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cIAP2 represses IKKα/β-mediated activation of MDM2 to prevent p53 degradation

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Cellular inhibitor of apoptosis proteins (cIAP1 and cIAP2) function to prevent apoptosis and are often overexpressed in various cancers. However, mutations in cIAP1/2 can activate the alternative NFκB pathway through IBα-kinase-α (IKKα) and are associated with hematopoetic malignancies. In the current study, we found that knockdown of cIAP2 in human mammary epithelial cells resulted in activation of MDM2 through increased SUMOylation and profound reduction of the pool of MDM2 not phosphorylated at Ser166. cIAP2 siRNA markedly decreased p53 levels, which were rescued by addition of the MDM2 inhibitor, Nutlin3a. An IAP antagonist, which induces cIAP degradation, transiently increased MDM2 mRNA. Simultaneous transfection of siRNA for cIAP2 and IKKα reduced MDM2 protein, while expression of a kinase-dead IKKβ strongly increased non-Ser166 P-MDM2. Inhibition of either IKKα or -β partially rescued p53 levels, while concomitant IKKα/β inhibition fully rescued p53 after cIAP2 knockdown. Surprisingly, IKKα knockdown alone increased SUMO-MDM2, suggesting that in the absence of activation, IKKα can prevent MDM2 SUMOylation. cIAP2 knockdown disrupted the interaction between the MDM2 SUMO ligase, PIAS1 and IKKα. Partial knockdown of cIAP2 cooperated with ^{V12}*H-ras*-transfected mammary epithelial cells to enhance colony formation. In summary, our data identify a novel role for cIAP2 in maintaining wild-type p53 levels by preventing both an NFκB-mediated increase and IKKα/-β-dependent transcriptional and post-translational modifications of MDM2. Thus, mutations or reductions in cIAP2 could contribute to cancer promotion, in part, through downregulation of p53.

Introduction

Proteins of the inhibitor of apoptosis protein (IAP) gene family have emerged as among the most important intrinsic inhibitors of apoptosis. They are characterized by the presence of a baculovirus IAP repeat (BIR) domain in one to three copies. Two cIAP members cIAP1 and -2 function as ubiquitin ligases for caspase-3 and -7.[1,2] cIAP1 and -2 are also required for NFκB signaling from the activated tumor necrosis factor receptor-1 (TNFR1) by mediating the ubiquitination of RIP1.[3-5] Both cIAP1 and -2 mediate K48-ubiquitination of the NFκB-inducing kinase NIK,[6] resulting in NIK proteasomal degradation, thereby preventing NFκB activation in unstimulated cells.[5-7]

The involvement of the IAPs in cancer is underscored by the frequent induction of these genes in several tumor types.[8] cIAP1 is an established oncogene seen in DNA amplicons (with cIAP2) in mice and human tumors, and can cooperate in transformation with YAP1[9] or with Myc.[10] Oncogenic activity of both cIAP1/2 can be attributed both to their anti-apoptotic role and their involvement TNF-mediated NFκB activation. As such, ongoing efforts directed at reducing cIAP levels in cancers are underway.[8,11-13] Reduction of both cIAP1 and -2 by small-molecule IAP antagonists results in stabilization and activation of NIK, leading to engagement of the alternative NFκB pathway and autocrine production of TNFα in some cells to result in cell death.[5,7]

NFκB transcription factors play an important role in many cancers.[14,15] There are two IκB kinases (IKKs) that activate NFκB: IKKα and IKKβ, as well as a regulatory subunit, IKKγ (NEMO). IKKβ preferentially phosphorylates the IκB proteins, resulting in their ubiquitination and degradation, followed by the release and activation of canonical NFκB p50/p65 (RelA) and c-Rel/p65 complexes. IKKα can be specifically activated by the NFκB-inducing kinase, NIK.[16] IKKα homodimer-mediated phosphorylation of cytoplasmic NFκB2/p100 results in partial proteasomal processing of p100 to produce mature p52, thereby activating the alternative pathway.[17]

The tumor suppressor p53 exerts governance over cellular responses to genotoxic stress, wherein it mediates a variety of outcomes including cell cycle arrest, senescence and apoptosis. Cellular levels of p53 are regulated, in large part, through the action of the E3-ubiquitin ligase, MDM2 (murine double minute 2). MDM2 is transcriptionally regulated by p53 and is subject to numerous posttranslational modifications. Among these, phosphorylation at Ser166 and Ser186 facilitate nuclear entry,[18] p300 binding[19] and enhance E3-ligase activity.[20,21] Nuclear

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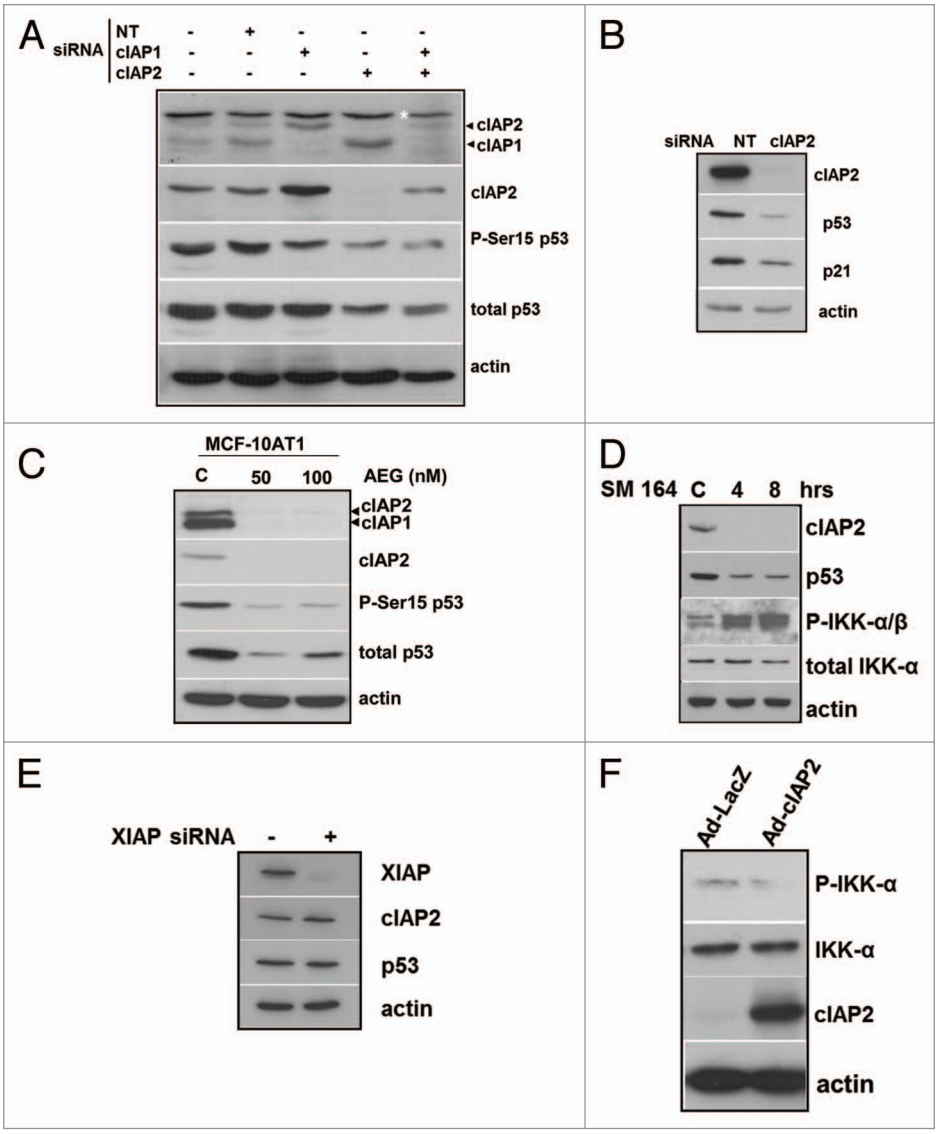

Figure 1. Downregulation of cIAPs in mammary epithelial cells reduces p53. (**A**) MCF-10AT1 cells were either non-transfected or transfected with non-targeting siRNA (NT) or cIAP1 and cIAP2 siRNAs, as indicated, for 48 h. Ten μg of cell lysate was immunoblotted for cIAP1 and -2 (top panel with anti-rat IAP (RIAP) antibody that recognizes both cIAP1 and -2, cIAP2 (Epitomics antibody), phospho-p53 (Ser15) and total p53. The asterisk indicates a non-specific immunoreactive band. (**B**) MCF-10AT1 cells transfected with non-targeting (NT) siRNA or cIAP2 siRNA for 48 h were immunoblotted for cIAP2, p53 and p21. (**C**) MCF-10AT1 cells were incubated with 50 or 100 nM AEG40730 for 6 h. Control cells were treated with vehicle for 12 h. Ten μg of cell lysate was subjected to immunoblotting for cIAP1/2 (RIAP), cIAP2, phospho-p53 (Ser15) and total p53. (**D**) MCF-10AT1 cells were exposed to vehicle (DMSO) or SM164 (5 nM) for 4 and 8 h. Immunoblotting was performed for cIAP2, total p53, phospho-IKKα/-β and total IKKα. (**E**) MCF-10AT1 cells were transfected with XIAP siRNA and lysates isolated after 48 h in culture and subjected to immunoblotting for XIAP, cIAP2 and p53. (**F**) MCF-10AT1 cells were infected with an adenovirus to overexpress human cIAP2 or a control LacZ adenovirus. Immunoblotting was performed on lysates 48 h following infection for P-IKKα, IKKα and cIAP2. Actin immunoreactivity was used as a loading control on all blots. Images shown are representative of independent experiments performed a minimum of three times.

MDM2 can be SUMOylated, which is mediated in part by PIAS proteins.[22] SUMOylation of MDM2 prevents autoubiquitination, resulting in MDM2 stabilization and p53-directed ubiquitination, while a SUMO-specific protease that removes SUMO from MDM2 stabilizes p53.[23]

Importantly, NFκB can negatively regulate p53. Specifically, reconstitution of IKKβ, but not IKKα, was shown to increase Mdm2 levels and decrease p53 in mouse IKKα/-β$^{-/-}$ mouse embryo fibroblasts (MEFs). The effects of IKKβ in these cells were inhibited by coexpression of a dominant-negative IκBα super repressor which suggests that canonical NFκB was responsible for regulating Mdm2 levels.[24]

Given the potential importance of the cIAPs as targets in cancer therapy and the known effect of IAP antagonism in activating NFκB, we sought to determine whether reduction of cIAP2 alone would induce NFκB and impact on p53 levels. Using cIAP2 siRNA, shRNA and IAP antagonists, we demonstrate that knockdown (KD) of cIAP2 using several methods downregulates p53 protein in both an IKKα- and IKKβ-dependent manner. Moreover, we uncover novel functions for both these kinases in regulating posttranslational modifications of MDM2. Lastly, we extended these findings by demonstrating that reduction or ablation of cIAP2 increases ^{V12}H-ras-induced colony formation.

Results

cIAP2 protein expression regulates p53 levels. Previous studies have demonstrated that IKKβ can regulate p53 protein stability by increasing MDM2 expression through the canonical NFκB pathway.[24] Since IAP antagonists reduce the cIAPs in conjunction with the activation of IKKα, we wished to determine if cIAP2 KD alone (1) would activate NFκB and (2) alter p53 protein expression. MCF-10AT1 cells (wild-type p53 MCF-10A human mammary epithelial cells transfected with ^{V12}H-ras[26]) were transfected with cIAP2 siRNA. **Figure 1A** shows that downregulation of cIAP2 alone resulted in a marked reduction in p53. Knockdown of cIAP1 weakly reduced p53 levels; however, this was accompanied by strong stabilization of cIAP2, likely a function of loss of cIAP1-mediated polyubiquitination of cIAP2.[27] Following transfection of both siRNAs, cIAP2 was again stabilized; however, a strong reduction in p53 was observed. The interaction between p53 and MDM2, and subsequent ubiquitination and degradation,

is regulated in part through phosphorylation of p53 at Ser15/20 and other phosphoacceptor sites. Moreover, phosphorylation at these residues promotes the transcriptional activity of p53.[28,29] To explore whether cIAP2 downregulation alone would impact on p53 phosphorylation, we performed immunoblotting for phospho-p53 Ser15 [P-p53(Ser15)]. Densitometry showed that while p53 was reduced by approximately 45% relative to control after cIAP downregulation, P-p53 (Ser15) was reduced by 60%, indicating a small net decrease in phosphorylation. Overall, however, the major effect of cIAP2 KD was on p53 levels, not its phosphorylation. **Figure 1B** shows that p21 levels were strongly decreased in association with cIAP2 KD and reduction of p53.

IAP antagonists simultaneously reduce cIAP1 and cIAP2 proteins.[30] The IAP antagonists SM164[30] and AEG40730[4] both reduced cIAP1 and cIAP2 and markedly reduced p53 in MCF-10AT1 cells as well as P-p53(Ser15) (**Fig. 1C and D**). Ablation of cIAP1 and -2 with SM164 also increased IKKα and -β phosphorylation (**Fig. 1D**). Bivalent SMAC mimetics such as SM164 have been shown to block XIAP interaction with caspases at higher concentrations.[31] To test the effect of blocking XIAP function on p53, we transfected MCF-10AT1 cells with a XIAP siRNA. p53 remained unchanged after KD of XIAP in **Figure 1E**, providing evidence that XIAP does not play a role in p53 regulation following IAP antagonist treatment. To see if overexpression of cIAP2 would have the reciprocal effect, we infected cells with a cIAP2 adenoviral expression vector or control adenovirus. The basal levels of phosphorylated IKKα were clearly reduced in cIAP2-overexpressing cells, consistent with the reciprocal ability of high levels of cIAP2 to block IKK activity (**Fig. 1F**).

Taken together, these data demonstrate that KD of either of the cIAPs, but in particular cIAP2, using RNAi and IAP antagonists, activates IKKs and negatively regulates p53 expression.

cIAP2 knockdown induces MDM2 Ser166 phosphorylation and SUMOylation to repress p53. Since MDM2 is the predominant E3 ligase that regulates p53, we first determined whether MDM2 was involved in the reduction of p53 in cIAP2-depleted cells. cIAP1 or cIAP2 siRNA-transfected MCF-10AT1 cells were treated with Nutlin-3a, which prevents MDM2-mediated ubiquitination.[32] Nutlin-3a completely rescued p53 from downregulation when cIAP1 or -2 were reduced (**Fig. 2A**). The MDM2 protein was also stabilized, since autoubiquitination is also blocked by Nutlin-3a. Thus, MDM2 activity is required for cIAP2 depletion induced p53 downregulation.

Ser166 phosphorylation promotes MDM2 nuclear translocation and interaction with p53;[18] however, the SMP14 antibody employed does not recognize MDM2 that has been phosphorylated at Ser166.[33] Using a P-MDM2(Ser166) antibody, **Figure 2B** shows that cIAP2 siRNA transfection resulted in an increase in the total P-MDM2 (Ser166) detected at both 95 kDa and 120 kDa compared with cells treated with the non-targeting siRNA. SUMO-1-conjugated MDM2 directly interacts with p53[34] and has an apparent molecular weight of 120 kDa.[23,35] In our experiments, we only detected the 120 kDa MDM2 with anti-P-MDM2 (Ser166) and not SMP14, consistent with a requirement for Ser166 phosphorylation for SUMOylation. Again, Nutlin-3a stabilized both p53 and MDM2 following cIAP2 KD. Next, cIAP2 siRNA-transfected cells were treated with actinomycin D for 3 h prior to harvest to prevent de novo transcription. This treatment completely eliminated both non-P-Ser166 MDM2 and the 95 kDa P-MDM2 (Ser166) but had little effect on the SUMOylated form of MDM2. In summary, these data show that cIAP2 KD induces an increase in phosphorylation of MDM2 at Ser166 and SUMOylation of the phosphorylated protein. Once SUMOylated, the MDM2 protein is highly stabilized.

Although cIAP2 KD increased P-MDM2 (Ser166) and SUMO-MDM2, the level of SMP14-reactive MDM2 was strongly reduced (**Fig. 2B**). This was surprising, given that previous studies have shown that NFκB positively regulates MDM2 transcription.[24] To assess if this was a result of reduced MDM2 stability, we treated cells for the final 2 h of culture with the proteasome inhibitor, MG132, after transfection with non-targeting (NT) or cIAP2 siRNA. **Figure 2C** shows that, as expected, MG132 stabilized both the Ser166 phosphorylated and non-phosphorylated forms of 95 kDa MDM2 but had no effect on the 120 kDa SUMOylated form. In contrast, MG132 had little effect on P-MDM2 (Ser166) when added to cIAP2 KD cells relative to cIAP2 KD alone and produced only a small increase in SMP14-reactive MDM2. The reduction in MDM2 protein following cIAP2 KD was therefore not the result of destabilization.

We next performed qRT-PCR to look for effects on MDM2 transcripts. A strong decrease in MDM2 transcripts was observed 48 h following siRNA-mediated cIAP2 KD (**Fig. 2D**), likely subsequent to the concurrent low levels of p53. In order to look at earlier time points after loss of cIAP2, we performed qRT-PCR after treatment with SM164 for short periods. **Figure 2E** shows that MDM2 mRNA levels transiently increased immediately following reduction of the cIAPs and then were rapidly reduced. To confirm that NFκB activation was required for the induction of MDM2 transcription after cIAP2 KD, we transiently transfected cells with the mutant IBα (IBα super repressor mutated at Ser 32/36), which blocks canonical NFκB activation. **Figure 2F** shows that inhibition of canonical NFκB strongly reduced MDM2 after cIAP2 KD.

Overall, these data indicate that cIAP2 KD transiently induces MDM2 transcription, in an NFκB-dependent manner followed by its phosphorylation at Ser166 and SUMOylation to mediate p53 reduction. Thereafter, MDM2 transcripts are decreased, correlating with the reduction in p53.

IKKα regulates MDM2 SUMOylation while IKKβ regulates MDM2 Ser166 phosphorylation. We next assessed the roles of IKKα and -β in MDM2 and p53 regulation following cIAP2 KD. IKKα siRNA was transfected into MCF-10AT1 cells in the presence of non-targeting or cIAP2 siRNA. **Figure 3A** shows that the sole KD of IKKα reduced SMP14-reactive MDM2. Moreover, we observed an increase in the 120 kDa SUMO-MDM2 associated with a reduction in p53 protein. Inhibition IKKβ activity by adenoviral expression of the IKKβKA kinase decreased both forms of P-MDM2 (Ser166) while markedly increasing the level of SMP14-reactive MDM2 (compare lanes 1 and 3). Thus, IKKβ appears to constitutively promote Ser166 phosphorylation of MDM2, although its activity is not necessary to maintain MDM2 protein under basal conditions. Predictably,

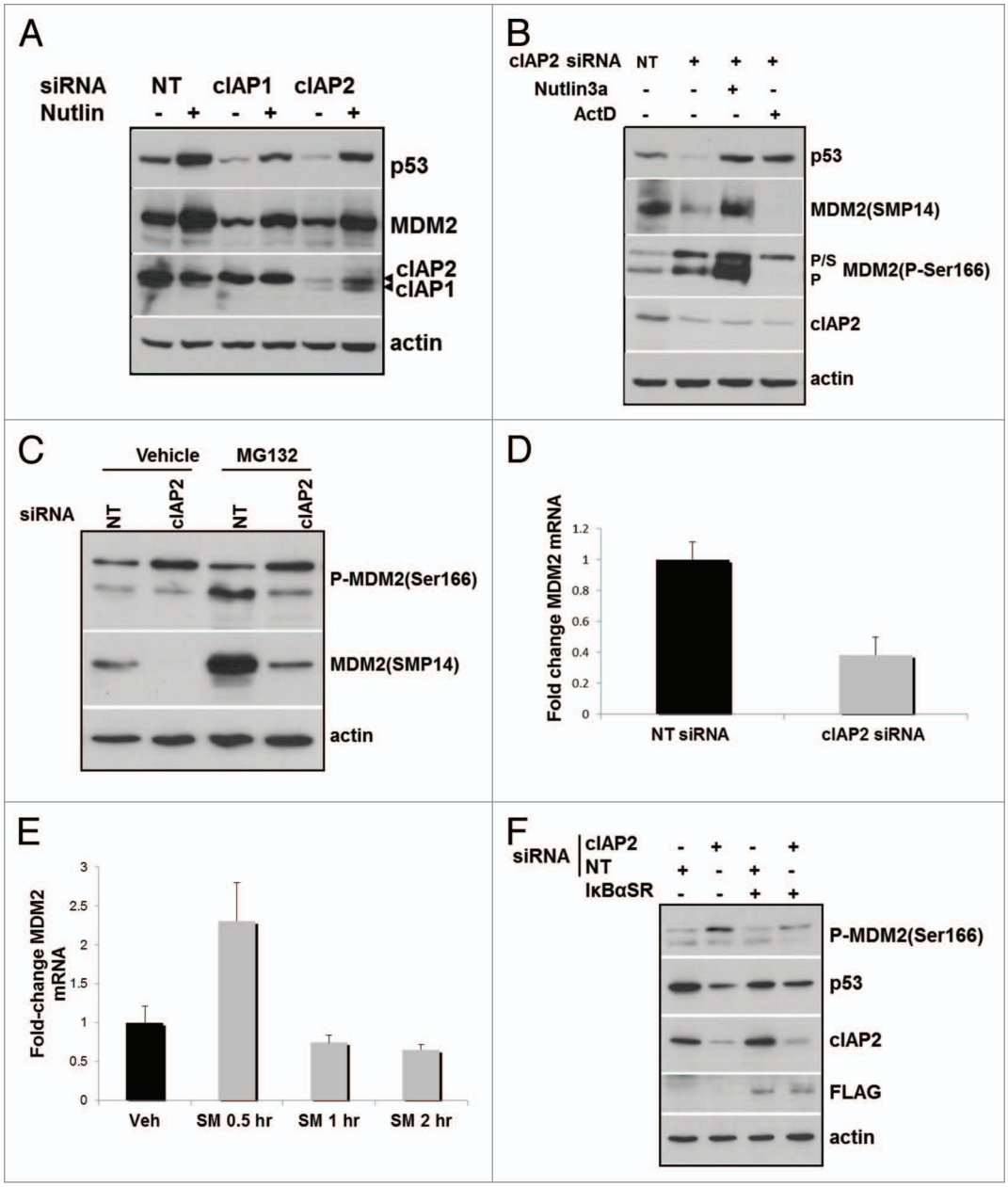

Figure 2. cIAP2 depletion results in posttranslational modifications of MDM2 (**A**) MCF-10AT1 cells were transfected with either a non-targeting (NT) or cIAP2 siRNA for 48 h as described in Materials and Methods. Some lanes contain protein lysate from cells incubated for 3 h prior to harvesting with Nutlin-3a (10 μM). Fifteen μg of cell lysate was subjected to immunoblotting for p53, MDM2 and cIAP1/2. (**B**) MCF-10AT1 cells were transfected with cIAP2 or NT siRNA as above. Some cells were treated with actinomycin D (ActD) (2 μg/mL) for the final 3 h. Antibodies for immunoblotting were p53, MDM2 (SMP14), which recognizes 95 kDa MDM2, P-MDM2 (Ser166) and cIAP2. (**C**) MCF-10AT1 cells were transfected with siRNA for cIAP2 or NT as indicated. Cells were then treated with MG132 or vehicle (DMSO) for the final 2 h. Lysates were immunoblotted for MDM2 (SMP14) and P-MDM2(Ser166). (**D**) MCF-10AT1 cells were transfected with either NT or cIAP2 siRNA as indicated. RNA was extracted 48 h later and qRT-PCR was performed to assess levels of MDM2 mRNA. (**E**) MCF-10AT1 cells were treated with SM164 for the indicated times. RNA was extracted and qRT-PCR was performed to assess levels of MDM2 mRNA and expressed as fold difference compared with vehicle (DMSO) treated cells. (**F**) Cells were transfected with cIAP2 siRNA or NT in the presence or absence of an expression construct for Flag-tagged IκBαSR and harvested after 48 h of culture. Protein lysates were used for immunoblotting as shown. The latter experiment was performed once. All other experiments were independently repeated three times. In all immunoblot experiments, actin was used as a protein loading control.

p53 levels remained unchanged. Consistent with a requirement for Ser166 phosphorylation prior to SUMOylation, the simultaneous IKKα ablation and IKKβ inhibition resulted in an overall decrease in P-MDM2 (Ser166) as well as the accumulation of SMP14-reactive MDM2 without a detectable change in the level of p53.

We next examined the effects of IKK inhibition in the context of cIAP2 KD. While a low basal level of NFκB activity is present

in MCF-10AT1 cells, cIAP2 KD activates both IKKα and -β. As in **Figure 2B**, cIAP2 siRNA transfection resulted in an increase in the 120kDa P-Ser166/SUMO-MDM2 form (**Fig. 3A**, compare lanes 1 and 5) while the level of SMP14-reactive MDM2 was sharply reduced. Co-transfection of siRNA for both IKKα and cIAP2 (lane 6) resulted in a reduction of both forms of P-MDM2 (Ser166) along with a similar loss of SMP14-reactive MDM2 seen after cIAP2 KD alone. Consistent with this, the reduction of postranslationally modified MDM2 was accompanied by a partial rescue of p53. Two separate IKKα siRNAs also partially rescued p53 after cIAP2 KD (**Fig. S1**). Inhibition of IKKβ activity in cIAP2-depleted cells again reduced P-MDM2 (Ser166) and resulted in a large increase in the steady-state levels of SMP14-reactive MDM2. The reduction in P-MDM2 (Ser166) was also associated with partial rescue of p53, which can be attributed to the requirement for Ser166 phosphorylation for activation of MDM2.[36] Simultaneous inhibition of both kinases in the presence of cIAP2 siRNA (lane 7) produced a response with elements of the individual effects, reducing both 120 kDa and 95 kDa-P-MDM2 (Ser166), increasing levels of SMP14-reactive MDM2 and fully rescuing p53.

These results combined with those in **Figure 2** demonstrate that negative regulation of p53 associated with cIAP2 downregulation is dependent on signaling to MDM2 by both IKKα and -β. IKKα activation following cIAP2 KD can increase MDM2 protein levels through activation of IKKβ and the canonical pathway. While IKKβ appears to constitutively promote Ser166 phosphorylation of MDM2, this is further increased following cIAP2 KD and IKK activation. Both the activation and absence of IKKα appear to be permissive for MDM2 SUMOylation suggesting that IKKα has a non-catalytic function in tonically repressing MDM2 SUMOylation.

Loss of cIAP2 releases SUMO ligase PIAS1 from IKKα. The SUMO-E3 ligase PIAS1 can mediate SUMOylation of p53 to inhibit its activity.[37] PIAS1 and other E3 ligases can also SUMOylate MDM2, which increases its ubiquitin ligase activity toward p53.[41] Interestingly, PIAS1 and IKKα have been shown to form a complex in macrophages. IKKα-mediated phosphorylation of PIAS1 at Ser90 disrupts this complex.[38] Thus, we reasoned that cIAP2 KD-associated IKKα activation would similarly release PIAS1, which might then SUMOylate MDM2. To explore this possibility, MCF-10AT1 cells were transfected with NT or cIAP2 siRNA and subjected to reciprocal immunoprecipitation (IP) and immunoblot of endogenous protein with anti-PIAS1 or anti-IKKα. **Figure 4A** demonstrates that PIAS1 and IKKα were present in IKKα and PIAS1 IPs, respectively, in control cells. Strikingly, this interaction was drastically inhibited by cIAP2 KD. Immunoblotting confirmed the cIAP2 KD in lysates of the cIAP2 siRNA-transfected cells used for IP and indicated that cIAP2 was not present in the IKKα:PIAS1 complex. In **Figure 4B**, non-immune IgG IPs confirmed the specificity of the IP antibodies. Thus, loss of cIAP2 and accompanying activation of IKKα disrupts its interaction with PIAS1.

To assess the role of PIAS1 in SUMOylation of MDM2 following KD of cIAP2, we co-transfected PIAS1 siRNA into MCF-10AT1 cells in the presence of cIAP2 siRNA. The immunoblots

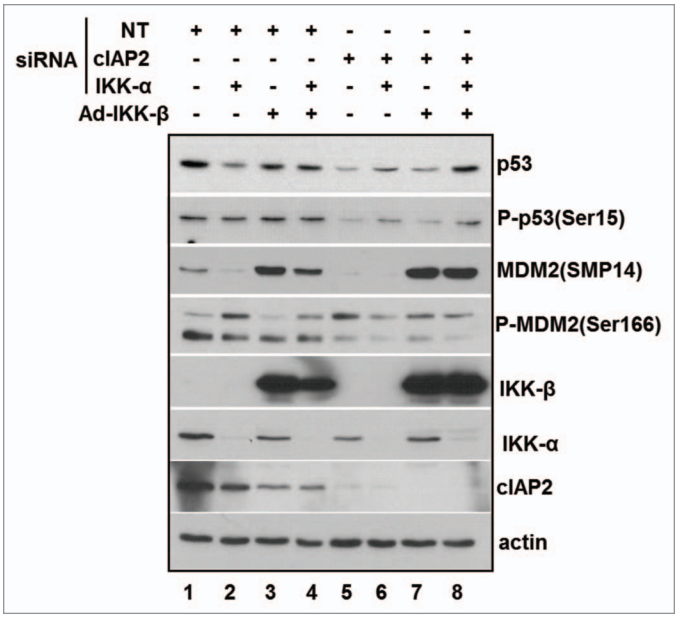

Figure 3. IKKα and IKKβ are both required for MDM2 activation and p53 downregulation following cIAP2 knockdown. MCF-10AT1 cells were transfected with cIAP2 or IKKα siRNA then infected with an adenovirus expressing a dominant-negative mutant of IKKβ. Non-targeting siRNA and adenovirus expressing GFP were used as transfection and infection controls. Protein extracts were immunoblotted for p53, P-p53 (Ser15), MDM2 (SMP14), P-MDM2 (Ser166), IKKβ, IKKα and cIAP2. The immunoblot shown is representative of three separate experiments.

in **Figure 4C** show that KD of cIAP2 again induced both phosphorylated forms of MDM2; however, although KD of both cIAP2 and PIAS1 resulted in a strong decrease in the 95kDa P-MDM2 (Ser166), SUMO-MDM2 underwent a smaller reduction relative to cIAP2 KD alone. As expected, p53 levels were reduced following cIAP2 KD, while transfection of siRNA for PIAS1 along with cIAP2 partially rescued p53 levels. Taken together, these results suggest that either IKKα KD and or its activation following cIAP2 KD may allow SUMOylation of MDM2, at least in part, through a mechanism involving disruption of a complex containing PIAS1 and IKKα.

Stable cotransfection of cIAP2 shRNA activates IKKα and cooperates with *^{V12}H-ras* transformation. Since IAP mutation has been associated with activation of NFκB and correlated with some cancers, we next investigated the effect of cIAP2 depletion following transformation with ^{V12}H-ras. Cells transfected with activated Ras can stabilize and activate wild-type p53 to result in senescence.[39] We therefore reasoned that p53 downregulation in cIAP2-depleted cells might facilitate transformation. To test this, MCF-10A cells were cotransfected with *^{V12}H-ras* and either control non-targeting or cIAP2 shRNA containing plasmids. Stably transfected cells were selected in G418 and clones were isolated and expanded. As shown in **Figure 5A**, cIAP2 shRNA expressing cells formed greater numbers of colonies relative to control clones when cotransfected with *^{V12}H-ras.* The expression of ^{V12}H-ras was confirmed by immunoblot analysis of isolated colonies (**Fig. 5B**). Vector-transfected cells (C) are also shown. All MCF-10A (*^{V12}H-ras*/NT shRNA) and (*^{V12}H-ras*/cIAP2 shRNA)

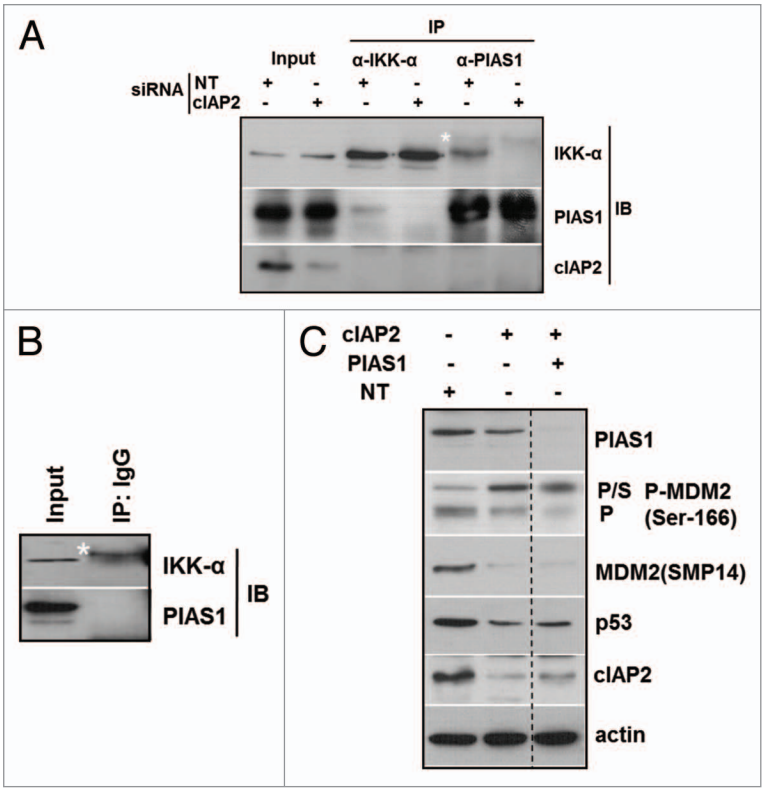

Figure 4. The IKKα interaction with PIAS1 SUMO ligase is disrupted following cIAP2 downregulation. (**A**) MCF-10AT1 cells were transfected with cIAP2 or NT siRNAs and 250 μg of protein were immunoprecipitated with anti-IKKα or anti-PIAS1. Complexes were separated by SDS-PAGE and immunoblotted with anti-IKKα or anti-PIAS1. Input was 5% of lysate used for IP and was also reacted with anti-cIAP2 to monitor KD. (**B**) To assess specificity of the IPs, lysate from NTsiRNA-transfected cells was immunoprecipitated with non-immune IgG and immunoreactivity compared with input lysate by immunoblot with anti-IKKα or anti-PIAS1. (**C**) MCF-10AT1 cells were transfected with NT, cIAP2 and PIAS1 siRNA as indicated. Whole-cell lysates were immunoblotted for PIAS1, P-MDM2 (Ser166) and MDM2 (SMP14). Note that cell lysates were run on the same gel but one lane was moved next to a non-adjacent lane as indicated for clarity. The asterisks in (**A and B**) indicate a higher mol wt anti-IKKα cross-reactive band. Actin was used as a loading control. Images shown are representative of two independent experiments.

clones expressed on average twice the level of H-ras relative to vector control cells, indicative of similar transfection efficiencies between colonies. Comparison of ^{V12}*H-ras* transfected cells with vector-transfected MCF-10A cells showed that the expression of cIAP2 was strongly increased in ^{V12}*H-ras*-transfected cells, consistent with a previous report.[40] Although p53 levels were not different in ^{V12}H-ras expressing cells relative to vector-transfected cells, p53 Ser15 phosphorylation was increased. Transfection of ^{V12}*H-ras* and cIAP2 shRNA in isolated colonies resulted in an average decrease in cIAP2 protein of approximately 40% in cIAP2 relative to control MCF-10A (^{V12}*H-ras*/NT shRNA). This partial KD cIAP2 may be due in part to the strong counterinduction of cIAP2 by both ^{V12}H-ras and NFκB signaling and is similar to the reduction of cIAP2 achieved by others with a different cIAP2 targeted duplex.[41] However, even stable partial reduction of cIAP2 expression consistently resulted in reduced p53/P-p53 (Ser15). Downregulation of cIAP2 also resulted in an increase in both total and phosphorylated NIK, consistent with the role of cIAP2 as a ubiquitin ligase for NIK, targeting it for degradation.[6] The DNA damage-induced protein GADD45α is negatively regulated by NFκB and positively regulated by p53.[42] Immunoblotting with anti-GADD45α demonstrated a strong reduction of this protein in cells expressing cIAP2 shRNA. cIAP2 shRNA had no effect on cIAP1 protein levels, indicating that the observed reduction in p53 was attributable to the reduced cellular levels of cIAP2 protein.

Since cIAP downregulation has been shown to activate NFκB through stabilization of NIK,[5,7] we next assessed activation of IKKs using phospho-IKK antibodies. ^{V12}H-ras expression alone induced IKKβ phosphorylation (P-IKKβ); however, stable KD of cIAP2 resulted in the differential appearance of P-IKKα (**Fig. 5C**). No change in the expression of either IKKα or IKKβ was observed. To assess NFκB, we performed EMSA using a canonical NFκB DNA probe on nuclear extracts from two pooled ^{V12}*H-ras*/NTshRNA clones and three pooled ^{V12}*H-ras*/cIAP2 shRNA clones. **Figure 5D** shows that ^{V12}*H-ras* expression in MCF-10A cells produced a small induction of NFκB activity; however, NFκB was markedly increased in cells expressing cIAP2 shRNA. Supershift analysis indicated that DNA-binding activity was entirely attributable to canonical p50/NFκB1 as either a homodimer (lower complex) or heterodimer (upper complex). Replicate samples of the same nuclear extracts were used to shift an SP1 probe to provide evidence of equivalent loading (**Fig. 5E**). Thus, cIAP2 downregulation activates the canonical NFκB pathway in ^{V12}H-ras-transformed cells.

Together, these results show that partial reduction in cIAP2 is sufficient to activate NFκB and this activation is associated with enhanced ^{V12}*H-ras*-induced colony formation.

Discussion

The anti-apoptotic survival functions of the cIAPs have been intensively researched, and their ability to either directly or indirectly inhibit caspase activation is well-described. From this standpoint, cIAP proteins are attractive targets for facilitating apoptosis in cancer cells. However, the presence of biallelic deletions, mutations and gene arrangements involving proteins within the TNFR1 complex have been associated with carcinogenesis in both multiple myeloma[43,44] and splenic marginal zone lymphoma.[45] It has been hypothesized that cIAP inactivation supports tumor progression through NIK-mediated activation of IKKα and the alternative NFκB pathway.[43,44] The activity of the alternative NFκB pathway is largely dependent on the steady-state level of NIK, which is constitutively targeted for degradation by a multi-subunit ubiquitin ligase complex composed of TNF receptor-associated factor 3 (TRAF3), TRAF2 and cIAP1/2.[6,46,47] Thus, mutations or alterations in any of these proteins could lead to accumulation of NIK, activation of IKKα and

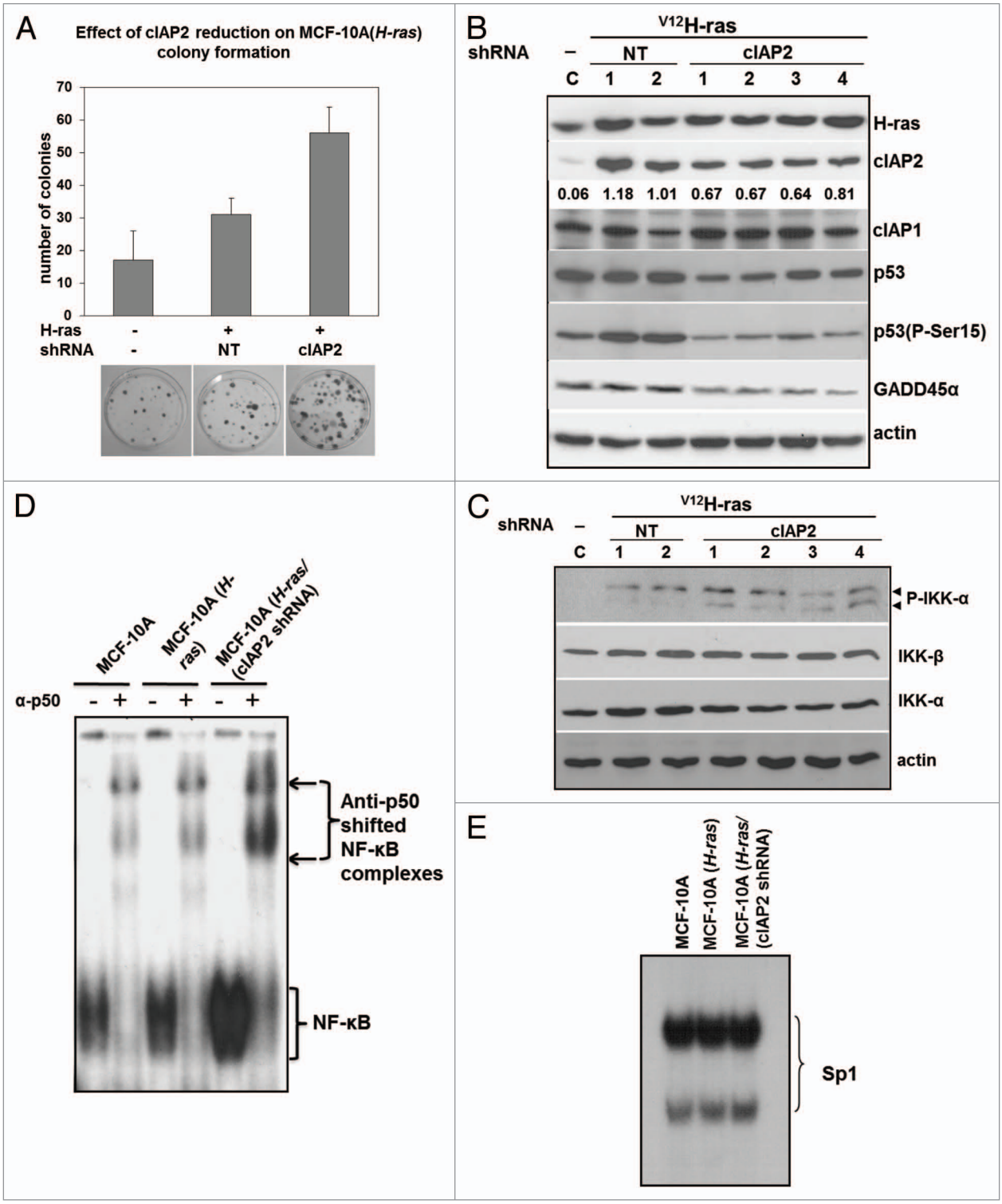

Figure 5. Reduction in cIAP2 protein levels in vitro increases colony formation in ^{V12}H-ras-expressing human mammary epithelial cells, activates IKKα and canonical NFκB. MCF-10A human mammary epithelial cells were cotransfected with ^{V12}*H-ras* with either cIAP2 shRNA or a control shRNA or transfected with vector only (pcDNA3). (**A**) Colonies were selected in G418 in triplicate and stained in 0.2% crystal violet for enumeration and results presented for cells transfected with the indicated plasmids. Bars represent mean ± SD of triplicate plates. (**B**) Immunoblot analysis of protein lysates derived from stable clones of MCF-10A cells transfected with ^{V12}*H-ras* + control shRNA or ^{V12}*H-ras* + cIAP2 shRNA. Lysates were immunoblotted for H-ras, cIAP2, NIK, P-NIK, p53, P-p53 (Ser15) and GADD45α. Densitometry is shown for cIAP2 protein. (**C**) Stable clones were immunoblotted for phospho-IKKα/β and total IKKα and IKKβ. Actin was used as a control for protein loading. (**D**) Nuclear extracts from the indicated stable clones were subjected to EMSA using an NFκB oligonucleotide as a probe. Supershift analysis was performed using anti-p50 antibody. Shifted and supershifted complexes are indicated. (**E**) The same nuclear extracts shifted on probe for Sp1 as a loading control. Images shown are representative of two independent experiments.

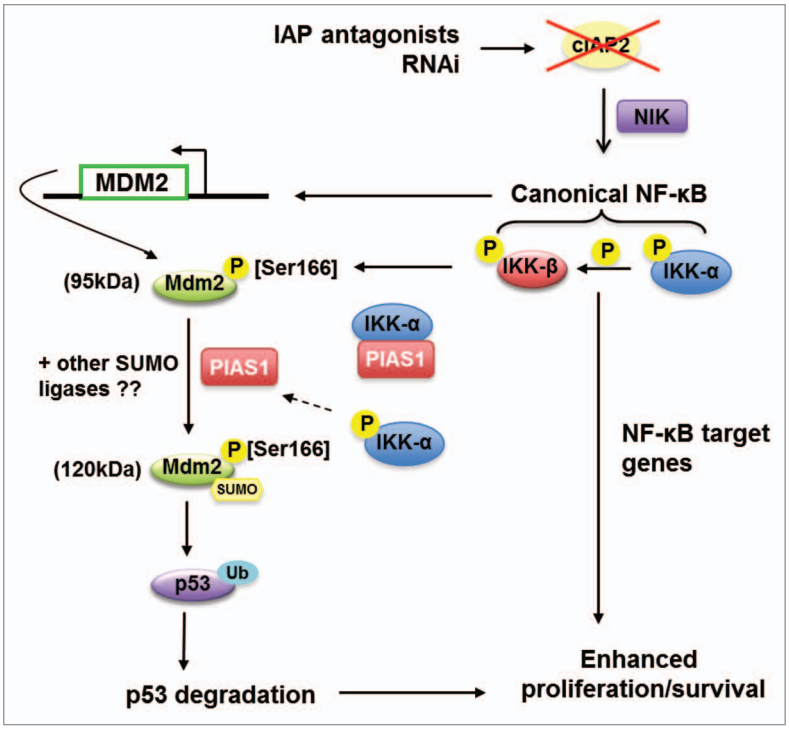

Figure 6. Hypothetical model of cIAP2-dependent regulation of p53. cIAP2 reduction results in the phosphorylation of IKKα which then activates IKKβ resulting in canonical NFκB activity. NFκB promotes a transient increase in MDM2 transcription, increasing the pool of MDM2 while IKKβ promotes MDM2 phosphorylation at Ser166. An IKKα:PIAS1 interaction is disrupted after cIAP2 KD and IKKα activation, which may contribute to the SUMOylation of MDM2. Induction and activation of MDM2 promotes the degradation of p53, which cooperates with NFκB to support cellular proliferation and survival.

the alternative pathway.[6,48-50] Our results suggest that the cellular level of cIAPs are, in fact, precisely set to prevent stabilization of NIK. In MCF-10A (^{V12}H-ras/cIAP2 shRNA) stable clones, in which only a partial reduction of cIAP2 was obtained after long-term culture, both NIK protein and NFκB activity were increased. Moreover, cIAP2 KD alone was sufficient to induce NFκB and inhibit p53. Consistent with this result, cIAP deletions in splenic marginal zone lymphomas are invariably monoallelic, indicative of the haploinsufficiency of these genes.[45]

Although NIK was phosphorylated after cIAP2 KD, EMSA analysis of cIAP2 shRNA transfected cells demonstrated activation of the canonical pathway. Canonical pathway activation following treatment with IAP-antagonists has also been previously reported.[7] In IKKα/β$^{-/-}$ MEFs, MDM2 levels were shown to be regulated by IKKβ and canonical pathway signaling but not by IKKα.[24] Given this, cIAP2 KD and subsequent IKKα activation might not be expected to affect p53. It is likely that cIAP2 KD induced the appearance of canonical NFκB complexes as a result of IKKα-mediated IKKβ activation. Indeed, activated P-IKKα can mediate activation of IKKβ through transphosphorylation, resulting in strong activation of the heterodimeric kinase complex and the canonical pathway.[16,51]

MEFs lacking IKKα would represent similar conditions to our experiments involving KD of IKKα alone, which resulted in increased SUMO-MDM2. Thus, in IKKα$^{-/-}$ MEFs it would appear that IKKα does not have a role in regulation of MDM2 and p53. On the other hand, IKKβ would be indispensable in these cells for phosphorylation of MDM2 (Ser166) and induction of canonical NFκB. Importantly, IKKα KD alone increased SUMO-MDM2 and reduced SMP14-reactive MDM2 and p53 levels. This finding, combined with the ability of cIAP2 KD to activate IKKα, suggests that IKKα KD and activation of IKKα/-β have qualitatively similar effects on MDM2. Thus, IKKα can repress MDM2 SUMOylation in a non-catalytic manner. Interestingly, the MDM2 SUMO ligase PIAS1 can also be phosphorylated by activated IKKα on Ser90, which results in dissociation of a PIAS1:IKKα complex.[38] Immunoprecipitation of both PIAS1 and IKKα showed that these proteins interact in control transfected cells but not in cIAP2-depleted cells in which IKKα is activated. Therefore, either activation of IKKα or IKKα KD would eliminate the IKKα:PIAS1 interaction and potentially facilitate SUMOylation of MDM2. When both cIAP2 and PIAS1 were knocked-down, we observed a small decrease in SUMO-MDM2 accompanied by a substantial reduction in 95kDa P-MDM2 (Ser166) compared with cIAP2 siRNA alone. There are several possibilities that might account for these results. First, our results indicate that the 95 kDa P-MDM2 (Ser166) is less stable relative to SUMO-MDM2, since it was highly sensitive to transcriptional inhibition. Moreover, depending on the kinetics of each siRNA-mediated KD, it is possible that significant levels of P-MDM2 (Ser166) are SUMOylated and stabilized before the effect of PIAS1 KD occurs. Nonetheless, we cannot rule out that since multiple SUMO E3 ligases have been identified for MDM2,[22] PIAS1 KD alone may not be sufficient to prevent the SUMOylation of the entire pool of MDM2.

MCF10-AT1 cells have a constitutive level of NFκB activity, and our experiments suggest that IKKβ actively promotes the phosphorylation of MDM2 at Ser166 with or without cIAP2 KD in these cells. The underlying mechanism is not clear, but could involve IKKβ itself or a downstream kinase. Pim-2, a kinase capable of MDM2-Ser166 phosphorylation in response to growth factor signaling,[52] is regulated by NFκB[53] and could be a candidate kinase for NFκB-inducible MDM2-Ser166 phosphorylation.

^{V12}*H-ras*-induced colony formation was enhanced after knockdown of cIAP2 in association with increased NFκB and decreased p53 protein. However, it is important to note that the interaction between p53 and IKKs as well as NFκB is complex and context-dependent. For example, the RelA and p52 subunits can directly interact with p53 to function as transcriptional co-regulators.[54] Key targets of this integrated transcriptional regulation include genes involved in control of cell death and proliferation, including SKP2 and p21^{WAF1} [55] and p53AIP1.[56] In each case, the regulation of the gene by wtp53 was modified by the presence of NFκB, and co-regulation resulted in the augmentation of growth inhibitory

and/or proapototic gene expression. NFκB transcriptional coactivation with wtp53 has most often been shown in cells in which p53 has been activated as part of the DNA damage respons.e[55,57] DNA damage can result in post-translational modifications of NFκB subunits that augment the p53/NFKB2 (p52) collaborative response.[55] In contrast, NFκB has previously been shown to potentiate *V12H-ras*-mediated transformation of mouse fibroblasts through a mechanism involving co-regulation of ras-induced genes.[58] Moreover, similar to our results in non-transformed MCF-10A cells, constitutive NFκB activity delays *V12H-ras*-induced premature senescence.[59] Overall, the outcome of increased constitutive NFκB activity following cIAP2 knockdown, resulting in the downregulation of p53, would be reduction of cooperative growth inhibitory signaling between p53 and NFκB.

While our results show that the IKKs can serve to activate MDM2 and reduce p53 protein levels, competing mechanisms associated with oncogene expression also induce p53. Oncogene-mediated induction of p14ARF-/p19ARF inhibits MDM2 function. Moreover, oncogene-induction of stress-associated ribosome biogenesis can result in ribosomal protein binding to MDM2 resulting in its inactivation.[60,61] Interestingly, the latter pathway appears to be specifically activated following oncogenic *c-myc* but not *H-ras*-mediated transformation[62] and therefore not a factor in the *V12H-ras* transformed MCF-10A cells in our study. Clearly, induction of the IKKs, in this instance resulting from knockdown of cIAP2, can override the MDM2 inactivating pathways to reduce p53 which would explain, at least in part, the NFκB contribution to cellular transformation.

In summary, we present a model in **Figure 6** that depicts the roles of IKKα and IKKβ following KD of cIAP2 in promoting the transcriptional activation and posttranslational modification of MDM2 to facilitate the ultimate degradation of p53. Our in vitro evidence shows that a reduction in cellular cIAP2 is sufficient to promote oncogene-induced colony formation in MCF-10AT1 cells that express wild-type p53. Consistent with this cIAP2 disruption has been found most often in high-risk chronic lymphocytic leukemia in which p53 is wild-type.[63] Thus, cIAP2 mutation or partial reduction could have ramifications on the promotion of various cancers resulting not only from IKKα-initiated NFκB activation, but also the downregulation of wild-type p53.

Materials and Methods

Cells culture. MCF-10A human mammary epithelial cells were purchased from the American Type culture collection. MCF-10AT1 cells (derived from xenograft of *V12H-ras* transfected MCF-10A cells[26]) were obtained from Dr. L. Murphy, University of Manitoba. MCF-10A and MCF-10AT1 cells were maintained in Ham's F12:DMEM (1:1) (GIBCO), 20 ng/mL epidermal growth factor (EGF) (Sigma), 10 μg/mL insulin (Sigma), 500 ng/mL hydrocortisone (Sigma) and 5% horse serum (GIBCO).

Transfections. Reverse transfections were performed for siRNA transfections. In 60 mm dishes, 1 mL of serum-free medium was mixed with 5 nM of the indicated siRNA and 5 μL of Dharmafect I (Dharmacon), then incubated at room temperature for 20 min. Cells were seeded on top of the transfection mixture. Cells were grown for 48 h prior to harvesting. The IκBαSR (the kind gift of Dr. A.S. Baldwin) plasmid was transfected into MCF-10AT1 cells using Fugene6 (Roche) and 18 h later, cells underwent reverse transfection with cIAP2 siRNA as described above.

siRNA sequences. siRNA targeted at cIAP1 (SMARTpool #M-004390–02–0005), cIAP2 (SMARTpool #M-004099–02–0005), XIAP (SMARTpool #M-004098–01–0005, IKKα #4 (siGENOME #D-003473–04–0005), IKKα #5 (siGENOME #D-003473–05–0005), PIAS1 (SMARTpool #M-008167–01–0005), non-targeting siRNA #1 (siGENOME #D-001210–01–05) were purchased from Dharmacon.

Colony-forming assay. MCF-10A cells were cotransfected using Fugene6 with oncogenic *V12H-ras* cDNA (gift of Dr. D. Gray, Ottawa Regional Cancer Centre) with either a control NT shRNA or a human cIAP2-shRNA construct. Cells were selected in 500 μg/ml G418 and colonies with a minimum of 50 cells were enumerated after staining with 0.2% crystal violet. Separate plates were used for colony isolation. Clones with reduced cIAP2 and overexpression of H-ras were expanded.

Treatments. MCF-10AT1 cells were treated with 10 μM Nutlin-3a (Sigma) for 3 h and/or 1 μg/mL Actinomycin D (Life Technologies) for 3 h prior to harvesting protein in RIPA buffer. For some experiments, 2 μM MG132 (Sigma) was added for the final 2 h of culture.

Adenovirus infection. Cells were plated in complete medium in 60 mm dishes. Twenty-four hours later, cells were infected with adenovirus expressing a kinase-dead mutant (K44A) of IKKβ[64] at 25 MOI. Adenovirus expressing GFP was used as an adenoviral control. Protein was extracted 24 h later in RIPA buffer and analyzed by western blotting.

Immunoblots and antibodies. Whole-cell extracts were prepared in RIPA buffer and western blotting was performed as previously described.[65] Tumor samples were processed as whole tumor extracts for immunoblot analysis as previously described.[65] The following primary antibodies were used for western blot analysis: anti-actin #A-2066, and anti-Mdm2 SMP14 #M4308 and anti-FLAG (M2) were from Sigma; anti-cIAP1 #AF818 was from R&D Systems; anti-cIAP2 #S2700 was from Epitomics; anti-GADD45-α (C-4) #sc-6850, anti-phospho-NIK (Thr559) #sc-12957, anti-p21 (C-19) #sc-397, anti-p53 (BP-53) #sc-263 were from Santa Cruz; anti-IKKα #2682, anti-IKKβ #2684, phospho-IKKα/β (Ser176/180) #2697, anti-phospho-Mdm2 (Ser166) #3521, anti-NIK #4994, and anti-phospho-p53 (Ser15) #9284 were from Cell Signaling; anti-PIAS1 #ab32219 and anti-H-Ras #ab32417 were from Abcam; anti-rIAP1to detect cIAP1 and cIAP2, and anti-rIAP3 to detect XIAP were the kind gift of Dr. R. Korneluk and described.[66]

To quantify the bands obtained via western blot analysis, ImageJ software based analysis (www.rsb.info.nih.gov/ij/) was used. The area under curve (AUC) of the specific signal was corrected for the AUC of the loading control. The data are representative of independent experiments performed at a minimum of three times.

q-RT-PCR. Cells were seeded in triplicate on 35 mm dishes. Total RNA extraction was performed using Trizol (Invitrogen) according to the manufacturer's instructions. Reverse transcription-quantitative PCR (RT-qPCR) reactions were performed using the QuantiTect SYBR Green RT-PCR kit (Qiagen) with 100 ng of RNA and 0.5 uM of the respective primers, according to manufacturer's protocol. Forward and reverse primers for MDM2 and GAPDH were purchased from Invitrogen. Primer sequences for MDM2 were: forward 5'-GAC CCT GGT TAG ACC AAA GC-3' and reverse-5'-CAC GCC AAA CAA ATC TCC TA-3' and for GAPDH: forward-5'-CTC TCT GCT CCT CCT GTT CG-3' and reverse-5'-ACG ACC AAA TCC GTT GAC TC-3'. Reverse transcription was performed at 50°C for 30 min followed by initial PCR activation at 95°C for 15 min. Forty cycles consisting of a denaturing step at 94°C for 15 sec, an annealing step at 55°C for 30 sec, then an extension step at 72°C for 30 sec were performed.

Co-immunoprecipitation (co-IP). MCF-10AT1 cells transfected with siRNA were harvested in co-IP buffer (25 mM Tris-Cl pH7.5, 150 mM NaCl, 50 mM NaF, 0.5 mM EDTA pH8, 0.5% Triton-X, 5 mM β-glycerophosphate, 5% glycerol, 1 mM DTT, 1 mM PMSF and 1 mM $NaVO_3$). Three hundred μg of protein was incubated with 2 μg of the indicated antibody, or rabbit IgG as a control for 2 h at 4°C with rotation. Protein A/G agarose beads (Roche) were added and incubated overnight at 4°C with rotation. Agarose beads bound with immunoreactive complexes were washed four times with co-IP buffer. Following the final wash, immunoprecipitated complexes were eluted with 60 μL of 2x sample buffer and boiled for 5 min. Twenty-five μL of each sample was analyzed using SDS-PAGE as described above with 15 μg of input protein.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were isolated and subjected to EMSA using NFκB site oligonucleotides obtained from Promega as previously described.[65] Equivalence of extract loading was demonstrated by EMSA with a DNA fragment corresponding to the consensus Sp1 binding site (Promega). Supershift analysis was performed by incubating 2 μg of α-p50 (Millipore) with nuclear extract prior to addition of the probe.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cc/article/22223/

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