cells among MSTO-211H cells overexpressing ROR1-WT but not ROR1-KD (Fig. 6c). Together, the present findings showed that ROR1 physically interacts with ASK1 and that ROR1 kinase activity is required to reduce that activation of ASK1, leading to repression of pro-apoptotic ASK1-p38 signaling in lung adenocarcinoma cells.

## Discussion

We previously reported that ROR1 sustains a favorable balance between pro-survival and pro-apoptotic signaling, thus playing a role as a key downstream molecule of the NKX2-1/TTF-1 lineage-survival oncogene.<sup>(6)</sup> In contrast to significant advances in understanding of the molecular mechanisms related to ROR1 pro-survival signaling, how ROR1 represses pro-apoptotic signaling remains elusive. The present findings clearly show that ROR1 is involved in negative regulation of the pro-apoptotic ASK1-p38 axis through its physical association with ASK1. ROR1 overexpression inhibited activation of ASK1 in response to oxidative stress in an ROR1 kinase-dependent manner. Intriguingly, ASK1 phosphorylation was also repressed in a steady state condition without exogenous ROS-inducing stress, suggesting the existence of ROR1-mediated repression of ASK1 activation in response to intrinsic cellular stresses. Thus, our results suggest that ROR1 may

be part of an important cellular machinery for repression of irrelevant ASK1-p38 activation.

ASK1 activity is known to be tightly regulated by phosphorylation, as it has been observed at a representative Thr845 residue within the activation loop of the kinase domain,<sup>(20)</sup> while there are additional residues phosphorylated by various kinases, including Ser83,<sup>(21)</sup> Ser967,<sup>(22)</sup> Ser1034,<sup>(23)</sup> and Tyr718.<sup>(24)</sup> Nevertheless, it remains to be elucidated which amino acid(s) of ASK1 among its more than 40 possible phosphorylation sites is affected either directly or indirectly by ROR1. Since ASK1 is also known to be both positively<sup>(25-27)</sup> and negatively<sup>(13-18)</sup> regulated through interaction with various other proteins, it is also possible that ASK1 activation may be inhibited by ROR1 by its effects on such ASK1-interacting proteins or a yet unidentified one.

NKX2-1/TTF-1 is crucially involved in maintenance of physiological functions in normal lungs, thus it cannot be considered as a therapeutic target, even though its genetic aberrations are among the most frequent in lung adenocarcinoma.<sup>(6)</sup> ROR1 is a crucial mediator of lineage-survival signaling of NKX2-1/TTF-1 and ROR1 inhibition represses lung cancer cells, which even include EGFR tyrosine kinase inhibitor-resistant ones due to the presence of T790M EGFR, MET amplification, and/or HGF overexpression.<sup>(10)</sup> Thus, ROR1 appears to be a promising molecular target for a future novel therapeutic method to treat this devastating cancer. Since the present study indicates that ROR1 counteracts oxidative stress-induced ASK1 activation and apoptosis, enhanced treatment efficacy of an ROS-inducing chemotherapeutic agent when combined with ROR1 inhibition is anticipated. In addition to lung adenocarcinoma development, ROR1 has been suggested to be involved in the pathogenesis of various other human cancers, such as ovarian, colon, lung, skin, pancreatic, testicular, bladder, uterus, prostate, gastric cancers, melanoma, and lymphomas.<sup>(28-34)</sup> In addition, onco-fetal expression of ROR1 is another attractive feature.<sup>(10, 28, 29)</sup>

In conclusion, ROR1 directly interacts with ASK1, repressing ASK1 phosphorylation and resultant ASK1-p38 pro-apoptotic signaling. A future study is awaited for development of a therapeutic means to inhibit this attractive molecular target. It would also be interesting to investigate whether ROR1 has a role as a mediator of outside-in signaling to the ASK1-p38 axis.

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### **Disclosure Statement**

The authors have no conflict of interest.

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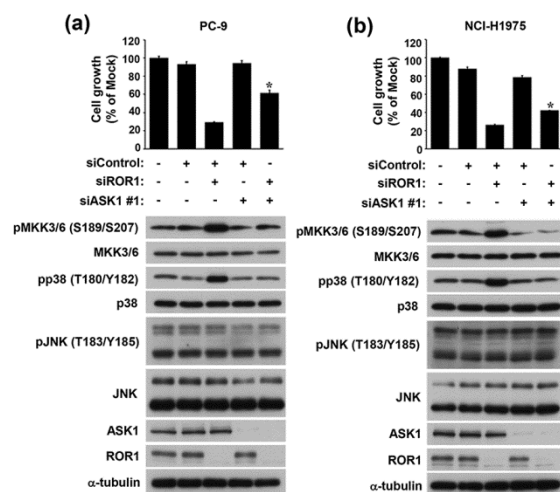
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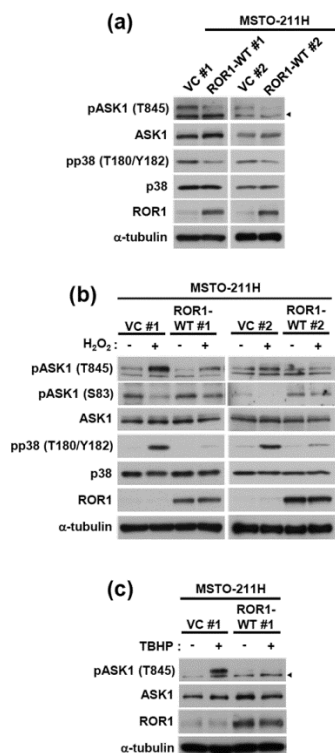




**Figure 1. ASK1-mediated signaling is involved, at least in part, in siROR1-induced growth inhibition.**

Effects of co-treatment with siASK1 and siROR1 in (a) PC-9 and (b) NCI-H1975 cells.

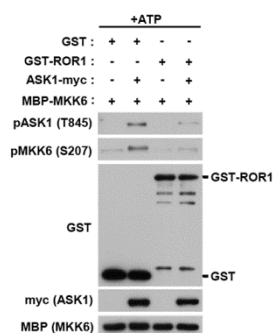
Colorimetric (top panel) and WB (bottom panel) analyses were performed at 5 and 3 days, respectively, after co-transfection. Data are shown as the mean  $\pm$  S.E.M. (n=3) \*P<0.05



**Figure 2. ROR1 suppresses ROS-induced ASK1 and p38 phosphorylation.**

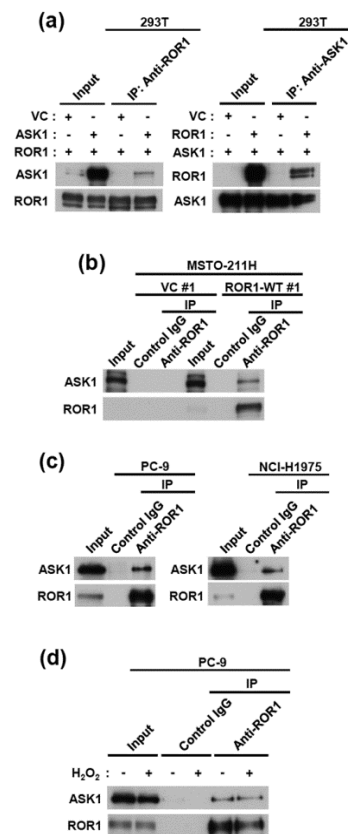
(a) WB analysis of ASK1 and p38 phosphorylation in MSTO-211H cells stably transfected with an empty vector (VC#1 and VC#2), or an expression construct of wild-type ROR1 (ROR1-WT#1 and ROR1-WT-#2). (b) WB analysis of ASK1 and p38 phosphorylation in the presence or absence of H<sub>2</sub>O<sub>2</sub> in MSTO-211H cells stably transfected with an empty or ROR1 expression vectors. (c) WB analysis of tertiary-butyl hydroperoxidase (TBHP)-induced ASK1 and p38 phosphorylation in vector control or ROR1-introduced MSTO-211H cells.

Arrowheads indicate non-specific bands.



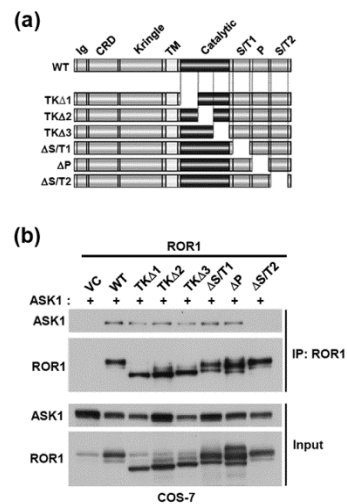
**Figure 3. ROR1 inhibits ASK1 kinase activity.**

Results of *in vitro* ASK1 kinase assays with use of myc-tagged ASK1 immunoprecipitated from ASK1-transfected 293T cells, as well as recombinant GST-ROR1 and MBP-MKK6 proteins purified from *E. coli*.



**Figure 4. ROR1 physically interacts with ASK1.**

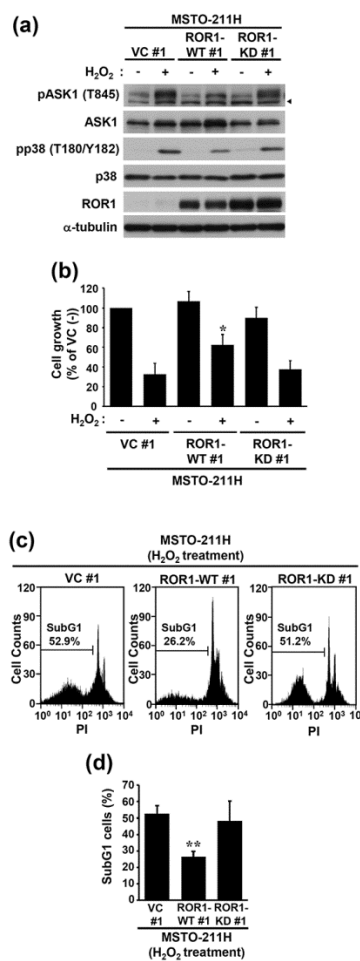
(a) IP-WB analysis of interaction between ROR1 and ASK1 co-introduced into 293T cells. (b) IP-WB analysis of interaction of endogenous ASK1 with ROR1 introduced into MSTO-211H cells. (c) IP-WB analysis of interaction between endogenous ROR1 and ASK1 using lysates of PC-9 and NCI-H1975 cells. Also, see Figure S2 for results with NCI-H441 and SK-LC-5 cells. (d) IP-WB analysis of interaction of endogenous ROR1 and ASK1 in presence or absence of H<sub>2</sub>O<sub>2</sub> in PC-9 cells.



**Figure 5. Fine mapping of ASK1-interacting domain of ROR1.**

(a) Schematic diagram of deletion mutants of ROR1. IP-WB analysis of COS-7 cells co-introduced with ASK1 and various forms of ROR1. WT, wild-type ROR1; TKΔ1, TKΔ2, and TKΔ3, mutants lacking one-third of the kinase domain; ΔS/T1 and ΔS/T2, mutants lacking one of the two serine/threonine-rich regions; ΔP, mutant lacking a proline-rich region.

(b) IP-WB analysis for fine mapping of ASK1-interacting domain of ROR1.



**Figure 6. ROR1 kinase activity is required for inhibition of ROS-elicited phosphorylation of ASK1 and p38.**

(a) WB analysis of ASK1 and p38 phosphorylation in presence or absence of H<sub>2</sub>O<sub>2</sub> in MSTO-211H cells stably expressing wild-type or kinase-dead ROR1. Arrowheads indicate non-specific bands. (b) Colorimetric assay of cell proliferation in MSTO-211H transfectants in presence or absence of H<sub>2</sub>O<sub>2</sub>. (c) Representative results of flow cytometric analysis of apoptosis induction in ROR1-transfected MSTO-211H cells treated with H<sub>2</sub>O<sub>2</sub>. (d) Flow cytometric analysis of subG1 cells. Representative data from 5 independent experiments are shown as the mean  $\pm$  S.E.M. \*p < 0.05, \*\*P < 0.01 vs. VC, as determined by Student's t test.