Ubiquitin-dependent regulation of MEKK2/3-MEK5-ERK5 signaling module by XIAP and cIAP1

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Abstract

Mitogen-activated protein kinases (MAPKs) are highly conserved protein kinase modules, and they control fundamental cellular processes. While the activation of MAPKs has been well studied, little is known on the mechanisms driving their inactivation. Here we uncover a role for ubiquitination in the inactivation of a MAPK module. Extracellular-signal-regulated kinase 5 (ERK5) is a unique, conserved member of the MAPK family and is activated in response to various stimuli through a three-tier cascade constituting MEK5 and MEKK2/3. We reveal an unexpected role for Inhibitors of Apoptosis Proteins (IAPs) in the inactivation of ERK5 pathway in a bimodal manner involving direct interaction and ubiquitination. XIAP directly interacts with MEK2/3 and competes with PB1 domain-mediated binding to MEK5. XIAP and cIAP1 conjugate predominantly K63-linked ubiquitin chains to MEK2 and MEK3 which directly impede MEK5–ERK5 interaction in a trimeric complex leading to ERK5 inactivation. Consistently, loss of XIAP or cIAP1 by various strategies leads to hyperactivation of ERK5 in normal and tumorigenic cells. Loss of XIAP promotes differentiation of human primary skeletal myoblasts to myocytes in a MEKK2/3-MEK5-dependent manner. Our results reveal a novel, obligatory role for IAPs and ubiquitination in the physical and functional disassembly of ERK5–MAPK module and human muscle cell differentiation.

Keywords ERK5, IAP, MAPK, myoblasts, ubiquitin

Subject Categories Development & Differentiation; Post-translational Modifications; Proteolysis & Proteomics; Signal Transduction

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Introduction

Mitogen-activated protein kinases (MAPKs) are highly conserved, ubiquitously expressed enzymes that are activated in response to a plethora of extracellular stimuli to regulate a variety of cellular processes (Chang & Karin, 2001; Coulombe & Meloche, 2007; Raman et al., 2007). Typically, MAPKs are activated by dual phosphorylation in the Thr-X-Tyr motif in their activation loop by a MAPKK. MAPKKs, on the other hand, are activated by phosphorylation of Ser-Thr residues by upstream MAPKKKs, thus constituting a three-tier kinase cascade (Zhang & Dong, 2007). RAF-MEK1/2-ERK1/2 is the first discovered, well-studied classical MAPK cascade. In mammalian cells, four distinct classical MAPK cascades are recognized: ERK1/2, JNK1/2/3, p38 MAPK α/β/γ/δ, and the ERK5 pathways (Chang & Karin, 2001). ERK5, also called the Big MAPK 1 (BMK1), was cloned by two groups in 1995. It is a unique member of this family as it is double the size of ERK1/2 with a kinase domain in the N-terminus and a long C-terminus (Nithianandarajah-Jones et al., 2012). The N-terminal kinase domain is 66% identical to the kinase domain of ERK2, and the C-terminus encompasses a nuclear localization signal (NLS), two proline-rich domains, a myocyte enhancer factor 2 (MEF2)-interacting region, and a transcriptional activation domain (Nithianandarajah-Jones et al., 2012). MEK5 was identified as an upstream MAPKK through yeast two-hybrid analysis, phosphorylating the activation loop of ERK5 at Thr218 and Tyr220 leading to its activation (Zhou et al., 1995). Activated ERK5 undergoes autophosphorylation at numerous residues in its C-terminus, which causes conformational change so that the NLS is exposed, facilitating its nuclear translocation (Mody et al., 2003; Nithianandarajah-Jones et al., 2012). MEK2 and MEK3 kinase domains are 90% identical, and they form heteromers with MEK5 through their PB1 (Phox, Bemp1) domains, an evolutionarily conserved protein–protein interaction motif (Seyfried et al., 2005; Moscat et al., 2006; Sumimoto et al., 2007). MEK2/3 and MEK5 interact through their PB1 domains in a front-to-back orientation where the basic residues in the front end of MEK2/3 PB1 domain mediate electrostatic interactions with the acidic residues in the
back end of MEK5 PB1 domain (Nakamura & Johnson, 2003, 2007). ERK5, though lacks the PB1 domain, interacts through its N-terminus (a.a. 78–139) with the PB1 domain and the following C-terminal stretch (a.a. 98–131) of MEK5 (Nithianandarajah-Jones et al., 2012). Like many MAPKs, ERK5 possesses a common docking domain (a.a. 350–358) with negatively charged amino acids, with which it interacts with its substrates and MEK5 consequently maintaining the signaling fidelity and efficiency (Tanoue & Nishida, 2002). The ERK5 cascade is activated in response to various growth factors (EGF, PDGF, VEGF, FGF-2, NGF, and BDNF), cytokines (like IL-6), and other stimuli like oxidative stress and ischemia. Gene knockout studies have revealed a crucial role of this cascade in the development of cardiovascular system (Wang & Tournier, 2006). There is mounting evidence for the role of ERK5/MAPK pathway in regulating cancer as ERK5 amplifications (17p11) are detected in hepatocellular carcinoma and high MEK5 and ERK5 levels are associated with bone and lymph node metastases in prostate cancer and oral squamous cell carcinoma, respectively (Lochhead et al., 2012). Despite the physiological and pathophysiological importance of the ERK5 signaling cascade, relatively less is known on the upstream signaling machinery as well as the factors contributing to the activation/inactivation of this pathway. Here we identify Inhibitors of Apoptosis Proteins (IAPs) as negative regulators of ERK5 activation.

IAPs are a class of evolutionally conserved, multifunctional proteins (Srinivasula & Ashwell, 2008). In mammals, eight IAPs exist distinguished by the presence of their signature BIR (Baculoviral IAP Repeat) domain, a conserved protein–protein interaction motif. Many of them possess a RING domain with E3 ubiquitin ligase activity (Srinivasula & Ashwell, 2008). X-linked inhibitor of apoptosis protein (XIAP, also called BIRC4) is considered as a legitimate inhibitor of caspases, and cellular IAPs (cIAPs) were shown to control both canonical and non-canonical pathways of NF-xB activation (O’Riordan et al., 2008). Apart from their well-established role in apoptosis, inflammation and innate immunity, emerging evidence revealed a role for IAPs in the regulation of cell shape, cell migration, and tumor metastases (Gyrd-Hansen & Meier, 2010). XIAP and cIAPs regulate classical MAPK activation as they can promote the proteasomal degradation of C-RAF through the Hsp90 protein quality-control machinery (Dogan et al., 2008). Further, XIAP and cIAP1 can function as the direct E3 ubiquitin ligase of RhoGTPase Rac1, thus controlling cell shape and migration (Oberoi et al., 2011). Here we uncover that XIAP and cIAP1 function as negative regulators of ERK5 signaling cascade by physically interacting with and ubiquitinating MEK2 and MEK3. As a consequence, loss of XIAP enhances ERK5 activation and MEF2C activity. This in turn promotes human myoblast differentiation in a MEK2/3-ERK5-dependent manner. Our results reveal a thus far unknown role of IAPs and ubiquitination in the functional disassembly of a classical MAPK module and human myocyte generation.

Results

Loss of IAPs leads to hyperactivation of ERK5 in normal and tumor cells

We have recently shown that XIAP and cIAPs can modulate ERK1/2 activation by directly regulating the stability of C-RAF kinase (Dogan et al., 2008). To test whether XIAP can influence other related classical MAPKs, we checked for the activation of ERK5 (pERK5) in IAP-depleted cells. Interestingly, depletion of XIAP with different siRNAs led to a striking increase in the phosphorylation of ERK5 at steady state in HeLa cells (Fig 1A and B). Loss of XIAP did not lead to any significant alterations in the protein levels of ERK5 (Fig 1A and B). Similarly, depletion of cIAP1 but not cIAP2 led to an increase in ERK5 phosphorylation in these cells (Supplementary Fig S1A and B). These effects were evident in other tumor cell lines as well as in human primary tracheal epithelial cells (Supplementary Fig S1C and D and data not shown). To exclude any potential off target effects, we performed complementation experiments. Expression of XIAP in trans-reduced pERK5 levels in HeLa cells transfected with 3’UTR-XIAP siRNA (Supplementary Fig S1E), thus confirming the specificity of the observed phenotype. To corroborate these observations, we checked for MEF2 transcriptional activity in HeLa cells stably expressing MEF2 luciferase reporter constructs. Consistent with the previous observations, depletion of XIAP led to a significant increase in MEF2 activity in an ERK5-dependent manner (Fig 1C). Further, we checked for the kinetics of ERK5 activation in HeLa cells and MEFs. Loss of XIAP increased ERK5 activation in HeLa cells and MEFs in response to FCS and FGF-2 stimulation, respectively (Fig 1B and D). This suggests that loss of XIAP influences the intensity of ERK5 activation. As XIAP loss can lead to stabilization of C-RAF and Rac1 protein levels, we checked for their possible role in the activation of ERK5. Co-knockdown experiments in HeLa cells revealed that depletion of XIAP led to an increase in pERK5 levels despite the absence of C-RAF and Rac1 (Supplementary Fig S1F–H). As MEK2/3 and MEK5 are the cognate upstream MAPK members, we checked for their role in activating ERK5 in XIAP-depleted cells. As expected, co-knockdown of MEK2, MEK3, or MEK5 prevented activation of ERK5 in XIAP-depleted cells (Fig 1E–G). These results confirmed that XIAP and cIAP1 negatively regulate ERK5 activation. Further, depletion of both MEK2 and MEK3 led to reduced ERK5 activation unveiling functional non-redundancy between these two MAP3Ks in activating MEK5–ERK5 pathway (Fig 1E and F).

XIAP directly binds to MEK2/3 and competes with MEK5 for PB1-mediated interaction

As XIAP has been shown to interact with MEK2 (Winsauer et al., 2008), we checked whether this is a direct interaction. In vitro interaction experiments with purified recombinant proteins revealed a direct interaction between XIAP and MEK2 or MEK3 (Fig 2A). Consistently, we could detect constitutive interaction between XIAP and MEK2 at endogenous levels in HeLa cells. However, we failed to detect MEK2 or MEK3 in XIAP immunoprecipitates (Fig 2B and data not shown). We then checked for the role of various domains of XIAP in mediating the interaction with MEK2. In vitro interaction experiments with purified proteins encompassing various domains of XIAP (Supplementary Fig S2A) revealed that the RING domain of XIAP is dispensable for binding to MEK2 and that this interaction can possibly be mediated through BIR1 and BIR2 domains (Supplementary Fig S2B). Next, we investigated the role of PB1 domain of MEK2 in mediating the interaction with XIAP,
although XIAP did not possess any PB1 domain. Interestingly, mutating the conserved basic lysine residue in the PB1 domain (K47A) severely impaired the direct interaction between XIAP and MEKK2 (Fig 2C). As XIAP could bind to the PB1 domain of MEKK2, we expected a potential competition between XIAP and MEKK2 (Fig 2C). XIAP failed to bind directly to MEK5 (Fig 2D). In vitro competition experiments with recombinant full-length proteins revealed that XIAP could directly compete with MEK5 in binding to MEKK2 (Fig 2E). Consistent with these observations, we could detect increasing amounts (∼1.5-fold) of MEK5 co-precipitating with MEK2 at endogenous levels in XIAP-depleted cells (Fig 2F and Supplementary Fig S2C). These results revealed that XIAP could directly bind to MEKK2/3 and compete with MEK5 interaction.

Figure 1. Depletion of XIAP leads to enhanced activation of the MEKK2/3-MEK5-ERK5 pathway.

A Depletion of XIAP increases the basal phosphorylation of ERK5. HeLa cells were transiently transfected with Control or XIAP siRNAs. Total lysates were analyzed by Western blotting with various antibodies.

B Depletion of XIAP enhances the strength of ERK5 activation. HeLa-MEF2 cells were transiently transfected with siControl or siXIAP or siERK5 and then stimulated with 10% FCS after serum starvation. Total lysates were analyzed by Western blotting.

C Depletion of XIAP enhances MEF2 transcriptional activity. HeLa cells stably expressing the MEF2 luciferase (luc) reporter gene were transiently transfected with siRNAs against XIAP, ERK5, or both. Cells were then lysed and the luciferase activity was measured and normalized to the Control activity as mentioned in the methods section (shown is the quantification of five independent experiments with *P < 0.05 and **P < 0.01, Student’s t-test).

D Loss of XIAP in MEFs also enhances ERK5 phosphorylation. WT, and XIAP−/− MEFs were stimulated with 25 ng/ml of FGF-2. Total lysates were analyzed by Western blotting.

E–G XIAP-mediated effect on ERK5 phosphorylation is dependent of MEKK2, MEK3, and MEK5. HeLa cells were co-transfected with siRNAs against XIAP and/or MEKK2 (E), MEK3 (F), or MEK5 (G). Total lysates were analyzed by Western blotting as indicated.

Source data are available online for this figure.
XIAP regulates ERK5 activation in a RING-dependent manner

As XIAP possesses a RING domain with E3 ubiquitin ligase activity, we tested for the role of RING domain in regulating ERK5 activation. To check for potential ubiquitination of MEKK2, we immunoprecipitated endogenous MEKK2 from control and XIAP-deficient MEFs stimulated with FGF-2. Upon FGF-2 stimulation, ERK5 phosphorylation increased and was inactivated at 15 min post-induction in control cells. Interestingly, XIAP-deficient cells exhibited pronounced ERK5 phosphorylation at 15 min post-induction (Fig 3A). Intriguingly, the MEKK2 antibody detected a smear at 15 min post-induction in control cells, which was clearly absent in the XIAP-deficient cells (Fig 3A). Furthermore, the appearance of MEKK2 smear correlates with the inactivation phase of ERK5. We then checked for direct ubiquitination of MEKK2/3 by XIAP or cIAP1. Ubiquitination experiments revealed that XIAP and cIAP1
can directly ubiquitinate MEKK2 and MEKK3 in vitro (Fig 3B, Supplementary Fig S3A–C). In addition, we have also detected autoubiquitination of the respective IAPs in these reactions (Supplementary Fig S3C). As the ubiquitin smears were detected in the absence of any proteasomal inhibitors (Fig 3A), we suspected that XIAP might conjugate non-degradative ubiquitin chains on MEKK2 and MEKK3. Recent studies revealed that several kind of ubiquitin chains (K-63, K-11, M0, K27/29, and K6) are involved in signaling and in the assemblage of protein complexes (Fulda et al., 2012). By employing linkage-specific antibodies and
ubiquitin mutants, we detected that XIAP and cIAP1 conjugate predominantly K63-linked ubiquitin chains to MEKK2 and MEKK3 both in vitro and in vivo (Fig 3C–E and Supplementary Fig S3D–F). To confirm these observations, we employed K-63 ubiquitin-specific DUB AMSH [associated molecule with the Src homology 3 domain of signal transducing adaptor molecule (STAM)] (Huang et al., 2013). As expected, AMSH treatment led to deubiquitination of MEKK2 similar to Usp2, suggesting that the chains synthesized on MEKK2 by XIAP are predominantly K-63 linked chains (Supplementary Fig S3G). Consistently, MEF2 luciferase activity induced by MEKK2 is impaired by co-expression of XIAP (Supplementary Fig S3H). Subsequently, we examined the role of ubiquitination in regulating the activation dynamics of ERK5. To pursue these experiments, we employed MEFs derived from XIAP RING knock-in mice or MEFs derived from XIAP knockout mice complemented with either wild-type or XIAP-H467A, a RING mutant of XIAP. Interestingly, the activation of ERK5 is sustained in XIAP ΔRING MEFs as loss of the RING domain of XIAP enhanced the basal as well as FGF-2-mediated activation of ERK5 (Fig 3F). Similarly, stable expression of wild-type XIAP, but not XIAP-H467A, rescued FGF-induced ERK5 overactivation in the XIAP-deficient MEFs (Fig 3G). Finally, we attempted mass spectrometry-based approach to identify the ubiquitination sites on MEKK2 and MEKK3 (Supplementary Fig S4). Interestingly, most of the ubiquitination sites identified in this screen were primarily localized in the kinase domains of MEKK2 or MEKK3. Mutation of these individual lysine residues failed to prevent direct ubiquitination of MEKK2 or MEKK3 by XIAP, suggesting that these sites are probably redundant (KR, TKO1 unpublished observations). However, transfection of MEKK2-R450R mutant increased pERK5 levels at steady state, suggesting that ubiquitination at this site is possibly involved in the activation dynamics of ERK5 (Supplementary Fig S4C).

**Ubiquitination of MEKK2 and MEKK3 does not impair their kinase activity but impedes ERK5 activation**

As IAPs directly ubiquitinate the kinase domain of MEKK2 and MEKK3, we then tested whether ubiquitination of MEKK2 or MEKK3 directly alters its kinase activity or its interaction with the downstream MAP2K, MEK5. We conducted in vitro ubiquitination-coupled phosphorylation assays to measure the activity of ubiquitin-conjugated or non-ubiquitinated MEKK2. Ubiquitination of MEKK2 did not impair the direct phosphorylation of MEK5 (Fig 4A). Similar results were obtained in in vitro kinase assays employing 32P with MEKK3 using myeline basic protein (MyBP), a common kinase substrate (Fig 4B). As XIAP-mediated ubiquitination fails to impair the kinase activity of MEKK2/3 directly, we performed in vitro reconstituted kinase assays with the entire MEKK2/3-MEK5-ERK5 kinase module components. Mutationally, inactivated ERK5, ERK5_K1 D182A, was used in all kinase experiments. Consistent with the previous observations, we detected efficient ERK5 phosphorylation with non-ubiquitinated MEKK2 or MEKK3 but not with ubiquitinated kinases, suggesting that ubiquitination of MEKK2 or MEKK3 by IAPs has a direct influence on the activation of ERK5 in a trimeric complex (Fig 4C). We employed concentrations of XIAP that will not compete with the interaction between MEKK2/3 and MEK5 in these experiments. Further, in the absence of E1 and E2 (or ubiquitination), ERK5 phosphorylation is not inhibited by the presence of XIAP (Supplementary Fig S5A). In addition, we detected that phosphorylation of ERK5 but not MEK5 is impaired in these coupled assays, suggesting that ubiquitination of MEKK2/3 has a direct effect on the activation of ERK5 (Fig 4D). Further, XIAP auto-ubiquitination fails to prevent ERK5 phosphorylation in the presence of constitutively active MEK5 (MEK5DD) and thus XIAP requires ubiquitination of MEKK2/3 for impairing ERK5 phosphorylation (Fig 4E). MEKK2/3 kinases also activate the JNK pathway by directly phosphorylating and activating MKK7. To confirm whether the observed effect of ubiquitination is specific for the ERK5 kinase module, we conducted similar experiments with purified MEKK2/3, MKK7, and JNK1. Interestingly, ubiquitination of MEKK2/3 by XIAP failed to prevent JNK1 phosphorylation in these coupled assays (Supplementary Fig S5B). These results revealed a direct, specific role for ubiquitinated MEKK2 and MEKK3 in negatively regulating the activation of ERK5 but not JNK1.

**Ubiquitination promotes MEKK2/3 dimerization but directly impairs MEK5–ERK5 interaction**

As ubiquitination is not influencing the kinase activity of MEKK2 and MEKK3, we checked for the complex formation between the members of this kinase cascade. MEKK2 and MEKK3, like many kinases, are activated by dimerization via their catalytic domain, and they form weak homodimers. Consistent with the kinase activity of ubiquitin-conjugated MEKK2, ubiquitination directly promoted homo- as well as heterodimerization of MEKK2 with...
Figure 4. XIAP-mediated MEKK2/3 ubiquitination directly impedes ERK5 activation.

A MEKK2 activity on MEK5 is not affected by ubiquitination. In vitro ubiquitination of MEKK2 was performed (lower panel) and then was immunoprecipitated out from the ubiquitination mix. Beads with non-ubiquitinated (-NU) and ubiquitinated (-(Ubi)n) MEKK2 were employed for kinase assay with recombinant MEK5 as substrate (higher panel). Western blotting with phospho-specific MEK5 antibody was used as readout for the experiment.

B MEKK3 activity on a general kinase substrate (myelin basic protein, MyBP) is not affected by ubiquitination. In vitro ubiquitination of MEKK3-MBP was performed as described in Materials and Methods and checked by Western blot (right panel). Non-ubiquitinated (-NU) and ubiquitinated (-(Ubi)n) MEKK3 was incubated with 20 μM MyBP in the presence of ATP(γ-32P) and MyBP phosphorylation was monitored by phosphorimaging (left panel).

C XIAP-mediated ubiquitination interferes with ERK5 phosphorylation in the reconstituted MEKK2-MEK5-ERK5 MAPK module (higher panel). Similar results were found in MEKK3-MEK5-ERK5 reconstitution experiments (lower panel). ERK5 phosphorylation by MEK5 was monitored by Western blots (mutationally inactivated ERK5 was used in all experiments, ERK5, KI D182A). MEKK2 and MEKK3 were ubiquitinated as described for (B) and then an in vitro kinase assay was started by adding additional 0.5 mM ATP and recombinantly expressed and purified MEK5 and ERK5 (1 μM and 5 μM, respectively).

D XIAP-mediated ubiquitination interferes with ERK5 phosphorylation in the reconstituted MEKK3-MEK5-ERK5 MAPK module without any effect on MEK5 activation. MEK5 and ERK5 phosphorylation were monitored by Western blots (mutationally inactivated ERK5 was used in all kinase assays, ERK5, KI D182A). MEKK3 was ubiquitinated and then an in vitro kinase assay was performed as described for (C).

E Ubiquitination does not interfere with MEK5-mediated phosphorylation of ERK5, as the constitutively activated form of MEK5 (MEK5DD) activates ERK5 independent of the presence of XIAP (in all assays, the following concentrations were employed: MEKK2/3, 0.2 μM, MEK5: 1 μM, ERK5: 5 μM).

Source data are available online for this figure.
MEKK3 (Fig 5A and B). We then checked whether ubiquitination impairs the interaction between MEK5 and MEKK2/3. Ubiquitination of MEK2 or MEK3 by XIAP did not impair the interaction with MEK5 (Fig 5C and D). The PB1 domain of MEK5 coordinates the interaction between the PB1 domain of MEKK2/3 and the N-terminus of ERK5 to form a kinase competent complex (Fig 5E) (Glatz et al., 2013). While the PB1-mediated interaction between MEKK2/3 and MEK5 is mediated via electrostatic interactions, ERK5 interaction with MEK5 is non-canonical as ERK5 lacks a PB1 domain (Nithianandarajah-Jones et al., 2012). We envisaged that ubiquitination of MEKK2/3 might compete with MEK5–ERK5 interaction as PB1 domain also presents an ubiquitin-like β-grasp fold (Sumimoto et al., 2007). We then repeated the assays with all three kinases and checked for the formation of the entire kinase module. As expected, ubiquitinated MEKK2/3 prevented the direct interaction between MEK5 and ERK5 in a trimeric complex (Fig 5F and G and Supplementary Fig S6A). To confirm these observations in cells, we have attempted to immunoprecipitate the entire kinase module from cells stably expressing ERK5 and MEK5. Interestingly, increasing amounts of MEK5 were co-precipitating with ERK5 in the absence of XIAP (Supplementary Fig S6B). Taken together, these data suggest that ubiquitin chains conjugated to MEK5/2/3 directly prevented the non-canonical interaction between MEK5 and ERK5, thus inactivating ERK5-MAPK module (Fig 5G). This effect is specific for ERK5 module as ubiquitination of MEKK2/3 kinases failed to prevent MKK7 or JNK1 activation under identical settings (Supplementary Fig S5B).

Loss of XIAP promotes MEF2 activity and human myoblast differentiation

As XIAP negatively regulates ERK5 activation by direct interaction and ubiquitination, we investigated the physiological significance of these observations. ERK5 regulates neuronal and muscle differentiation through the activation of MEF2 transcription factors (Dinev et al., 2001; Hindi et al., 2013). Consequently, we explored the possible role of XIAP in the regulation of human primary skeletal myoblasts (HSM) differentiation. Human primary skeletal myoblasts differentiate to myocytes in a span of 6–7 days as evidenced by the formation of polynucleated myotubes and expression of differentiation markers like Myosin Heavy Chain (MHC), MEF2C, MyoD, and p21. We initially checked whether depletion of XIAP modulates ERK5 activation in HSM cells. As expected, depletion of XIAP led to an increase in pERK5 signals in the immunoblots and MEF2 activity in HSM cells (Fig 6A and B). Consistently, loss of XIAP strongly promoted (~10- to 12-fold increase) the differentiation of myoblasts as evidenced by the presence of polynucleated muscle fibers with a strong staining of Myosin Heavy Chain (Fig 6C and D). Immunoblot analysis revealed that loss of XIAP led to a consistent increase in pERKS levels with a concomitant increase in the appearance of various differentiation markers including MHC, p21, Myogenin, and MEF2C (Fig 6E and F). As cIAP1 loss also leads to ERK5 activation in tumor cells, we initially tested for the role of cIAP1 in regulating human myoblast differentiation. However, single depletion of cIAP1 failed to enhance myocyte formation possibly due to cross-stabilization of XIAP in these cells (ANT, KR unpublished observations). These results confirmed that loss of XIAP promotes ERK5 activation and myogenesis in human cells.

XIAP regulates human myogenic differentiation in MEKK2/3–ERK5-dependent manner

We then investigated whether the enhanced differentiation observed in XIAP-depleted HSM cells was indeed due to the hyperactivation of ERK5 signaling. To address this issue, we performed several co-knockdown experiments. Interestingly, knockdown of ERK5, MEK2, and MEK3 completely prevented basal as well as siXIAP-mediated differentiation of myoblasts (Fig 7A–G and Supplementary Fig S7A and B). To corroborate these observations, we employed ERK5-specific kinase inhibitor XMD 8–92. Consistent with the observations made with ERK5 siRNAs, treatment of HSM cells with XMD 8–92 prevented both normal and XIAP-depletion-induced differentiation as evidenced by the lack of polynucleation or expression of differentiation markers (Fig 7C and D and Supplementary Fig S7C). These results confirmed the physiological role of XIAP–ERK5 interface in human myogenic differentiation.

Discussion

MAPKs are activated in response to various stimuli to perform fundamental cellular processes (Raman et al., 2007). The signaling fidelity and efficiency of MAPK modules are controlled by scaffolding proteins and docking interactions occurring in a spatiotemporal manner (Tanoue & Nishida, 2002). Dephosphorylation by phosphatases is one of the major means of inactivating MAPKs (Raman et al., 2007). The cross-talk between phosphorylation and ubiquitination is multilayered, and there is a lot of evidence for the role of ubiquitination in the regulation of kinase activity (Hunter, 2007). To the best of our knowledge, this is the first evidence where non-degradative ubiquitination (predominantly K63-linked ubiquitin) is involved in the physical and functional disassembly of a “classical” MAPK module, thus adding another layer in the inactivation of MAPKs. ERK5 is a relatively less studied MAPK despite its importance in vertebrate development and in pathogenic conditions such as cardiac hypertrophy and cancer (Wang & Tournier, 2006). The MEKK2/3–MEK5–ERK5 cascade is activated by numerous stimuli and assembled by distinct PB1 domain-mediated protein–protein interactions, though a role for scaffold proteins such as Lad has been suggested (Sun et al., 2001; Drew et al., 2012). This study reveals a novel role for IAP-mediated ubiquitination events in directly uncoupling a MAPK from its cognate MAPKK.

XIAP and cIAP1 are highly conserved RING domain-containing E3 ubiquitin ligases, and they can catalyze the conjugation of ubiquitin chains of various kinds to their growing list of substrates. We have previously shown that XIAP can regulate Rac1 protein stability (Oberei et al., 2011). While we fail to detect a significant role for Rac1 in ERK5 activation (Supplementary Fig S1F and G), an upstream, cell type-specific role in activating MEKK2/3 cannot be ruled out as chemical inhibition of Rac1 has been shown to reduce MEK3 activation in T cells (Wang et al., 2011). XIAP and cIAP1 negatively regulate ERK5 activation, and as both IAPs are required, XIAP–cIAP1 complex is possibly functional in vivo in regulating the ubiquitination of MEKK2/3.
In the same lines, both MAP3Ks are functionally non-redundant as depletion of either MEKK2 or MEKK3 led to an identical reduction in pERK5 levels and inhibition of myocyte formation. These data suggest that MEKK2 and MEKK3 might function as heterodimers (like B-RAF and C-RAF kinases) and that this interaction could be stabilized by ubiquitination. In fact, ubiquitination of MEKK2 by XIAP promotes homodimerization and heteromerization with MEKK3 (Fig 5A and B). IAPs directly conjugate ubiquitin predominantly to the kinase domains of MEKK2 and MEKK3, and ubiquitination at Lys450 in MEKK2 contributes to the dynamics of ERK5 activation (Supplementary Fig S4). Mutation of two or more lysine sites severely compromises the stability and expression of MEKK2,
Figure 5. Functional significance of MEKK2/3 ubiquitination.

A. Ubiquitination of MEKK2 promotes its homodimerization. Purified GST-tagged MEKK2 was subjected to in vitro ubiquitination. In vitro transcribed and translated MEKK2 was added to the non-ubiquitinated (−NU) and ubiquitinated (−(Ubi)n) MEKK2. In vitro ubiquitination mix and GST pull down were performed as stated in Materials and Methods. Homodimerization of MEKK2 was analyzed by Western blotting.

B. Ubiquitination of MEKK2 promotes its heterodimerization with MEKK3. Purified MEKK2 protein was subjected to in vitro ubiquitination. Purified MEKK3 was added to the non-ubiquitinated (−NU) and ubiquitinated (−(Ubi)n) MEKK2 samples, and MEKK2 was pulled down. Co-precipitated MEKK3 was detected using Western blotting.

C. Ubiquitination of MEKK2 does not impair MEK5 binding. In vitro ubiquitination of MEKK2 by XIAP was performed as stated above. His-tagged ubiquitin was pulled down from non-ubiquitinated (−NU) and ubiquitinated (−(Ubi)n) MEKK2 samples using Ni-NTA beads as detailed in Materials and Methods section. Co-precipitated MEK5 was analyzed using Western blotting.

D. Ubiquitination of MEKK3 does not impair MEK5 binding. Ubiquitination of MBP-MEKK3 was carried out after the protein was bound to amyllose resin and non-ubiquitinated (−NU), and ubiquitinated (−(Ubi)n) MBP-MEKK3 was used for pulling down GST-MEKS. Samples were subjected to SDS-PAGE and stained with Coomassie.

E. MEKK2/3 and MEK5 binding is mediated through their PB1 domains in the MEKK2/3-MEK5-ERK5 ternary complex. The structural model of the ternary complex is shown in surface representation (Glazt et al., 2013).

F. The trimeric complex formation between MEKK2-MEKS-ERK5 was analyzed using non-ubiquitinated and ubiquitinated MEKK2. GST-tagged MEK5 was used for immunoprecipitating GST-MEKS and GST-ERK5 using rabbit monoclonal antibody against MEK5. Difference between binding of ERKS to MEKS in the presence of ubiquitinated and non-ubiquitinated MEKK2 was analyzed by Western blotting.

G. Schematic representation of the MEKK2/3-MEKS-ERK5 module. Ubiquitin conjugated to MEKK2/3 competes with the non-canonical interaction between the MEKS PB1 domain and MEK5. The XIAP–cIAP1 complex promotes MEKK2/3 K63-ubiquitination, which, in turn, interferes with the recruitment of ERK5 to the MEKK2/3-MEKS binary complex. (Kinases are depicted schematically with their classical N- and C-terminal bipartite lobe structure where “P” indicates activation loop phosphorylation sites, * marks the position of the kinase active site, and the rectangle at the back of the ERK5 kinase domain shows the MEK5-docking motif binding to the MAPK-docking groove (Glazt et al., 2013).

Source data are available online for this figure.
in different outcomes depending on the external stimuli, that is TNF-α triggering late-phase of NF-κB activation, sorbitol stimulating JNK, and growth factors leading to ERK5 inactivation and regulation of cellular differentiation (Fig 7H). In this context, it is interesting to point out that p62 can bind to MEKK3 to mediate NF-κB activation (Nakamura et al., 2010). However, XIAP was reported not to control the early phase of NF-κB activation which is mediated by MEKK3 (Winsauer et al., 2008). Further, ubiquitination of MEKK2/3 failed to prevent the activation of JNK1 directly unlike ERK5 (Supplementary Fig S5).

Consistent with the negative regulatory role of XIAP in ERK5 activation, we uncover that depletion of XIAP augments activation...
of ERK5 and MEF2, thus promoting the formation of human myotubes. Further, loss of MEKK2, MEKK3, and ERK5 led to a complete block in myogenic differentiation in control and XIAP-depleted cells. These data reveal the physiological significance of XIAP–ERK5–cascade interactions in regulating human myogenic differentiation.

While the role of IAPs in regulation of apoptosis, inflammation, and immune signaling is well established, their role in regulation of cell migration and differentiation is just emerging (Kenneth & Duckett, 2012). cIAP1 has been previously shown to regulate monocyte to macrophage differentiation and NAIP, to regulate neuronal differentiation. TWEAK and cIAP1 have been shown to regulate myoblast fusion through non-canonical pathway of NF-κB (Enwere et al., 2012). While cIAP1 functions as a direct E3 ligase of NIK, loss of XIAP does not lead to constitutive NF-κB activation, negating the possible involvement of this pathway in XIAP-regulated human myogenic differentiation. In this context, XIAP antagonists might be valuable in treating muscular degenerative diseases and for promoting muscle regeneration. Taken together, our data reveal a novel role of IAPs and IAP-mediated non-degradative ubiquitination in the regulation of ERK5-MAPK module and myogenic differentiation (Fig 7H). It would be indeed interesting to investigate whether the assemblage and the activation dynamics of other MAPK modules are also directly influenced by non-degradative ubiquitination.

Materials and Methods

Cell culture

HeLa and A549 cells were cultured in RPMI-1640 medium (Gibco BRL) supplemented with 10% FCS (Gibco BRL) and 0.2% penicillin (100 U/ml)/streptomycin (100 µg/ml) (Gibco BRL) at 37°C in 5% CO2. HEK293T, A431, and BT474 were cultured in DMEM medium (Gibco BRL), 1 mM non-essential amino acids, 1 mM β-mercaptoethanol at 37°C in 5% CO2.

SKB are Human Skeletal Muscle Myoblast obtained from ZenBio (Cat #: SKB-F (Lot 1 #: SK052009, Lot 2 #: SK051810)). SKB were cultured in the Skeletal Muscle Cell Growth Medium (Cat #: SKM-M, ZenBio) and were differentiated with the Skeletal Muscle Cell Differentiation Medium (Cat #: SKM-D, ZenBio) at 37°C in 5% CO2.

In order to obtain the HeLa MEKK2-wt, K450R, and ERK5/MEK5 stable cell lines, we first transfected HEK293T with pHAGE-CMV-N-Flag-HA-GAW-IREs-Puro-MEK2 wt or pHAGE-CMV-N-Flag-HA-GAW-IREs-Puro-MEK2-K450R or pHAGE-CMV-N-Flag-HA-GAW-IREs-PROERK5, and pLenti4TO/V5-DEST-MEK5 together with the pLenti package (HDM-VSV-G; HDM-tat/h; HDM-Hgprn2 (gag-pol); RC-CMV-Rev1b). The media containing the virus were sterile filtered and then added to Hela cells in presence of 8 µg/ml of polybrene. After 24 h, cells were selected with 2.5 µg/ml puromycin or in the case of ERK5/MEK5 overexpression with 2.5 µg/ml puromycin and 100 µg/ml Zeocin. pHAGE plasmid was a kind gift from Dr. Behrends.

Wherever appropriate, cells were stimulated with FGF-2 (human FGFb147 from eBiosence, Cat. No. 14-8986-80) in presence of serum for the indicated times at a final concentration of 25 ng/ml. In case of FCS stimulation, cells were starved for 15 h in FCS-free medium and then stimulated with media containing 10% FCS.

Transfection of siRNAs

In order to silence XIAP, cIAP1, cIAP2, MEKK2, MEKK3, MEK5, ERK5, and C-RAF expression by siRNA interference, approximately 75,000 cells/well were seeded in a 12-well plate at least 20 h prior to transfection. siRNAs directed against various genes and scrambled control siRNA as negative control were transfected at a final concentration of 60 nM using Lipofectamine™ RNAiMAX (Invitrogen, Cat. No. 13778) transfection reagent. For complementation experiments, siRNA and plasmid were co-transfected using HiPerFect (QIAGEN, Cat. No. 301705) transfection reagent. For complementation experiments, siRNA and plasmid were co-transfected using HiPerFect (QIAGEN, Cat. No. 301705) transfection reagent. Unless otherwise mentioned, cells were lysed at 48 h post-transfection. The following siRNAs and shRNAs were employed in this study (sense strand sequence):

siControl: 5’-UUCUCCGAACGUGACUCAU-3’ (QIAGEN, Cat. No. 1027310)

siXIAP #1 (3’utr): 5’-GACGUGAUCUAUUAUUAUATT-3’ (QIAGEN)
siXIAP #2: 5’-AAGTCCTTTACCTGTGGAGGA-3’ (QIAGEN, Hs_BIRC4_5_HP Validated siRNA, Cat. No. SIO0299446)
siXIAP #3: 5’-ACACUCGCGACGCGAGGGUUUCUUU-3’ (Invitrogen Stealth, XIAPHSS100564)
siXIAP #4: 5'-GAAGGAGAUACCGUGCGGUGCUUUA-3' (Invitrogen Stealth, XIAPHS100565)
sicIAP1 #1: 5'-GAUUGAAAGGCCAAGAGUU-3' (Thermo Scientific, ON-TARGET plus siRNA human BIRC2 (329), Cat No. J-004390-13-0020)
sicIAP1 #2: 5'-CAUGAGCUUUAGCAAGAGU-3' (QIAGEN, Hs_BIRC2_12, Cat No. SI05067258)
sicIAP1 #3: 5'-CAUAGUAGCUUGUUUCAGUGTT-3' (QIAGEN, Hs_BIRC2_7, Cat No. SI02654435)
The plasmids employed for the expression of indicated proteins are listed here:


The various point mutations in MEKK2 (K47A, K384M, K360R, K450R), XIAP (H467A), and cIAP1 (H588A) were generated with a Site-Directed Mutagenesis Kit (Stratagene) following manufacturer’s instructions.

**SDS–PAGE and Western blot**

For SDS–PAGE, cells were lysed in 5× Laemmli buffer and boiled at 100°C for 5 min before loading on polyacrylamide gels. The proteins were then transferred to nitrocellulose membranes by Western blotting. After transfer, membranes were stained with Ponceau (AppliChem). For immunoblot analysis, membranes were blocked with 5% low-fat milk in phosphate-buffered saline for 1 h at room temperature and then incubated with various primary antibodies overnight at 4°C. For K63 linkage-specific antibody, the nitrocellulose membrane was incubated for 2 h at room temperature in the primary antibody and then proceeded as stated above. Antibody–antigen complexes were detected by horseradish peroxidase-coupled secondary antibodies followed by enhanced chemiluminescence (Amersham Biosciences, Millipore, Thermo Scientific). Quantification of Western blots was performed by densitometry (ImageJ software, NIH). The following antibodies have been employed in this study:

- Phospho-ERK5 (Thr218/Tyr220; Cat. No. 3371) and total-ERK1/2 (p44/42 MAPK; Cat. No. 9102) rabbit polyclonal from Cell Signaling; total-ERK5 (C-term, Cat. No. 1719-1), MEKK2 (Cat. No. 1662-1), MEKK3 (N-term, Cat. No. 1672-1) rabbit monoclonal and MEF2c (T300, Cat. No. T1324), MyoD (Cat. No. S0549) rabbit polyclonal antibodies from Epitomics; XIAP (Cat. No. 610673), total-MEK5 (Cat. No. 610956) and Rac1 (Cat. No. 616050) mouse monoclonal antibodies from BD Biosciences; Actin rabbit polyclonal (Cat. No. A2066) and Tubulin (Cat. No. T9026), Flag (M2, Cat. No. F3165), GAPDH (clone GAPDH-71.1, Cat. No. G795) mouse monoclonal antibodies from Sigma-Aldrich; cIAP1 (E1-1-10, Cat. No. ALX-803-335) and cIAP2 (16E6-3, Cat. No. ALX-803-341) rat antibody from ENZO; normal mouse IgG (Cat. No. sc-3877), normal rabbit IgG (Cat. No. sc-3888), phospho-MEK5 (Ser311/Thr315; Cat. No. sc-135702), C-RAF (F-12, Cat. No. sc-133), p21 (C-19, Cat. No. sc-397) rabbit polyclonal, GST (B-14, Cat. No. sc-133) and HA (12CA5; Cat. No. sc-57592) mouse monoclonal antibodies from Santa Cruz Biotechnology; Ubiquitin mouse monoclonal antibody from Invitrogen (Cat. No. 13-1600); Goat anti-human IgG-HRP from Invitrogen (Cat. No. AH0704); MHC (MF20) mouse monoclonal antibody from DSHB; Myogenin mouse monoclonal antibody from Millipore (Cat. No. MAB3876); Cy3-conjugated AffiniPure Goat Anti-Mouse IgG (Jackson ImmunoResearch, Code No. 115-165-146). The antibody against K63-linked polyubiquitin was a kind gift from Genentech (San Francisco, CA, USA).
Luciferase assay

HeLa cells were transduced with the Cignal Lentiviral particles using Cignal Lentivector (luc) Kit (Qiagen, Cat. No. CLS-4042L). Following transduction, the cells were cultured under puromycin selection to generate a homogenous population of transduced cells. Briefly, transduced and selected HeLa cells were seeded on a 12-well plate and transfected with XIAP, ERK5, and/or MEKK2 siRNAs using Lipofectamine RNAliter.

HEK293T cells were transiently transfected with the Cignal MEF2 Reporter (luc) Kit (Qiagen, Cat. No. CCS-7024L). Cells were transfected using GeneJuicer (MERCK Millipore, Cat. No. 70967) with 500 ng of each of the following plasmids: the luciferase reporter gene, MEKK2-myc/his, and XIAP-flag. As negative and positive controls, we used the control plasmids provided by the Kit.

In both case, the cells were harvested in Passive Lysis Buffer 48 h after transfection. The MEF2 luciferase assay was performed using the Luciferase Reporter Assay System (Promega, Mannheim, Germany) in accordance with manufacturer’s protocol.

Ubiquitination experiments

For detecting the ubiquitination of MEKK2 in vivo, 293T cells were transfected with flag-MEKK2 in combination with HA-Ubiquitin wt, or with or without myc/his-XIAP and XIAP-H467A, using GeneJuicer transfection reagent (Novagen, Merck Millipore, Cat. No. 70967) at a final concentration of 1 g/ml for 48 h. The cells were lysed and immunoprecipitated with Flag antibody for 15 h. The bound proteins were analyzed by SDS–PAGE and immunoblotting.

In vitro ubiquitination of MEKK2 or MEKK3 was performed in the presence of ubiquitination buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 2.5 mM MgCl2, 1 mM DTT), 100 nM XIAP or 100 nM c-IAP1 (R&D Systems), 100 nM E1 (Boston Biochem), 150 mM UbcH5a (Boston Biochem), 107 mM His-Ubiquitin (Boston Biochem), 50 mM EDTA, 100 mM NaCl, 5 mM MgCl2, 0.05% IGEPAL, 5% glycerol, 2 mM DTT. Wherever specified, in vitro ubiquitination was performed using K48-only, K63-only, or K29-only ubiquitin (Boston Biochem). The reaction was incubated at 37°C for 1 h. Western blot analyses, the reaction was stopped using Laemmli buffer and MEKK2 was visualized using MEKK2 rabbit monoclonal antibody (Epitomics).

For GST and His-ubi pull down after in vitro ubiquitination, 300 µl of GST pull-down buffer (GPB) was added to the in vitro ubiquitination reaction mix with 50 µl of either equilibrated glutathione sepharose or Ni-NTA beads. We then proceeded for GST pull down. For immunoprecipitation after in vitro ubiquitination, 300 µl of lysis buffer (constituents described above) was added to the in vitro ubiquitination reaction mix with 2 µg of MEKK2 antibody or MEKK3 antibody and immunoprecipitated. For the deubiquitination experiments, we treated in vitro ubiquitinated MEKK2-GST (SignalChem) with 5 µM Amsk (K63 linkage specific) or 5 µM Usp2 (linkage unspecific) for 60 min at 30°C. The incubation was terminated by denaturation in Laemmli Buffer.

Kinase assay

For kinase assay, in vitro ubiquitination of MEKK2 was performed as described above, with or without the addition of E3 ligase, XIAP. MEKK2 is immunoprecipitated from these samples using MEKK2 antibody and sepharose-coupled protein A/G beads according to procedure described above. Beads were washed three times with lysis buffer and then the lysis buffer was removed using insulin syringe. To the beads, added 4 µl of 10x kinase buffer (100 mM MgCl2, 250 mM β-glycerophosphate, 250 mM HEPES pH 7.5, 50 mM benzamidine, 5 mM DTT, 10 mM sodiumorthovanadate), 2 µl of 20x Mg-ATP (Enzo Lifesciences), 400 ng of GST-MEK5 (Abcam), and distilled water up to 40 µl of reaction mix. The reaction was incubated at 30°C for 30 min and then stopped by adding 8 µl of 5x Laemmli. The entire reaction mix was loaded on the SDS–PAGE gel for immunoblot analysis.

Proteins for the MEKK2/3-MEK5-ERK5 and MEKK2/3-MKK7-JNK1 MAPK module reconstitution experiments were produced in E. coli or in SF9 insect cells (with the baculoviral expression system) as N-terminal GST or MBP fusions which contained a C-terminal His6 tag (apart from JNK1 that contained an N-terminal His6-tag only). Samples were normally purified by double-affinity chromatography or by ion exchange close to homogeneity checked by SDS–PAGE. In vitro ubiquitination and phosphorylation reactions were carried out in 50 mM HEPES pH 7.5, 100 mM NaCl, 5 mM MgCl2, 0.05% IGEPAL, 5% glycerol, 2 mM DTT. MEKK3 was expressed with the Bac-to-Bac baculoviral expression system with a C-terminal His6-tag and an N-terminal maltose binding protein (MBP) tag and purified by double-affinity chromatography (using Ni-NTA and amylose resin, New England Biolabs). GST-MEKK2 was purchased from Signalchem. MAPKKks (0.2 µM) were first ubiquitinylated in the presence of ubiquitin (25 µM, Boston Biochem) at room temperature for 30’ using E1, E2 (0.1 µM, Boston Biochem) and GST-XIAP (0.2 µM, expressed in E. coli and purified) similarly as described in the ubiquitination experiments section. Phosphorylation assays were started by adding MAPKKs (1 µM) and MAPks (5 µM) and additional 0.5 mM fresh Mg-ATP. For non-ubiqu reactions, samples were treated exactly the same, but XIAP was not added to the reaction mix. Reactions were stopped with protein-loading sample buffer complemented with 20 mM EDTA, boiled and then subjected to SDS–PAGE. Gels were dried before analysis by phosphorimaging or by Western blots on a Typhoon Trio+ scanner (GE Healthcare). Western blots were done by using anti-phospho ERKS antibody, anti-phospho MEK5, anti-phospho JNK1 antibody (9251S, Cell Signaling), and anti-phospho MKK7 antibody (4171S, Cell Signaling). The constitutive active form of MEK5 (MEK5DD: S311D, T315D) and the kinase inactive version of ERK5 (ERK5_KI: D182A)—that was used as substrate in the in vitro kinase assays—were generated by QuickChange Site-Directed Mutagenesis or by PCR, and proteins were expressed and purified as described earlier (Glatz et al, 2013). JNK1 kinase dead (KD) was expressed with N-terminal His6-tag in E. coli Rosetta (DE3) plS5 (Novagen), and purification was done with nickel affinity and ion-exchange chromatography (ResourceQ 1 ml column, GE Healthcare). Dey phosphorylated MKK7 (with N-terminal GST- and C-terminal His6-tag) was co-expressed with λ phage phospatase in E. coli (MG950 vector) and purified by double-affinity chromatography (on Ni-NTA and glutathione sepharose). For the myelene basic protein (MyBP) phosphorylation experiments, the reaction was started by adding 0.5 mM ATP with ~5 µCi of ATP(γ-32P).
Expression of GST-tagged proteins and their purification

XIAP-GST (M1-S497), XIAP-BIR1-GST (M1-S123), XIAP-BIR2-GST (L121-S261), XIAP-BIR3-GST (S261-E350), XIAP-BIR1+2+3-GST (M1-E350), XIAP-DRING-GST (M1-L449), or GST alone were transformed into BL21-CodonPlus competent cells and transformation, and protein purification was done according to manufacturer's instructions. Briefly, the transformed cells were grown in a liquid culture at 37°C overnight with chloramphenicol and ampicillin with constant shaking. Part of this overnight culture was then added to fresh LB media and incubated at 37°C till it reached OD600 0.6. Transformed bacteria were then induced with 1 mM IPTG for 4 h at 37°C. The cells were pelleted and lysed in GST lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 5% glycerol, 0.1% NP-40), 1 mM DTT and 1 mM protease inhibitor cocktail (Calbiochem). The GST proteins were bound to glutathione–sepharose beads (GE Healthcare). MEKK2-GST and MEKK3-GST were obtained from SignalChem (active MEKK2, Cat. No. M10-10G-20; active MEKK3, Cat. No. 11-10G-20) and MEK5-GST from Abnova (Cat. No. H00005607-P01).

In vitro translation

In vitro translation was performed using the TNT T7 Coupled Reticulocyte Lysate Systems from Promega following the manufacturer’s protocol (Cat. No. LA610). Briefly, 1 µg of template DNA was mixed in the following reaction: 25 µl TNT lysates, 2 µl TNT Reaction Buffer, 1 µl TNT T7 RNA Polymerase, 1 mM Amino Acids Mixture, minus Leucine, 1 mM Amino Acids Mixture, minus Methionine and 40 units of RNasin Ribonuclease Inhibitor, and incubated at 30°C for 90 min. pcDNA3.1B-myc/his-MEKK2-wt, pcDNA3.1B-myc/his-MEKK2-K47A, pcDNA3.1B-myc/his-MEKK2-R384M, pcDNA3.1B-myc/his-XIAP plasmids were used as template.

Pull-down experiments

Glutathione Sepharose™ (GE Healthcare, Cat. No. 17-0756-05) was washed and equilibrated in the so-called "GST pull-down Buffer" (GPB): 50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM DTT. For each condition, 50 µl of beads were resuspended in 300 µl of GPB and incubated on rotator for 2 h at 4°C with 1 µg of GST protein. The beads were then washed three times with GPB and incubated on rotator for 1 h at 4°C with 100 µg/ml BSA-GPB solution. After washing three times, beads were finally incubated on rotator for 2 h at 4°C with purified XIAP protein or in vitro translated protein. The final washing was performed with GPB containing either 150 mM or 250 mM NaCl. Buffer was completely removed using an insulin syringe, and samples were then prepared for SDS–PAGE.

For MBP pull-down experiments, first the amylose resin (New England BioLabs) was equilibrated with binding buffer (20 mM Tris, 100 mM NaCl, 0.1% IGEPAL, 2 mM β-mercaptoethanol) and 10 µg immobilized MBP-fusion protein (and MBP protein as negative control) was incubated in the presence of 10 µM prey in 200 µl binding buffer for 30 min at room temperature. Binding reactions typically contained 10–20 µl resin saturated with baits. Amylose beads were pelleted with centrifugation and washed three times. Retained proteins were eluted from the resin with SDS-loading buffer. Samples were subjected to SDS–PAGE and stained with Coomassie staining.

Immunoprecipitation

To immunoprecipitate endogenous proteins, HeLa or MEFs cells were seeded on 10-cm dishes and if required, transfected after 24 h and then lysed 48 h post-transfection. Next day, 80% confluent cells were lysed with lysis buffer containing 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 0.5% Triton X-100, 1 mM NaVO₄, 10 mM Na-pyrophosphate, 1 mM NaF, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 1 µg/ml aprotinin, 0.5 µg/ml leupeptin, 10 µM pepstatin for 30 min on ice. Lysates were cleared by centrifugation for 15 min at 14,000 rpm. Endogenous MEKK2, MEKK3, or XIAP was then immunoprecipitated overnight. The antigen–antibody complexes were precipitated by sepharose-coupled protein A/G beads (Roche, Cat. No. 11-134-515-001 and 11-243-233-001). The beads were washed with the lysis buffer, and bound proteins were analyzed by SDS–PAGE and immunoblotting.

For immunoprecipitation of co-expressed proteins in 293T cells, we have transfected 293T cells with various plasmids using GeneJuice transfection reagent (Novagen, Merck Millipore, Cat. No. 70967). The cells were lysed at 48 h post-transfection, and proteins were immunoprecipitated as mentioned above. Control IPs were performed with IgG isotype control antibodies (Santa Cruz).

For immunoprecipitation of the entire protein complex (MEKK2–MEK5-ERK5), HeLa cells stably overexpressing ERK5-FLAG and MEKK5-V5 were employed. Two days post-transfection with siXIAP 3'UTR using RNAiMAX, the cells were lysed in RIPA buffer (250 mM NaCl, 50 mM Tris pH 7.5, 10% glycerine, 1% Triton X-100, 1 mM DTT, 1% of Protease Inhibitor Cocktail Set I (Calbiochem)) for 30 min at 4°C. Lysates were cleared by centrifugation for 15 min at 14,000 rpm. FLAG-ERK5 fusion protein was isolated using ANTI-FLAG M2 Magnetic beads (Sigma). For each condition, 10 µl magnetic beads were used and incubated with the lysate for 4 h at 4°C. The immunoprecipitation and subsequent elution with FLAG peptides were performed according to the manufacturer’s instructions (Sigma).

Mass spectrometry

Samples from the in vitro ubiquitination assay were cleaned and fractionated using SDS–PAGE and digested in-gel using trypsin as described by earlier with minor modifications. The sample was alkylated with chloroacetamide to reduce formation of lysine modifications with the same atomic composition as the di-glycine modification (Nielsen et al., 2008). Before performing LC-MS analysis, peptides were desalted using C₁₈ StageTips (Rappsilber et al., 2002). Peptides were loaded with Solvent A (0.5% acetic acid) to an Easy-nLC II coupled to an Orbitrap Elite (Thermo Fisher Scientific) and separated using a C₁₈ fused silica emitter packed in-house. Elution was performed using a segmented gradient of 5–50–90% of solvent B (80% ACN in 0.5% acetic acid) with a constant flow of 200 nL/min over 87 min. Full-scan MS spectra were acquired in the Orbitrap mass analyser in the range of 300–2,000 Th at a resolution of 120,000. The 20 most intense ions were successively isolated for CID fragmentation in the linear ion trap at a target value of 5,000 charges. Ions selected for fragmentation were included in a dynamic exclusion list for 60 s.

The acquired data were processed using the MaxQuant software suite (Cox & Mann, 2008) which contains the Andromeda search.
engine (Cox et al., 2011). Mass spectra data was searched against a human database containing 73,929 entries (www.uniprot.org). Protein data set in FASTA format, downloaded on the 18.01.2012) and 248 frequently, seen laboratory contaminants. Carbamidomethylation (Cys) was defined as fixed, and protein N-terminal acetylation, oxidation (Met), and GlyGly (Lys) were defined as variable modifications. Initial mass tolerance was set to 7 ppm for precursor ions, and to 0.5 Da for fragment ions. False discovery rate was set to 1% at the protein, peptide and modification levels.

Myoblast differentiation

Human Skeletal Muscle Myoblast (HSM) was seeded in a 12-well plate at 10,000 cells per well. When immunofluorescence analysis was required, cells were seeded out on glass coverslips, previously coated with purified bovine collagen solution from Nutragen (Advanced BioMatrix, Cat. No. 5010-D) for at least 2 h. At 70–80% confluency, cells were transfected with siRNAs as explained above. The following day differentiation was started by first rinsing residual growth medium with PBS and then by adding differentiation medium (ZenBio, see above). Transfection of siRNA was performed every 3 days to ensure the knockdown of the genes of interest. In order to inhibit ERK5 activation, cells were treated with 5 μM of XMD 8–92 (R&D Systems, Cat. No. 4132) or with the equivalent amount of DMSO, every 2 days while renewing the differentiation medium. Cells were lysed at various time points by adding 5× Laemmli and prepared for SDS–PAGE and immunoblotting. For the immunofluorescence, cells were fixed with 2% PFA for 20 min at room temperature (RT). After washing three times with PBS, cells were permeabilized 7 min at RT with 0.1% Triton X-100 diluted in PBS. Cells were then blocked in 5% BSA-PBS overnight at 4°C. After washing three times with PBS, cells were incubated with the secondary anti-mouse-Cy3 antibody at 4°C. The DAPI staining was obtained by incubating the fixed cells in 1/5,000 Hoechst 33342 solution for 10 min at RT. Finally, cover slips were fixed on slides using Mowiol 4-88 reagent (Calbiochem, Cat. No. 475904). The images were acquired using a Leica fluorescent microscope (DMI2RE2) fitted with a digital camera with both high NA and low NA 10× objectives.

Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions

ANT and TKO performed most of the experiments, analyzed and interpreted data, and prepared figures. GC, KS, and RPS performed additional experiments, analyzed and interpreted data, and prepared figures. AC and BM performed mass spectrometric analysis. AR designed experiments, and analyzed and interpreted data. KR conceived and designed the project, analyzed and interpreted data, coordinated the study, and wrote the paper with input from all authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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