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The Kelch Repeat Protein KLHDC10 Regulates Oxidative Stress-Induced ASK1 Activation by Suppressing PP5

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SUMMARY

Reactive oxygen species (ROS)-induced activation of Apoptosis signal-regulating kinase 1 (ASK1) plays crucial roles in oxidative stress-mediated cell death through the activation of the JNK and p38 MAPK pathways. However, the regulatory mechanism of ASK1 in the oxidative stress response remains to be elucidated. Here, we identified the kelch repeat protein, Slim, as an activator of ASK1 through a *Drosophila* misexpression screen. We also performed a proteomics screen and revealed that Kelch domain containing 10 (KLHDC10), a mammalian ortholog of Slim, interacted with Protein phosphatase 5 (PP5), which has been shown to inactivate ASK1 in response to ROS. KLHDC10 bound to the phosphatase domain of PP5 and suppressed its phosphatase activity. Moreover, KLHDC10 was required for H_2O_2-induced sustained activation of ASK1 and cell death in Neuro2A cells. These findings suggest that Slim/KLHDC10 is an activator of ASK1, contributing to oxidative stress-induced cell death through the suppression of PP5.

INTRODUCTION

During the process of aerobic metabolism in cells, reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide (H_2O_2), and hydroxyl radicals, are produced by intracellular enzymatic systems, including the mitochondrial electron transport chain. Stressors from the exogenous environment, such as ultraviolet radiation, ionizing radiation, and anticancer drugs, also cause the formation of ROS. Excessive and/or ectopic generation of ROS results in oxidative stress, in which ROS induce nonspecific oxidation of nucleic acids, lipids, and proteins, leading to profound damage to the cells and eventually cell death. Accordingly, ROS have been considered to be the cause of various human diseases and aging. To cope with oxidative stress and maintain the intracellular homeostasis of redox status, living organisms are equipped with a wide variety of antioxidant proteins and redox-sensitive signaling systems (Finkel and Holbrook, 2000).

The mitogen-activated protein kinase (MAPK) cascades are evolutionarily conserved signaling pathways that play crucial roles in cellular responses to environmental changes (Kyriakis and Avruch, 2001; Widmann et al., 1999). Apoptosis signal-regulating kinase 1 (ASK1) is a member of the MAP kinase kinase kinases (MAP3K) that activates the c-Jun N-terminal kinase (JNK) and p38 MAPK pathways in response to a wide variety of pathophysiological stressors, including oxidative stress, endoplasmic reticulum (ER) stress, and inflammatory cytokines (Ichijo et al., 1997; Takeda et al., 2008). ASK1 has been demonstrated to be involved in ROS-induced cell death in various types of cells and has been implicated in the pathogenesis of oxidative stress-related diseases such as neurodegenerative diseases, cardiovascular diseases, and cancers (Nagai et al., 2007; Tobiume et al., 2001). The molecular mechanism of ASK1 activation by ROS has been revealed mainly through analyzing ASK1-binding proteins. Under conditions in which there is no oxidative stress, the antioxidant protein thioredoxin (Trx) forms a complex with ASK1 through the N-terminal region of ASK1, and inhibits ASK1's kinase activity (Saitoh et al., 1998). Under oxidative stress conditions, the reactive cysteine residues within Trx are oxidized and form an intramolecular disulfide bond. The oxidized form of Trx is released from ASK1, and reciprocally tumor necrosis factor (TNF) receptor-associated factor (TRAF) family proteins, such as TRAF2 and TRAF6, are recruited to ASK1 (Fujino et al., 2007; Nishitoh et al., 1998; Noguchi et al., 2005). The interaction of TRAFs with ASK1 appears to enhance

the transautophosphorylation of the threonine residue within the activation loop of the kinase domain of ASK1, which is essential for ASK1 activation (Nishitoh et al., 1998). Although Trx and TRAF family proteins are pivotal molecules in the regulation of ROS-induced activation of ASK1, we have also demonstrated that Protein phosphatase 5 (PP5) is a negative regulator of ASK1 (Morita et al., 2001). PP5 is a serine/threonine phosphatase that belongs to the Phosphoprotein phosphatase (PPP) family. It has been reported that PP5 is involved in hormone and stress responses by dephosphorylating various substrates (Golden et al., 2008; Hinds and Sánchez, 2008). PP5 interacts with the activated form of ASK1 in response to H_2O_2 and dephosphorylates the essential phosphothreonine residue, thereby inhibiting the kinase activity of ASK1 and oxidative stress- and ASK1-dependent apoptosis. Thus, PP5 is one of the key molecules that determine the cell fate in the oxidative stress response.

The kelch repeat domain consists of five to seven tandem repeats of the kelch motif and forms a β-propeller structure (Adams et al., 2000; Prag and Adams, 2003). The kelch repeat protein family members exist across species and have been reported to play roles in a wide range of cellular processes, including signal transduction, transcription, DNA repair, and protein degradation. Because the kelch repeat domain is important for protein-protein interactions, the kelch repeat proteins function mainly through interaction with their binding partners. Recently, several lines of evidence have demonstrated that the kelch repeat proteins interact with the complex-type ubiquitin E3 ligases called the Cullin-RING ligases (CRLs) and serve as the substrate recognition subunits of the CRL complex, which determine the substrate specificity of this complex (Bennett et al., 2010). However, the molecular functions and physiological roles of most kelch repeat proteins remain unknown.

Here, we identified the kelch repeat protein Slim and its mammalian ortholog KLHDC10 as an activator of ASK1 by taking advantage of *Drosophila* genetics. Slim/KLHDC10 was found to activate ASK1 through the suppression of PP5 and thereby to contribute to oxidative stress-induced cell death.

RESULTS

A Misexpression Screen for Activators of ASK1 using *Drosophila*

To identify genes that regulate activation of the ASK1-MAPK cascades, we took advantage of a *Drosophila* misexpression screen, using the GAL4/UAS system that has been extensively used to induce ectopic gene expression in flies (Brand and Perrimon, 1993). We have recently reported that an N terminus-lacking mutant of *Drosophila* ASK1 (DASK1 ΔN) activated the *Drosophila* p38 (Dp38) pathway more strongly than did DASK1 wild-type (WT) (Sekine et al., 2011). Ectopic expression of DASK1 ΔN, but not DASK1 WT, under the control of *pannier* (*pnr*)-*GAL4*, which is expressed along the dorsal midline of adult flies, resulted in a Dp38-dependent increase in melanization (increase in black and brown pigments) in the thorax cuticle of flies (Figures 1A and 1B) (Sekine et al., 2011). To establish a misexpression screening system for activators of ASK1, we used this melanization as a visible marker for activation of the ASK1-p38 pathway in flies and sought to screen for genes that could induce melanization in the thorax in a *pnr-GAL4*-dependent manner. The Gene Search (GS) lines have a GS vector that contains UAS, which is randomly inserted in the fly genome of each GS line, thereby allowing ectopic expression of endogenous genes after crossing with the GAL4 driver strains (Toba et al., 1999). We generated *pnr > DASK1WT* flies and crossed them with approximately 4,500 GS lines. The melanization of thoraxes of the F1 progeny was examined, and nine lines were found to exhibit enhanced melanization in a *pnr-GAL4*-dependent manner. We designated those lines as *kuma* (*key upswing in melanin accumulation*) lines.

Slim Expression in *pnr > kuma1* Flies Induces Melanization

Of the nine *kuma* lines, we focused on the *kuma1* (GS10238) line. All of the *pnr > kuma1* flies exhibited strong melanization, similar to *pnr > DASK1ΔN*, even without coexpression of DASK1 WT (Figure 1C). These results suggest that *kuma1*-dependent activation of endogenous DASK1-Dp38 may be sufficient to melanize the thorax in these conditions. When we crossed the flies at 18°C (a temperature that weakly induced the gene misexpression in *kuma1*), about half of the *pnr > kuma1* flies showed a weak phenotype that resulted in the loss of the upper half of melanization in the thoracic *pnr-GAL4* expression region (indicated by the white arrowhead in Figure 1D). Under these conditions, coexpression of DASK1 WT restored the melanization in *pnr > kuma1* flies, suggesting that exogenous DASK1 enhanced the melanization in *pnr > kuma1* flies (Figure 1D). The GS vector in the *kuma1* line was inserted 508 bases upstream of a gene, *scruin like at the midline* (*slim*) (Figure 1E), and we examined the expression levels of *slim* in *kuma1* lines under the control of heat shock (hs)-inducible GAL4. The expression of *slim* mRNA in *hs > kuma1* flies was elevated compared to that in *hs > GFP* flies, whereas the expression levels of *DASK1* or *CG5189*, which is located directly 3′ to *slim*, were not affected (Figure 1F). Slim protein levels were also increased in *hs > kuma1* flies (Figure 1G). Furthermore, coexpression of inverted repeat (IR) RNA targeting *slim* (*slim-IR*) strongly reduced the melanization in *pnr > kuma1* flies (Figures 2A and 2B). Based on these results, we concluded that *slim* is the gene responsible for melanization in the thorax of *pnr > kuma1*.

The DASK1-Dp38 Pathway Mediates Slim-Induced Melanization

Because DASK1 ΔN-induced melanization is dependent on the Dp38 pathway (Sekine et al., 2011), we examined the requirement of the DASK1-Dp38 pathway for Slim-induced melanization. IR RNA-mediated knockdown of *DASK1* or *licorne (lic)*, a *Drosophila* MAP2K gene in the Dp38 pathway, in *pnr > kuma1* flies partially inhibited melanization (Figures 2C and 2D). In addition, the expression of a dominant-negative mutant of Dp38a (Dp38a DN) also reduced melanization in *pnr > kuma1* flies (Figure 2E). By contrast, knockdown of *slim* did not affect DASK1 ΔN-induced melanization (Figures 2F and 2G), indicating that *slim* is genetically located upstream of *DASK1* and that Slim-induced melanization was mediated through the DASK1-Dp38 pathway.

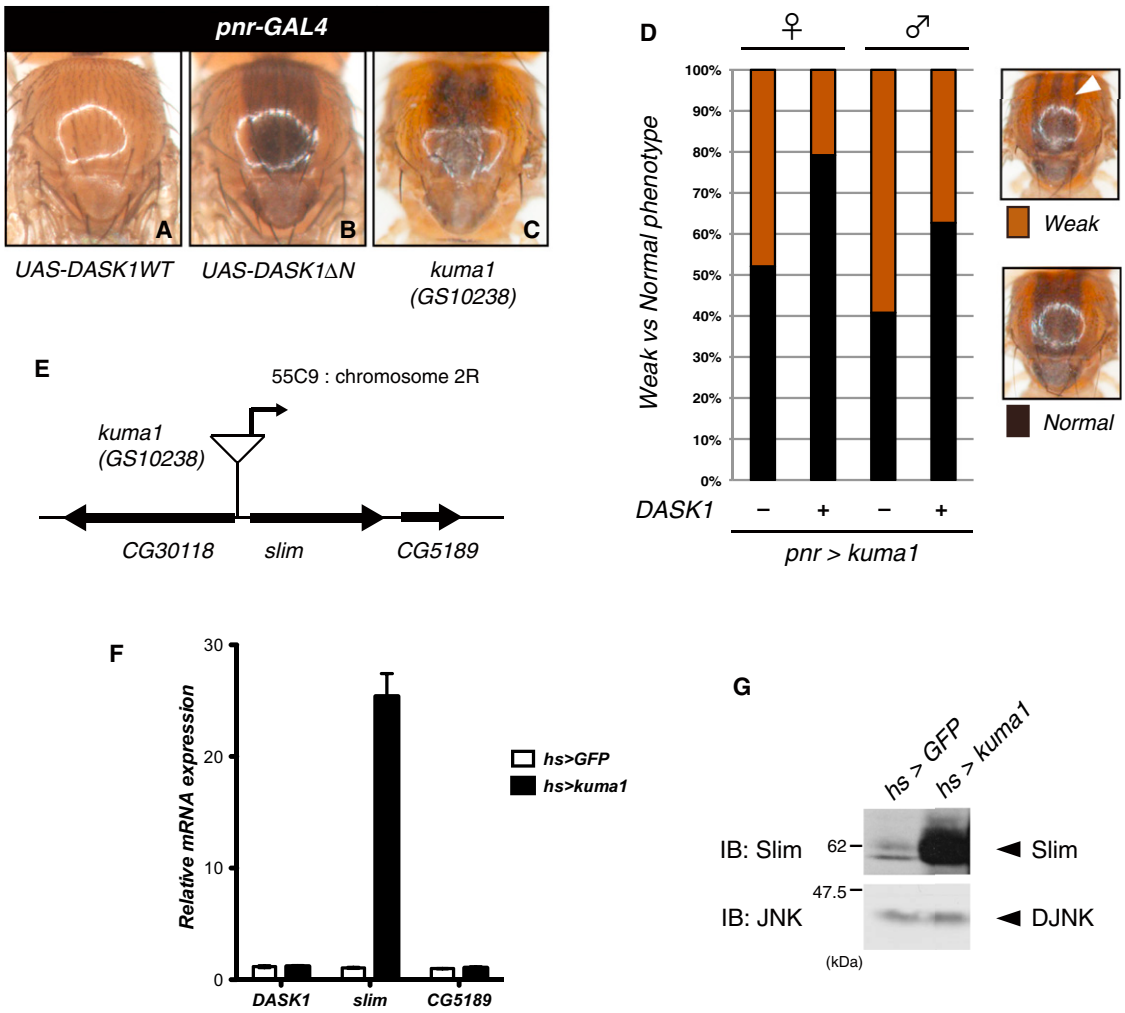

Figure 1. Ectopic Expression of Slim Induces Melanization in the Fly Thorax

(A and B) Thoraxes of *UAS-DASK1WT*/+; *pnr-GAL4*/+ (A) and *UAS-DASK1ΔN*/+; *pnr-GAL4*/+ (B) flies are shown.

(C) The thorax of a *kuma1*/+; *pnr-GAL4*/+ fly is shown.

(D) Flies were crossed and maintained at 18°C. Under these conditions, about half of all flies exhibited weakened melanization (weak), whereas the other half exhibited a similar extent of melanization to flies crossed at 25°C (normal). The number of flies with weak and normal melanization was counted in *kuma1*/+; *pnr-GAL4*/+ flies (48 females and 71 males) and *kuma1*/*UAS-DASK1WT*; *pnr-GAL4*/+ flies (48 females and 59 males). The white arrowhead indicates reduced melanization in the flies with the weak phenotype.

(E) A schematic representation of the GS vector insertion site within chromosome 2R of the *kuma1* (*GS10238*) line. The GS vector, in which the gene is expressed in the direction of the arrow, was inserted 508 base upstream of the *slim* gene.

(F) Total RNA isolated from the heat-shocked adult flies of *UAS-GFP^{S65T}*/*hs-GAL4* (*hs* > *GFP*) and *kuma1*/*hs-GAL4* (*hs* > *kuma1*) was analyzed by qRT-PCR analysis for the indicated genes. The results shown are the means of three independent RNA samples. Error bars indicate SEM.

(G) Heat-shocked adult males of *hs* > *GFP* and *hs* > *kuma1* were lysed and subjected to immunoblotting (IB).

The Kelch Repeat Protein Slim/KLHDC10 Activates ASK1

To examine whether the expression of Slim indeed activates DASK1, we transfected Flag-tagged Slim and DASK1 into *Drosophila* S2 cells and performed immunoblotting analysis. Activation of DASK1 was monitored using an antibody against the phosphothreonine residue within the kinase domain of ASK1 (Thr838 and Thr747 in human and *Drosophila* ASK1, respectively) that is essential for the activation of the ASK family proteins (Kuranaga et al., 2002; Tobiume et al., 2002). The coexpression of Slim and DASK1 induced the activation of DASK1, *Drosophila* JNK (DJNK), and Dp38 (Figure 3A), suggesting that Slim is an activator of the DASK1-MAPK cascades. In contrast, Slim did not activate coexpressed DASK1 ΔN (see Figure S1A online). This result seems to be consistent with the results showing that DASK1 ΔN by itself efficiently induced melanization in fly thorax (Figure 1B) and that knockdown of *slim* did not affect the DASK1 ΔN-induced melanization (Figure 2G).

Kelch domain containing 10 (KLHDC10) is the mammalian counterpart of Slim. The primary structure of both Slim and

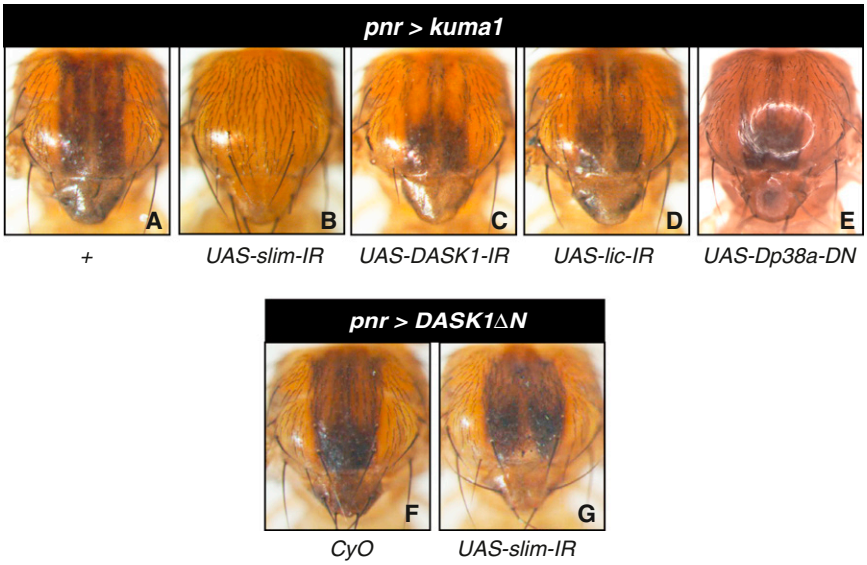

Figure 2. The DASK1-Dp38 Pathway Is Required for the Slim-Induced Melanization
Thoraxes of female flies with the following genotypes are shown: *kuma1*/+; *pnr-GAL4*/+ (A), *kuma1/UAS-slim-IR*; *pnr-GAL4*/+ (B), *kuma1*/+; *pnr-GAL4/UAS-DASK1-IR* (C), *kuma1/UAS-lic-IR*; *pnr-GAL4*/+ (D), *UAS-Dp38a-DN*/+; *kuma1*/+; *pnr-GAL4*/+ (E), *UAS-DASK1ΔN/CyO*; *pnr-GAL4*/+ (F), and *UAS-DASK1ΔN/UAS-slim-IR*; *pnr-GAL4*/+ (G).

KLHDC10 is mostly composed of the kelch repeat domain that consists of six repeated kelch motifs (Figure S1B). The amino acid sequence identity of the kelch repeat domain between Slim and KLHDC10 is 40.8%. Coexpression of Flag-tagged human KLHDC10 and human ASK1 in HEK293 cells also induced the activation of ASK1 and the subsequent activation of endogenous JNK and p38 (Figure 3B, compare lane 5 with lane 7). The fold increases in p-ASK1, p-p38, and p-JNK (upper band, p54) signals with KLHDC10 expression were shown in Figure 3C. These results suggest that Slim and KLHDC10 are evolutionarily conserved activators of the ASK1-MAPK cascades.

Slim/KLHDC10 Is a Substrate Recognition Subunit of the CRL2 Complex

Recent reports using mass spectrometry-based proteomic analyses revealed that several kelch domain-containing proteins including KLHDC2, KLHDC3, and KLHDC10 interacted with the Cullin2-RING ubiquitin ligase (CRL2) complex (Bennett et al., 2010; Mahrour et al., 2008). The CRL2 complex is composed of a large scaffold protein (Cullin2), a RING domain containing protein (Rbx1), adaptor proteins (Elongin B and Elongin C), a ubiquitin-like molecule (Nedd8), and a substrate recognition subunit (Bosu and Kipreos, 2008; Petroski and Deshaies, 2005). We confirmed the interaction of endogenous KLHDC10 with Cullin2 in Neuro2A cells (Figure S2A). Moreover, endogenous KLHDC10 was immunoprecipitated with Flag-Elongin B or Flag-Elongin C (Figure S2B), indicating that KLHDC10 is part of the CRL2 complex. KLHDC2 has been shown to possess the consensus sequence required for binding to Cullin2 or Elongin B and C, called the Cul2 box and the BC box, respectively, suggesting that KLHDC2 serves as one of the substrate recognition subunits of the CRL2 complex (Mahrour et al., 2008). We identified Cul2 box- and BC box-like sequences in the C-terminal region of Slim and KLHDC10 by comparison with those in KLHDC2 (Figure 4A) and constructed KLHDC10 mutants lacking the C-terminal region containing the Cul2 box and BC box sequences (ΔBC) or possessing a point mutation in the BC box (A409P; AP) (Figure 4B). We examined the ability of these mutants to bind to Cullin2. Endogenous Cullin2 was immunoprecipitated with Flag-tagged KLHDC10 WT, but not with Flag-KLHDC10 ΔBC or with AP mutants in HEK293 cells (Figure 4C). Cullin2 was detected as doublet bands; the upper and lower bands are known to correspond to the Nedd8-bound and unbound form of Cullin2, respectively (Jubelin et al., 2010). We also examined the interaction of Slim WT and the ΔBC mutant with the *Drosophila* CRL2 complex components, *Drosophila* Elongin C (dElongin C). Slim WT, but not the ΔBC mutant, interacted with dElongin C (Figure 4D). Moreover, we found that knockdown of *Cullin2* or *Drosophila Cullin2* (*dCul2*) resulted in the increase in protein levels of KLHDC10 or Slim, respectively (Figures S2C–S2E). The substrate recognition subunits of the CRL complex themselves have been known to be degraded through autoubiqutination by the CRL complex (Kamura et al., 2002), suggesting that Slim and KLHDC10 themselves are also regulated by the CRL2 complex. These results suggest that Slim and KLHDC10 function as one of the substrate recognition subunits of the CRL2 complex.

The CRL2 Complex Is Dispensable for Slim/KLHDC10-Dependent ASK1 Activation

To investigate whether Slim/KLHDC10-induced ASK1 activation requires the activity of Slim/KLHDC10 as the substrate recognition subunit in the CRL2 complex, we examined whether Slim/KLHDC10 mutants that failed to interact with the CRL2 complex could also activate DASK1. Interestingly, DASK1 was activated by the coexpression of Slim ΔBC as well as Slim WT (Figure 4E and Figure S2F). KLHDC10 AP mutant also activated ASK1 (Figure 4F), suggesting that Slim/KLHDC10 activates ASK1 in a CRL2 complex-independent manner. Because we have previously reported that ASK1 protein is degraded through ubiquitin-proteasome system upon H_2O_2 stimulation (Nagai et al., 2009), we also investigated the possibility that the CRL2-KLHDC10 complex regulates the stability of ASK1; however, knockdown of neither *KLHDC10* nor *Cullin2* affected the H_2O_2-dependent degradation of ASK1 (Figures S2G and S2H).

KLHDC10 Interacts with PP5 through Phosphatase Domain of PP5

To address the question of how Slim/KLHDC10 activates ASK1, we explored binding partners of KLHDC10 with a pull-down

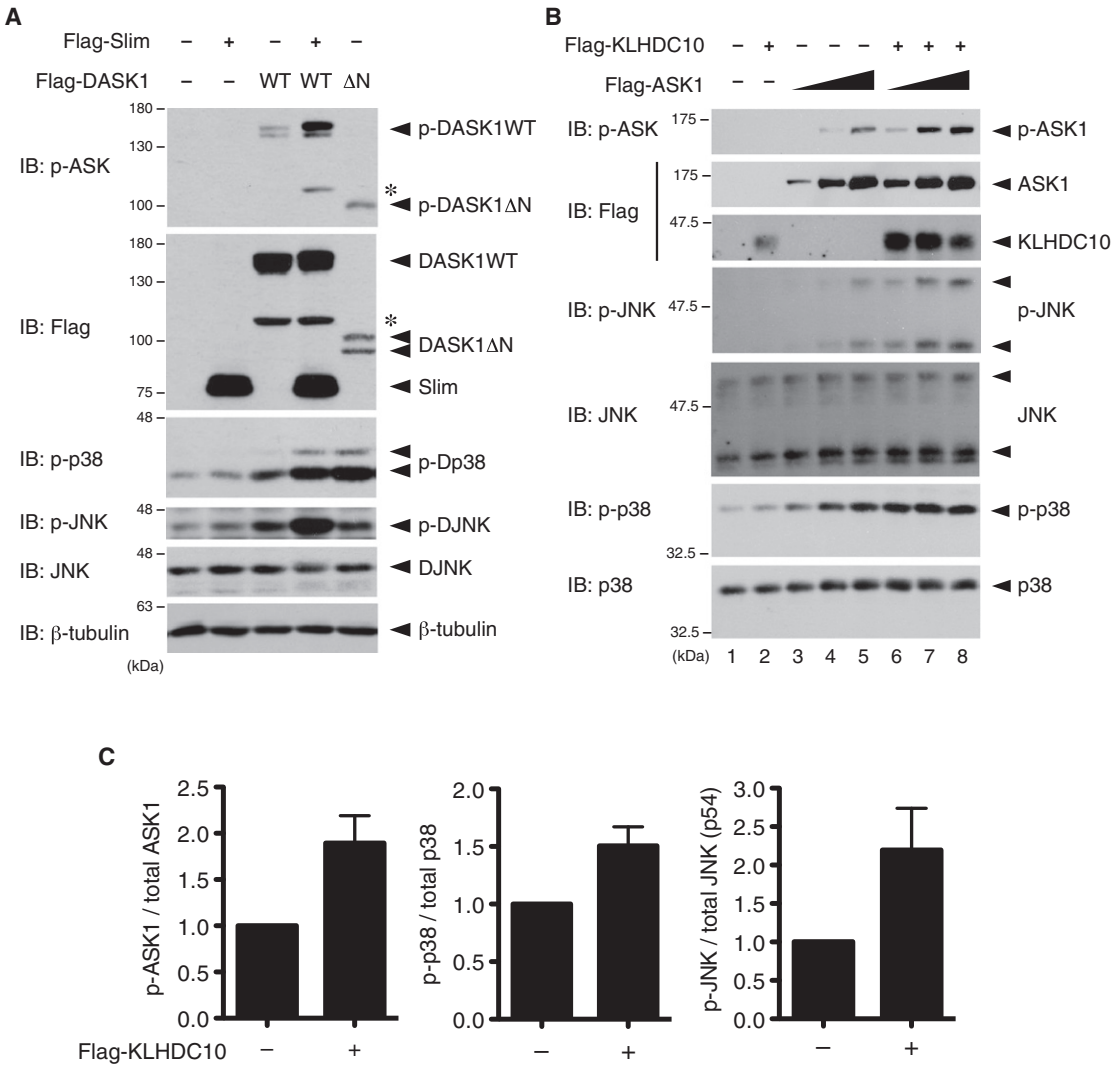

Figure 3. Slim/KLHDC10 Activates DASK1/ASK1

(A) S2 cells were transiently transfected with the indicated constructs and pWAGAL4. After 24 hr, cells were lysed and subjected to immunoblotting (IB). The asterisk indicates the putative C-terminal cleaved products of Flag-DASK1WT, which was observed when DASK1 WT was overexpressed in S2 cells. The lower band of Flag-DASK1 ΔN appeared to be a dephosphorylated form of DASK1 ΔN, because the upper but not lower band was detected by the phospho-ASK antibody.

(B) HEK293 cells were transiently transfected with the indicated constructs. After 24 hr, cells were lysed and subjected to IB.

(C) The ratios of phospho-ASK1, p38, and JNK (upper band; p54) to total ASK1, p38, and JNK (p54), respectively, with or without Flag-KLHDC10 expression were calculated and are shown as the fold changes. The results shown are the means of at least three independent experiments, and error bar indicates SEM.

screen using Flag-tagged KLHDC10 WT, ΔBC, or AP expressed in HEK293 cells as bait. As expected, multiple components of the CRL2 complex were pulled down with KLHDC10 WT, but not with the ΔBC and AP mutants (Table S1). We also identified several proteins that bound to the KLHDC10 ΔBC and AP mutants. Of these, we focused on a serine/threonine protein phosphatase, PP5, because we previously identified PP5 as a negative regulator of ASK1 (Morita et al., 2001). Coimmunoprecipitation analysis in HEK293 cells revealed that Flag-KLHDC10 WT, ΔBC, and A409P bound to coexpressed HA-PP5 (Figure 5A), suggesting that the interaction of KLHDC10 with the CRL2 complex is dispensable for the interaction of KLHDC10 with PP5. We also examined two other constructs; one is a deletion mutant of human KLHDC10 (KLHDC10 ΔN), which lacks the N-terminal 85 amino acids adjacent to the kelch repeat domain, and the other is a splicing variant of mouse KLHDC10 (KLHDC10 ΔE2), which skips exon 2 of the mouse *KLHDC10* gene and thus lacks the N-terminal 29 amino acids adjacent to the kelch repeat domain (Figure 4B). Both KLHDC10 ΔN and ΔE2 also bound to HA-PP5 (Figure 5A), suggesting that KLHDC10 interacted with PP5 through the six-repeated kelch motif. Because PP5 has been shown to localize to both nucleus and cytoplasm (Borthwick et al., 2001; Morita et al., 2001), we investigated the subcellular localization of Flag-KLHDC10 WT

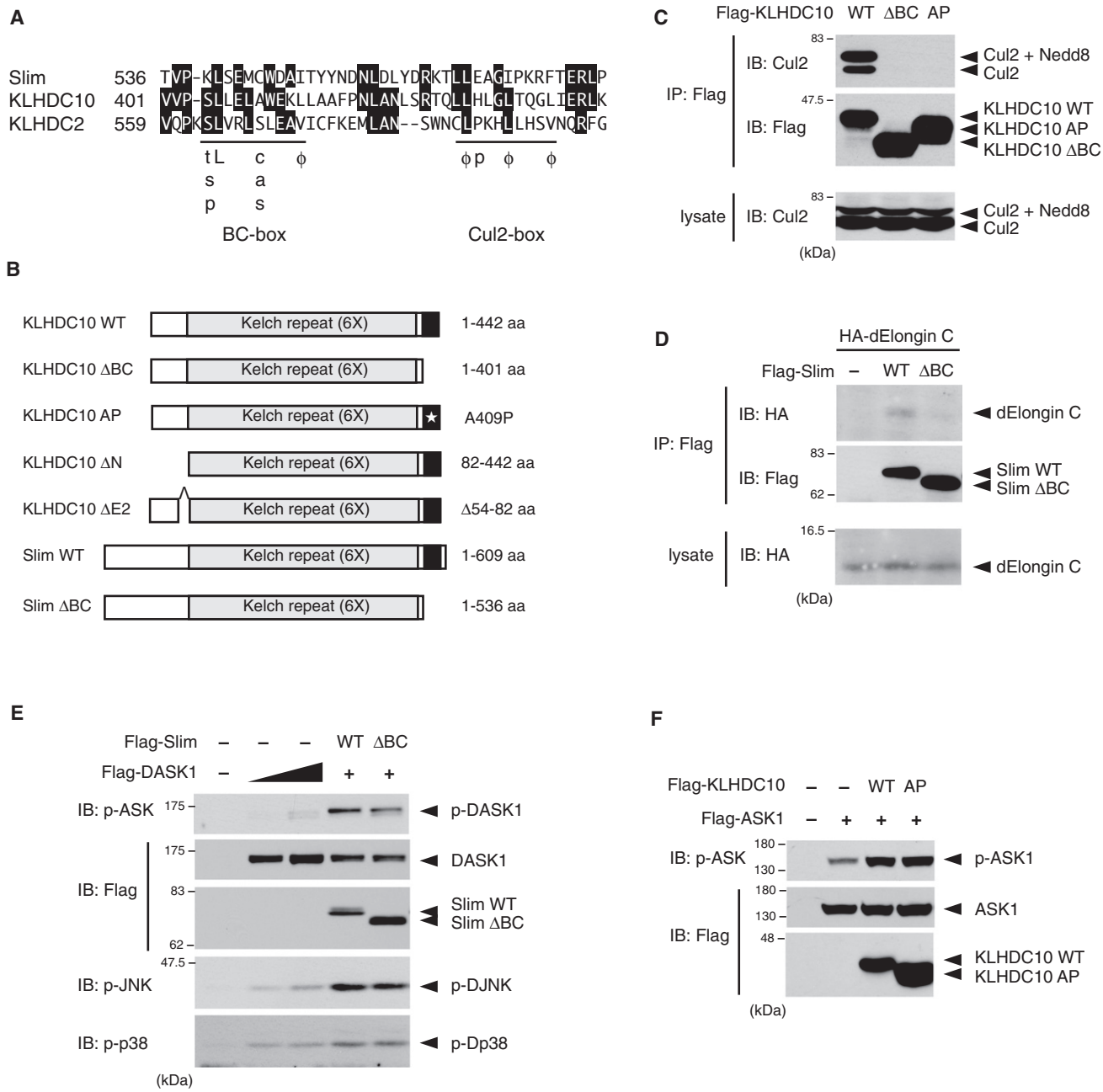

Figure 4. The CRL2 Complex Is Dispensable for ASK1 Activation by Slim

(A) ClustalW alignment of the amino acid sequences of the C-terminal regions of Slim, KLHDC10, and KLHDC2. Slim and KLHDC10 contain BC box- and Cul2 box-like sequences. ϕ indicates hydrophobic amino acids.

(B) Schematic representation of human KLHDC10 WT and its mutants ΔBC, AP, and ΔN and a splicing variant of mouse KLHDC10, ΔEx2, as well as Slim WT and ΔBC.

(C) HEK293 cells were transiently transfected with the indicated constructs. After 24 hr, cells were lysed and subjected to immunoprecipitation (IP) with anti-Flag antibody followed by immunoblotting (IB).

(D) Neuro2A cells were transiently transfected with the indicated constructs. After 24 hr, cells were lysed and subjected to IP with anti-Flag antibody followed by IB.

(E) S2 cells were transiently transfected with the indicated constructs and pWAGAL4. After 24 hr, the cells were lysed and subjected to IB.

(F) HEK293 cells were transiently transfected with the indicated constructs. After 24 hr, the cells were lysed and subjected to IB.

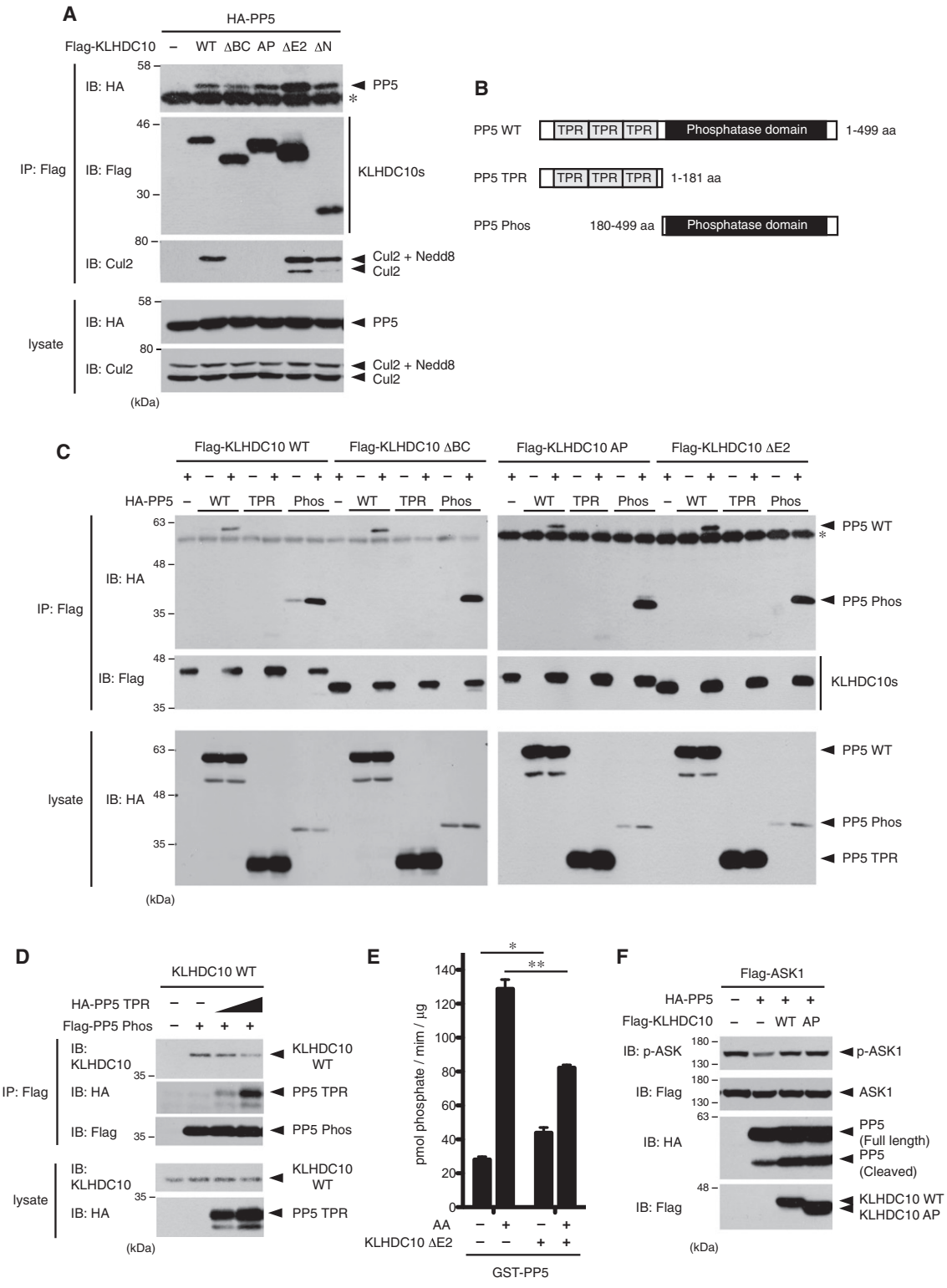

Figure 5. KLHDC10 Interacts with PP5 and Suppresses the Phosphatase Activity of PP5

(A) HEK293 cells were transiently transfected with the indicated constructs. After 48 hr, cells were lysed and subjected to immunoprecipitation (IP) with anti-Flag antibody followed by immunoblotting (IB).

(B) Schematic representation of human PP5 WT and its deletion mutants, TPR and Phos.

(C) HEK293 cells were transiently transfected with the indicated constructs. After 48 hr, cells were lysed and subjected to IP with anti-Flag antibody followed by IB.

and ΔE2. KLHDC10 WT localized predominantly to the nucleus but also to cytoplasm, whereas KLHDC10 ΔE2 exhibited cytoplasmic but not nuclear localization (Figures S3A and S3B), suggesting that nuclear localization of KLHDC10 is regulated via the amino acid sequence within the exon2 region. A bipartite basic amino acids cluster, KKKIRWDPVRRR, included in this region may function as a nuclear localization signal for KLHDC10 (Figure S3C). These data indicate that KLHDC10 interacts with PP5 in both nucleus and cytoplasm.

KLHDC10 Regulates Phosphatase Activity of PP5

PP5 contains three consecutive TPR domains within its N-terminal region that are known to function as the autoinhibitory domain covering the catalytic site within the C-terminal phosphatase domain of PP5 (Yang et al., 2005). Based on a coimmunoprecipitation analysis using PP5 deletion constructs (Figure 5B), we found that all KLHDC10 constructs we tested preferentially bound to the C-terminal fragment of PP5 including the phosphatase domain (PP5 Phos), but not to the N-terminal fragment containing the TPR domain (PP5 TPR) (Figure 5C). In addition, the interaction between KLHDC10 and PP5 Phos was suppressed by further coexpression of PP5 TPR, indicating the possibility that KLHDC10 and PP5 TPR competitively interact with PP5 Phos through the catalytic site within the phosphatase domain of PP5 (Figure 5D). These results prompted us to examine the effect of KLHDC10 on the phosphatase activity of PP5. Glutathione S-transferase (GST)-fused recombinant PP5 protein was purified from *E. coli*, and phosphatase activity was measured by an in vitro phosphatase assay using the phosphothreonine peptide as a substrate. It has been shown that PP5 is activated by arachidonic acid (AA) through interaction with the TPR domain of PP5 in vitro (Chen and Cohen, 1997; Kang et al., 2001; Skinner et al., 1997). We confirmed that incubation of PP5 with AA increased PP5 phosphatase activity and found that this AA-induced GST-PP5 activation was suppressed in the presence of GST-KLHDC10 ΔE2 protein (Figure 5E). These results suggest that KLHDC10 possesses the ability to suppress the phosphatase activity of PP5 by interacting with the PP5 phosphatase domain. In contrast, the basal activity of PP5 was slightly but significantly increased with KLHDC10 ΔE2 (Figure 5E), which might be attributed to the basal binding of KLHDC10 to the phosphatase domain of PP5, interrupting the TPR domain-dependent autoinhibition of PP5.

PP5 dephosphorylates the phosphothreonine (Thr838) in the activation loop of human ASK1 kinase domain, inactivating ASK1 in response to ROS (Morita et al., 2001). Thus, we examined whether KLHDC10 counteracts PP5-dependent inactivation of ASK1. Coexpression of ASK1 and PP5 decreased the phosphorylation of ASK1 Thr838, indicating that ASK1 is inactivated by PP5 (Figure 5F). Under these conditions, coexpression of KLHDC10 WT or AP inhibited the PP5-induced dephosphorylation of ASK1 (Figure 5F). KLHDC10 also counteracted the PP5-dependent inactivation of ASK1 in H_2O_2-treated cells (Figure S3D). Furthermore, knockdown of *PP5* in HEK293 cells reduced the KLHDC10-induced activation of ASK1 (Figure S3E), suggesting that PP5-dependent inactivation of ASK1 is required at least in part for the KLHDC10-induced ASK1 activation. These results indicate that KLHDC10 activates ASK1 by inhibiting PP5-dependent inactivation of ASK1.

H_2O_2-Dependent Interaction of KLHDC10 with PP5

Because ASK1 interacts with PP5 in an H_2O_2-dependent manner, we examined whether the interaction of KLHDC10 with PP5 was also affected by H_2O_2 stimulation. The interaction of coexpressed KLHDC10 WT or AP with PP5 WT increased in response to H_2O_2 (Figure 6A). TNF-α, which has been reported to induce the activation of ASK1 through ROS production and the interaction of ASK1 with PP5 (Liu et al., 2000; Morita et al., 2001), also induced the increase in binding between KLHDC10 and PP5 (Figure S4A). This binding was attenuated with pretreatment of antioxidant, N-acetylcysteine (NAC), suggesting that TNF-α increased the interaction between KLHDC10 and PP5 in an ROS-dependent manner. In contrast, the interaction of KLHDC10 WT with PP5 Phos was not altered by H_2O_2 treatment (Figure 6B). Given that KLHDC10 strongly bound to PP5 Phos rather than PP5 WT even under conditions of no oxidative stress (Figure 5C), these results imply that ROS might induce some conformational changes in PP5 that unlock the autoinhibitory structure of PP5, thereby leading to an increase in the interaction between KLHDC10 and PP5 WT. In these immunoblotting experiments, we noticed that, upon H_2O_2 stimulation, a fraction of PP5 WT bands migrating faster than the major bands of PP5 on SDS-PAGE was increased (Figures 6A and 6B, lysate). The lower bands of both exogenous and endogenous PP5 were not detected by an antibody against the C terminus of PP5 (Figures S4B and S4C), indicating that H_2O_2 induces the C-terminal cleavage of a fraction of PP5. The putative cleavage site in the C terminus of PP5 has been reported to be located between Arg425 and Ser426 (Zeke et al., 2005). Although the C-terminal cleaved form derived from HA-PP5 WT was hardly detected in coIP with KLHDC10, probably due to relatively low amount of cleaved form compared with that of full-length form (Figure 6A), we found that KLHDC10 WT bound to the coexpressed putative cleaved form mutant (PP5 Δ426-499) (Figure S4D), suggesting that KLHDC10 interacts with both full-length and the cleaved form of PP5. In addition, we detected the interaction between endogenous KLHDC10 and PP5 in Neuro2A cells (Figure 6C). The total amount of PP5 (full length and cleaved) coimmunoprecipitated with KLHDC10

(D) HEK293 cells were transiently transfected with the indicated constructs. After 24 hr, cells were treated with 0.5 μM MG132 to enhance transfected protein expression. After an additional 14 hr of culturing, cells were lysed and subjected to IP with anti-Flag antibody followed by IB.
(E) GST-tagged recombinant PP5 and KLHDC10 ΔE2 were purified from *E. coli*. GST-PP5 alone or in combination with GST-KLHDC ΔE2, incubated with or without 400 μM AA at 30°C for 20 min, was subjected to an in vitro phosphatase assay using a phosphothreonine peptide as a substrate. The results shown are the means of three independent experiments. Error bars indicate SEM (*$p < 0.02$, **$p < 0.01$, Student's t test).
(F) HEK293 cells were transiently transfected with the indicated constructs. After 24 hr, the cells were lysed and subjected to IB. The asterisks in (A) and (C) indicate the bands derived from immunoglobulin heavy chain.

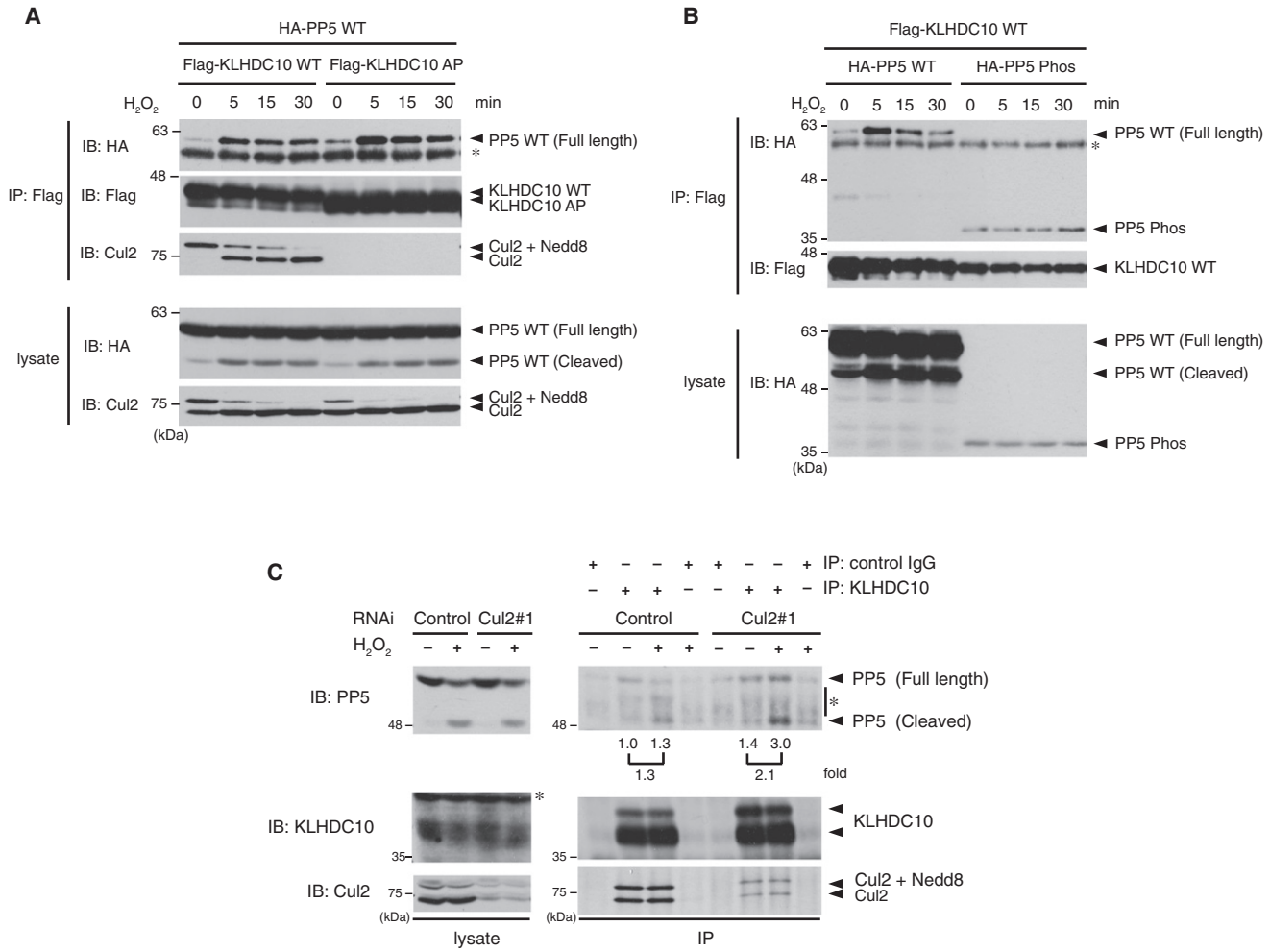

Figure 6. H_2O_2-Dependent Interaction of KLHDC10 with PP5

(A and B) HEK293 cells were transiently transfected with the indicated constructs. After 48 hr, the cells were treated with 5 mM H_2O_2 for the indicated periods and then lysed and subjected to immunoprecipitation (IP) with anti-Flag antibody followed by immunoblotting (IB).

(C) Neuro2A cells were transfected with a negative control siRNA or an siRNA that targets *Cul2*. After 48 hr, the cells were treated with 1 mM H_2O_2 for 1 hr and then lysed and subjected to IP with control IgG or KLHDC10 antibody followed by IB. The asterisk indicates the bands derived from immunoglobulin heavy chain or nonspecific bands. The total amount of PP5 (full length and cleaved) coimunoprecipitated with KLHDC10 was quantified and was shown as the fold changes.

was increased in response to H_2O_2. The interaction was also enhanced by knockdown of *Cullin2*, presumably reflecting the result that PP5 was isolated only from the pull-down of Cul2-unbound mutants of KLHDC10 (Figure 6C and Table S1).

KLHDC10 Is Required for H_2O_2-Induced Sustained Activation of ASK1 and Cell Death

Because the interaction of KLHDC10 with PP5 was induced by H_2O_2, we next examined the requirement for KLHDC10 in H_2O_2-induced ASK1 activation. Knockdown of *KLHDC10* in Neuro2A cells resulted in a decrease in H_2O_2-induced activation of ASK1, which was more remarkable in the late time course (60–120 min) than in the early time course (5–15 min) after H_2O_2 stimulation (Figure 7A and Figure S5A). H_2O_2-induced p38 activation was also decreased in *KLHDC10* knockdown cells. These results suggest that KLHDC10 is required for ROS-induced sustained activation but not for early (initial) activation of ASK1. Because the ASK1-p38 pathway is known to be crucial for ROS-induced cell death (Noguchi et al., 2008), we investigated the effect of knockdown of *KLHDC10* on H_2O_2-induced cell death in Neuro2A cells. LDH assay revealed that H_2O_2-induced cell death was significantly suppressed by knockdown of *KLHDC10* (Figure 7B), suggesting that KLHDC10 is required for ROS-induced cell death. Furthermore, to address the requirement of PP5 for KLHDC10-induced ASK1 activation and cell death, we examined the effect of *PP5* knockdown on H_2O_2-induced ASK1 activation in *KLHDC10* knockdown cells, in which ASK1 activation was supposed to be inhibited by "active" PP5. Knockdown of *PP5* restored the activation of ASK1 in H_2O_2-treated *KLHDC10* knockdown cells to the level of that in control knockdown cells (Figure 7C, Figures S5B and S5C), suggesting that suppression of ASK1 activation in *KLHDC10* knockdown cells is mostly dependent on PP5. H_2O_2-induced cell death was also restored by *KLHDC10* and

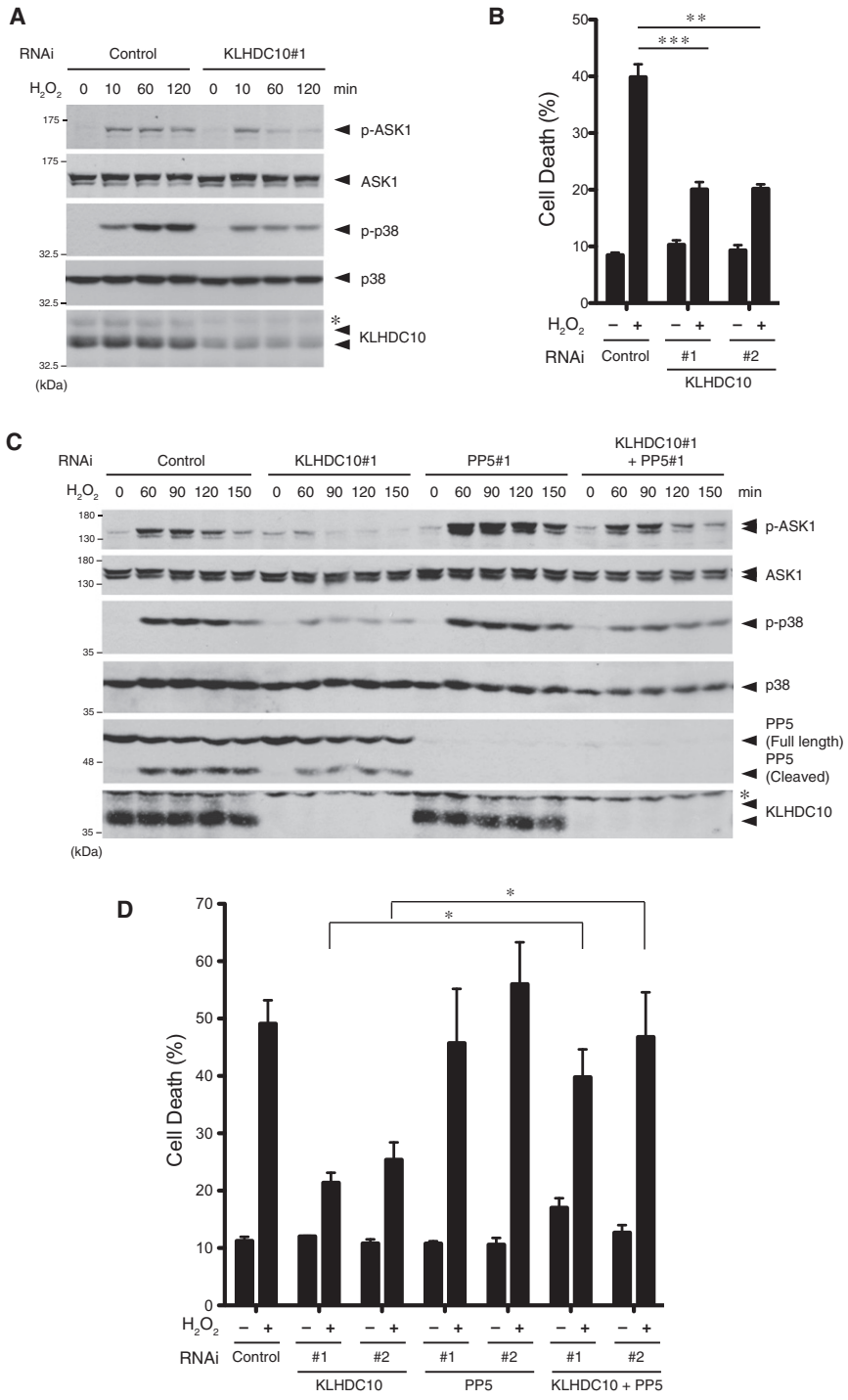

Figure 7. KLHDC10 Is Required for H_2O_2-Induced Activation of ASK1 and Cell Death

(A and B) Neuro2A cells were transfected with a negative control siRNA or an siRNA that targets *KLHDC10*. After 48 hr, the cells were treated with 0.5 mM H_2O_2 for the indicated periods, then lysed and subjected to immunoblotting (IB) with the indicated antibodies (A), or after 43 hr, the cells were treated with 1 mM H_2O_2 for 5 hr and then analyzed with an LDH assay (B). The results shown are the means of three independent experiments. Error bars indicate SEM (**p < 0.003, ***p < 0.007, Student's t test).

(C and D) Neuro2A cells were transfected with the indicated siRNAs. After 48 hr, the cells were harvested and replated. After 24 hr, the indicated siRNAs were transfected again. After 48 hr, the cells were treated with 0.5 mM H_2O_2 for the indicated periods and then lysed and subjected to IB (C), or after 43 hr, the cells were treated with 1 mM H_2O_2 for 5 hr and then analyzed with an LDH assay (D). The results shown are the means of at least five independent experiments. Error bars indicate SEM (*p < 0.05, Student's t test). The asterisk indicates nonspecific bands.

PP5 double knockdown (Figure 7D). These results strongly suggest that suppression of PP5 is crucial for the KLHDC10-induced ASK1 activation and cell death.

DISCUSSION

Recent reports have shed light on the role of the kelch repeat proteins as the substrate recognition subunits of the CRL complex (Bennett et al., 2010). We also confirmed the interaction of Slim/KLHDC10 with the components of the CRL2 complex through the BC box and Cul2 box sequences (Figures 4C and 4D), suggesting that Slim/KLHDC10 functions as one of the substrate recognition subunits of the CRL2 complex. However, interestingly, the function of Slim/KLHDC10 as the ASK1 activator appeared to be independent of the interactions of the CRL2 complex (Figures 4E and 4F). This finding suggests that Slim/KLHDC10 possesses at least two functions, one as a substrate recognition subunit that targets unidentified substrates for ubiquitination, and another as a signaling regulator through its interaction with PP5. A tumor suppressor gene product, the von Hippel-Lindau protein (pVHL), is one of the most characterized substrate recognition subunits of the CRL2 complex. It has been shown that pVHL binds to a transcription factor, hypoxia-inducible factor-1α (HIF-1α), in an oxygen concentration-dependent manner, allowing the CRL2-pVHL complex to mediate ubiquitination and degradation of HIF-1α (Kaelin, 2008). Moreover, pVHL is also reported to play a CRL2 complex-independent role via its interaction with various molecules, such as fibronectin, Collagen IV, and kinesin-2 (Frew and Krek, 2008). These findings suggest that pVHL is a multifunctional protein and that its function depends on its binding partners. Our findings also suggest that KLHDC10 might

possess multiple functions through the interaction with other binding molecules identified by the pull-down screen in this study (Table S1).

We demonstrated that KLHDC10 is an inhibitor of PP5. Given the importance of the balance between kinases and phosphatases in various intracellular signaling systems, it is indisputable that not only kinases but also phosphatases need to be tightly regulated by their regulatory molecules. It has been shown that PP5 is activated by heat shock protein 90 (HSP90) through an interaction of the C-terminal region of HSP90 and the TPR domain of PP5, and this interaction disrupts the autoinhibitory structure of PP5 (Yang et al., 2005). The HSP90-PP5 complex targets HSP90-associated proteins, such as the glucocorticoid receptor and heme-regulated eIF2α kinase, for dephosphorylation, thereby regulating their molecular functions (Golden et al., 2008). The G_{12} class of G protein members, $G\alpha_{12}$ and $G\alpha_{13}$, has also been shown to activate PP5 catalytic activity by associating with the TPR domain of PP5 (Yamaguchi et al., 2002). In contrast, although PP5 exhibits weak phosphatase activity, owing to its autoinhibitory structure, little is known about the negative regulatory mechanism of PP5 by inhibitory proteins. We revealed that KLHDC10 interacted with the PP5 Phos and that this interaction was suppressed by the TPR domain of PP5 (Figures 5C and 5D). Because the TPR domain of PP5 has been shown to directly associate with the catalytic site of the phosphatase domain (Yang et al., 2005), these findings suggest that KLHDC10 inhibits PP5 phosphatase activity by interacting with the catalytic site of PP5. Consistent with this notion, KLHDC10 suppressed the AA-activated phosphatase activity but not the basal activity of PP5 (Figure 5E). Because AA has been shown to induce the activation of PP5 by disrupting the autoinhibitory structure of PP5 through direct interaction with the TPR domain, this suggests that AA-induced conformational change of PP5 that opens up the catalytic site of PP5 triggers the KLHDC10-PP5 interaction to inhibit the catalytic activity of PP5.

H_2O_2 increased the interaction of KLHDC10 with PP5 WT, whereas KLHDC10 constitutively bound to PP5 Phos regardless of the presence of H_2O_2 (Figures 6A and 6B). In addition, H_2O_2 induced the C-terminal cleavage of a fraction of PP5 (Figure 6 and Figure S4C). The three-dimensional structure of PP5 has revealed that together with the TPR domain, the α helix structure at the C-terminal of PP5 is also involved in its autoinhibitory structure (Yang et al., 2005). Thus, the cleavage of the C terminus of PP5 indicates that the catalytic site of the phosphatase domain of PP5 is uncovered upon H_2O_2, which is consistent with the finding that KLHDC10 preferentially interacted with the cleaved form of PP5 (Figure 6C and Figure S4D). Although coexpression of KLHDC10 did not affect the cleavage of PP5 (Figure S4B), it would be interesting in the future to examine the effect of C-terminal cleavage on the phosphatase activity of PP5 by using deletion constructs. However, because KLHDC10 bound even to the full-length form of PP5 in an H_2O_2-dependent manner, the cleavage of PP5 itself seems to be dispensable for the H_2O_2-dependent interaction of KLHDC10 with PP5 (Figure 5 and Figure 6). Nevertheless, in response to H_2O_2, certain conformational change in PP5 that precedes the C-terminal cleavage might induce the open-up of PP5 phosphatase domain and eventually the interaction of KLHDC10 with PP5.

Knockdown of *KLHDC10* in Neuro2A cells resulted in a decrease in H_2O_2-dependent sustained activation of ASK1 and cell death (Figure 7). We have recently identified the deubiquitinating enzyme ubiquitin-specific peptidase 9, X-linked (USP9X) as an H_2O_2-dependent binding molecule of ASK1 (Nagai et al., 2009). USP9X interacts with ASK1 via its ubiquitin-like sequence (LRLRGG), which is identical to the ubiquitin C terminus. USP9X binds within the C-terminal region of ASK1 and removes the ubiquitin from the activated ASK1, thereby leading to stabilization of the activated ASK1. USP9X is also required for oxidative stress-induced cell death. These findings and the findings in this study raise the possibility that USP9X and KLHDC10 may coordinately regulate the strength and/or duration of ASK1 activation by counteracting both ubiquitination of ASK1 by ubiquitin ligases and dephosphorylation of ASK1 by PP5, leading to sustained activation of ASK1 and ultimately cell death. Because ROS-activated ASK1 has been known to mediate not only cell death but also various cellular responses including inflammatory responses and cardiac hypertrophy in a stimulus- and cellular context-dependent manner (Iriyama et al., 2009; Izumiya et al., 2003; Matsuzawa et al., 2005; Noguchi et al., 2008), such fine-tuning of the magnitude of ASK1 activation by USP9X and KLHDC10 seems to be a crucial determinant of ASK1-mediated cellular responses to oxidative stress. Further analyses of physiological and/or pathological contexts in which ROS-dependent KLHDC10-PP5 interaction regulates the activation of ASK1 will provide new insight into ROS- and ASK1-mediated stress responses and human diseases.

EXPERIMENTAL PROCEDURES

Additional information regarding plasmids, antibodies, cell culture, RNA interference, quantitative RT-PCR, immunostaining, and mass spectrometry analysis can be found in the Supplemental Experimental Procedures.

Fly Stocks and Generation of Transgenic Flies

Flies were raised on standard *Drosophila* medium at 25°C. The GS strains were provided by the *Drosophila* Genetic Research Center at Kyoto Institute of Technology. The following strains were used in this study: *UAS-DASK1ΔN*, *UAS-DASK1-IR* (Sekine et al., 2011), *UAS-Dp38a-DN* (Adachi-Yamada et al., 1999), *UAS-lic-IR* (Vienna *Drosophila* RNAi Center), *pnr-GAL4* (Calleja et al., 2000), *UAS-GFP^{S65T}*, and *hs-GAL4* (Bloomington *Drosophila* Stock Center). The transgenic strains harboring *UAS-DASK1WT* (Kuranaga et al., 2002) and *UAS-slim-IR* were generated through standard P element-mediated transformation (BestGene Inc.).

Immunoblotting Analysis

Cells were lysed in IP lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 10 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride [PMSF] in addition to 150 units/ml of aprotinin or 5 mg/ml leupeptin) supplemented with PhosSTOP (Roche). Flies were lysed with RIPA buffer (1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM PMSF, and 5 μg/ml leupeptin). The cell and fly extracts were clarified by centrifugation, and the supernatants were added to the same volume of SDS sample buffer (40 mM Tris-HCl [pH 8.8], 80 μg/ml bromophenol blue, 28.8% glycerol, 4% SDS, and 20 mM DTT with or without 5% 2-mercaptoethanol), boiled at 98°C for 5 min, resolved on SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and electroblotted onto polyvinylidene difluoride (PVDF) membranes. After blocking with 5% skim milk in TBS-T (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, and 0.05% Tween 20), the

membranes were probed with the indicated antibodies. The antibody-antigen complexes were detected using the ECL system (GE Healthcare). The optical density of bands was quantified using NIH ImageJ software. All immunoblotting data are representative of at least three independent experiments.

Coimmunoprecipitation Analysis

Cells were lysed in IP lysis buffer. After centrifugation, the supernatants were immunoprecipitated with Flag antibody (anti-Flag affinity M2 gel, Sigma). The beads were extensively washed with IP lysis buffer before immunoblotting analysis. For IP of endogenous KLHDC10, Neuro2A cell lysates were incubated with control rat IgG (Santa Cruz) or KLHDC10 antibody (clone S-20, Supplemental Experimental Procedures) and immunoprecipitated with protein G Sepharose (GE Healthcare).

In Vitro Phosphatase Assay

The GST-PP5 and GST-KLHDC10 ΔE2 proteins were purified from *E. coli* (BL21) as described previously (Saitoh et al., 1998). GST-PP5 (0.4 μg) alone or in combination with GST-KLHDC10 ΔE2 (5 μg) in 50 μl reaction mixture (approximately 0.1 and 1.4 μM, respectively) was incubated with 25 μM phospho-threonine peptide (RRApTVA, Promega) at 30°C for 20 min with or without 400 μM AA. The phosphatase activity of PP5 was measured using the Serine/Threonine Phosphatase Assay System (Promega), which determined the absorbance of a molybdate:malachite:phosphate complex.

LDH Assay

H_2O_2-induced cell death was monitored using the LDH-Cytotoxic Test Wako (Wako) according to the manufacturer's protocols. The released LDH activity into the culture media was quantified as a percentage of the total LDH activity.

Statistical Analysis

Statistical analyses were performed using Student's t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, one table, Supplemental Experimental Procedures, and Supplemental References and can be found with this article at http://dx.doi.org/10.1016/j.molcel.2012.09.018.

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REFERENCES

Adachi-Yamada, T., Nakamura, M., Irie, K., Tomoyasu, Y., Sano, Y., Mori, E., Goto, S., Ueno, N., Nishida, Y., and Matsumoto, K. (1999). p38 mitogen-activated protein kinase can be involved in transforming growth factor beta superfamily signal transduction in Drosophila wing morphogenesis. Mol. Cell. Biol. *19*, 2322–2329.

Adams, J., Kelso, R., and Cooley, L. (2000). The kelch repeat superfamily of proteins: propellers of cell function. Trends Cell Biol. *10*, 17–24.

Bennett, E.J., Rush, J., Gygi, S.P., and Harper, J.W. (2010). Dynamics of cullin-RING ubiquitin ligase network revealed by systematic quantitative proteomics. Cell *143*, 951–965.

Borthwick, E.B., Zeke, T., Prescott, A.R., and Cohen, P.T. (2001). Nuclear localization of protein phosphatase 5 is dependent on the carboxy-terminal region. FEBS Lett. *491*, 279–284.

Bosu, D.R., and Kipreos, E.T. (2008). Cullin-RING ubiquitin ligases: global regulation and activation cycles. Cell Div. *3*, 7.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401–415.

Calleja, M., Herranz, H., Estella, C., Casal, J., Lawrence, P., Simpson, P., and Morata, G. (2000). Generation of medial and lateral dorsal body domains by the pannier gene of Drosophila. Development *127*, 3971–3980.

Chen, M.X., and Cohen, P.T. (1997). Activation of protein phosphatase 5 by limited proteolysis or the binding of polyunsaturated fatty acids to the TPR domain. FEBS Lett. *400*, 136–140.

Finkel, T., and Holbrook, N.J. (2000). Oxidants, oxidative stress and the biology of ageing. Nature *408*, 239–247.

Frew, I.J., and Krek, W. (2008). pVHL: a multipurpose adaptor protein. Sci. Signal. *1*, pe30. http://dx.doi.org/10.1126/scisignal.124pe30.

Fujino, G., Noguchi, T., Matsuzawa, A., Yamauchi, S., Saitoh, M., Takeda, K., and Ichijo, H. (2007). Thioredoxin and TRAF family proteins regulate reactive oxygen species-dependent activation of ASK1 through reciprocal modulation of the N-terminal homophilic interaction of ASK1. Mol. Cell. Biol. *27*, 8152–8163.

Golden, T., Swingle, M., and Honkanen, R.E. (2008). The role of serine/threonine protein phosphatase type 5 (PP5) in the regulation of stress-induced signaling networks and cancer. Cancer Metastasis Rev. *27*, 169–178.

Hinds, T.D., Jr., and Sánchez, E.R. (2008). Protein phosphatase 5. Int. J. Biochem. Cell Biol. *40*, 2358–2362.

Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K., and Gotoh, Y. (1997). Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science *275*, 90–94.

Iriyama, T., Takeda, K., Nakamura, H., Morimoto, Y., Kuroiwa, T., Mizukami, J., Umeda, T., Noguchi, T., Naguro, I., Nishitoh, H., et al. (2009). ASK1 and ASK2 differentially regulate the counteracting roles of apoptosis and inflammation in tumorigenesis. EMBO J. *28*, 843–853.

Izumiya, Y., Kim, S., Izumi, Y., Yoshida, K., Yoshiyama, M., Matsuzawa, A., Ichijo, H., and Iwao, H. (2003). Apoptosis signal-regulating kinase 1 plays a pivotal role in angiotensin II-induced cardiac hypertrophy and remodeling. Circ. Res. *93*, 874–883.

Jubelin, G., Taieb, F., Duda, D.M., Hsu, Y., Samba-Louaka, A., Nobe, R., Penary, M., Watrin, C., Nougayrède, J.P., Schulman, B.A., et al. (2010). Pathogenic bacteria target NEDD8-conjugated cullins to hijack host-cell signaling pathways. PLoS Pathog. *6*, e1001128. http://dx.doi.org/10.1371/journal.ppat.1001128.

Kaelin, W.G., Jr. (2008). The von Hippel-Lindau tumour suppressor protein: O2 sensing and cancer. Nat. Rev. Cancer *8*, 865–873.

Kamura, T., Brower, C.S., Conaway, R.C., and Conaway, J.W. (2002). A molecular basis for stabilization of the von Hippel-Lindau (VHL) tumor suppressor protein by components of the VHL ubiquitin ligase. J. Biol. Chem. *277*, 30388–30393.

Kang, H., Sayner, S.L., Gross, K.L., Russell, L.C., and Chinkers, M. (2001). Identification of amino acids in the tetratricopeptide repeat and C-terminal domains of protein phosphatase 5 involved in autoinhibition and lipid activation. Biochemistry *40*, 10485–10490.

Kuranaga, E., Kanuka, H., Igaki, T., Sawamoto, K., Ichijo, H., Okano, H., and Miura, M. (2002). Reaper-mediated inhibition of DIAP1-induced DTRAF1

degradation results in activation of JNK in Drosophila. Nat. Cell Biol. *4*, 705–710.

Kyriakis, J.M., and Avruch, J. (2001). Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol. Rev. *81*, 807–869.

Liu, H., Nishitoh, H., Ichijo, H., and Kyriakis, J.M. (2000). Activation of apoptosis signal-regulating kinase 1 (ASK1) by tumor necrosis factor receptor-associated factor 2 requires prior dissociation of the ASK1 inhibitor thioredoxin. Mol. Cell. Biol. *20*, 2198–2208.

Mahrour, N., Redwine, W.B., Florens, L., Swanson, S.K., Martin-Brown, S., Bradford, W.D., Staehling-Hampton, K., Washburn, M.P., Conaway, R.C., and Conaway, J.W. (2008). Characterization of Cullin-box sequences that direct recruitment of Cul2-Rbx1 and Cul5-Rbx2 modules to Elongin BC-based ubiquitin ligases. J. Biol. Chem. *283*, 8005–8013.

Matsuzawa, A., Saegusa, K., Noguchi, T., Sadamitsu, C., Nishitoh, H., Nagai, S., Koyasu, S., Matsumoto, K., Takeda, K., and Ichijo, H. (2005). ROS-dependent activation of the TRAF6-ASK1-p38 pathway is selectively required for TLR4-mediated innate immunity. Nat. Immunol. *6*, 587–592.

Morita, K., Saitoh, M., Tobiume, K., Matsuura, H., Enomoto, S., Nishitoh, H., and Ichijo, H. (2001). Negative feedback regulation of ASK1 by protein phosphatase 5 (PP5) in response to oxidative stress. EMBO J. *20*, 6028–6036.

Nagai, H., Noguchi, T., Takeda, K., and Ichijo, H. (2007). Pathophysiological roles of ASK1-MAP kinase signaling pathways. J. Biochem. Mol. Biol. *40*, 1–6.

Nagai, H., Noguchi, T., Homma, K., Katagiri, K., Takeda, K., Matsuzawa, A., and Ichijo, H. (2009). Ubiquitin-like sequence in ASK1 plays critical roles in the recognition and stabilization by USP9X and oxidative stress-induced cell death. Mol. Cell *36*, 805–818.

Nishitoh, H., Saitoh, M., Mochida, Y., Takeda, K., Nakano, H., Rothe, M., Miyazono, K., and Ichijo, H. (1998). ASK1 is essential for JNK/SAPK activation by TRAF2. Mol. Cell *2*, 389–395.

Noguchi, T., Takeda, K., Matsuzawa, A., Saegusa, K., Nakano, H., Gohda, J., Inoue, J., and Ichijo, H. (2005). Recruitment of tumor necrosis factor receptor-associated factor family proteins to apoptosis signal-regulating kinase 1 signalosome is essential for oxidative stress-induced cell death. J. Biol. Chem. *280*, 37033–37040.

Noguchi, T., Ishii, K., Fukutomi, H., Naguro, I., Matsuzawa, A., Takeda, K., and Ichijo, H. (2008). Requirement of reactive oxygen species-dependent activation of ASK1-p38 MAPK pathway for extracellular ATP-induced apoptosis in macrophage. J. Biol. Chem. *283*, 7657–7665.

Petroski, M.D., and Deshaies, R.J. (2005). Function and regulation of cullin-RING ubiquitin ligases. Nat. Rev. Mol. Cell Biol. *6*, 9–20.

Prag, S., and Adams, J.C. (2003). Molecular phylogeny of the kelch-repeat superfamily reveals an expansion of BTB/kelch proteins in animals. BMC Bioinformatics *4*, 42.

Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998). Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. EMBO J. *17*, 2596–2606.

Sekine, Y., Takagahara, S., Hatanaka, R., Watanabe, T., Oguchi, H., Noguchi, T., Naguro, I., Kobayashi, K., Tsunoda, M., Funatsu, T., et al. (2011). p38 MAPKs regulate the expression of genes in the dopamine synthesis pathway through phosphorylation of NR4A nuclear receptors. J. Cell Sci. *124*, 3006–3016.

Skinner, J., Sinclair, C., Romeo, C., Armstrong, D., Charbonneau, H., and Rossie, S. (1997). Purification of a fatty acid-stimulated protein-serine/threonine phosphatase from bovine brain and its identification as a homolog of protein phosphatase 5. J. Biol. Chem. *272*, 22464–22471.

Takeda, K., Noguchi, T., Naguro, I., and Ichijo, H. (2008). Apoptosis signal-regulating kinase 1 in stress and immune response. Annu. Rev. Pharmacol. Toxicol. *48*, 199–225.

Toba, G., Ohsako, T., Miyata, N., Ohtsuka, T., Seong, K.H., and Aigaki, T. (1999). The gene search system. A method for efficient detection and rapid molecular identification of genes in Drosophila melanogaster. Genetics *151*, 725–737.

Tobiume, K., Matsuzawa, A., Takahashi, T., Nishitoh, H., Morita, K., Takeda, K., Minowa, O., Miyazono, K., Noda, T., and Ichijo, H. (2001). ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. EMBO Rep. *2*, 222–228.

Tobiume, K., Saitoh, M., and Ichijo, H. (2002). Activation of apoptosis signal-regulating kinase 1 by the stress-induced activating phosphorylation of pre-formed oligomer. J. Cell. Physiol. *191*, 95–104.

Widmann, C., Gibson, S., Jarpe, M.B., and Johnson, G.L. (1999). Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol. Rev. *79*, 143–180.

Yamaguchi, Y., Katoh, H., Mori, K., and Negishi, M. (2002). Galpha(12) and Galpha(13) interact with Ser/Thr protein phosphatase type 5 and stimulate its phosphatase activity. Curr. Biol. *12*, 1353–1358.

Yang, J., Roe, S.M., Cliff, M.J., Williams, M.A., Ladbury, J.E., Cohen, P.T., and Barford, D. (2005). Molecular basis for TPR domain-mediated regulation of protein phosphatase 5. EMBO J. *24*, 1–10.

Zeke, T., Morrice, N., Vázquez-Martin, C., and Cohen, P.T. (2005). Human protein phosphatase 5 dissociates from heat-shock proteins and is proteolytically activated in response to arachidonic acid and the microtubule-depolymerizing drug nocodazole. Biochem. J. *385*, 45–56.