Inhibition of cIAP1 as a strategy for targeting c-MYC-driven oncogenic activity

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Protooncogene c-MYC, a master transcription factor, is a major driver of human tumorigenesis. Development of pharmacological agents for inhibiting c-MYC as an anticancer therapy has been a longstanding but elusive goal in the cancer field. E3 ubiquitin ligase cIAP1 has been shown to mediate the activation of c-MYC by destabilizing MAD1, a key antagonist of c-MYC. Here we developed a high-throughput assay for cIAP1 ubiquitination and identified D19, a small-molecule inhibitor of E3 ligase activity of cIAP1. We show that D19 binds to the RING domain of cIAP1 and inhibits the E3 ligase activity of cIAP1 by interfering with the dynamics of its interaction with E2. Blocking cIAP1 with D19 antagonizes c-MYC by stabilizing MAD1 protein in cells. Furthermore, we show that D19 and an improved analog (D19-14) promote c-MYC degradation and inhibit the oncogenic function of c-MYC in cells and xenograft animal models. In contrast, we show that activating E3 ubiquitin ligase activity of cIAP1 by Smac mimetics destabilizes MAD1, the antagonist of MYC, and increases the protein levels of c-MYC. Our study provides an interesting example using chemical biological approaches for determining distinct biological consequences from inhibiting vs. activating an E3 ubiquitin ligase and suggests a potential broad therapeutic strategy for targeting c-MYC in cancer treatment by pharmacologically modulating cIAP1 E3 ligase activity.

Significance

Dysregulated expression of master transcription factor c-MYC has been shown to promote proliferation and cell survival programs in cancer cells to mediate resistance to anticancer therapies and promote metastasis. Development of pharmacological agents to inhibit c-MYC as an anticancer therapy is a longstanding but elusive goal in the cancer field. Our study provides a potential widely applicable pharmacological strategy for targeting c-MYC-driven oncogenic activity by inhibiting cIAP1 E3 ubiquitin ligase activity as a treatment for cancers. Furthermore, we demonstrate the pharmacological interference in the dynamic interaction of an E3 ubiquitin ligase with its E2s as a strategy for inhibiting ubiquitination reactions.

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Results
Smac Mimetics Stabilize c-MYC Through Promoting E3 Ubiquitin Ligase Activity of cIAP1. Since cIAP1 has been implicated in promoting the turnover of MAD1, which antagonizes c-MYC (14), we investigated the effect of two Smac mimetics, LCL161 (20) and Birinapant (21), on the protein levels of c-MYC in multiple cancer cell lines that do not die in the presence of Smac. As expected, the treatment with Smac mimetics promoted cIAP1 E3 ubiquitin ligase activity, which led to the degradation of cIAP1 (2–5). Interestingly, the treatment with Smac mimetics dose-dependently increased the levels of c-MYC protein, but not mRNA (Fig. 1 A and B and SI Appendix, Fig. S1 A and B). The effect of Smac mimetics on the levels of c-MYC was dependent on cIAP1 as Smac mimetics could no longer increase the levels of c-MYC in cells with cIAP1 knocked-down or knocked-out (Fig. 1 C and D and SI Appendix, Fig. S1C). We compared the effect of cIAP1 and cIAP2 on the levels of c-MYC individually. We found that knockdown of cIAP1 alone or knockout of both cIAP1/2 was sufficient to reduce levels of c-MYC (Fig. 1 E and F), whereas knockdown of cIAP2 alone had no effect on c-MYC levels (SI Appendix, Fig. S1D). Thus, cIAP1, but not cIAP2, positively regulates the levels of c-MYC.

Since Smac mimetics stimulate the E3 ligase activity of cIAP1 to promote its autoubiquitination and degradation (2–5), we next characterized the role of cIAP1 catalytic activity in regulating the abundance of c-MYC protein in cells overexpressing wild-type (WT) or H588A mutant cIAP1, which contains a mutation in the RING domain rendering it unable to maintain normal E3 ligase activity (22). Overexpression of WT cIAP1 led to increased levels of c-MYC protein whereas overexpression of catalytically inactive H588A cIAP1 did not (Fig. 1G). This finding suggests that promoting E3 ubiquitin ligase activity of cIAP1 can increase the levels of c-MYC.

MAD1 Promotes c-MYC Ubiquitination and Degradation. As MAD1, a key antagonist of c-MYC, is degraded by cIAP1-mediated K48 ubiquitination (14), we then examined if the increased levels of c-MYC protein in cells treated with Smac mimetics were dependent on MAD1. We found that the treatment with LCL161 dose-dependently decreased the levels of MAD1 protein in H299 cells (Fig. 2A). Moreover, in cells with MAD1 knockdown, LCL161 treatment could no longer increase the levels of c-MYC, suggesting that MAD1 is involved in mediating Smac mimetics-induced c-MYC up-regulation (Fig. 2B). We then asked if increasing the levels of MAD1 might regulate the stability of c-MYC. We found that overexpression of MAD1 reduced the level of c-MYC protein (Fig. 2C). Conversely, MAD1 knockdown increased the levels of c-MYC (Fig. 2D). Consistently, c-MYC was more extensively modified by polyubiquitination when coexpressed with MAD1 than when expressed alone in 293T cells (Fig. 2E).

Because MAD1 competes with c-MYC for dimerization with MAX (15, 23), we hypothesized that MAX/c-MYC dimerization might protect c-MYC from proteasomal degradation. Indeed, overexpression of MAX increased the levels of c-MYC protein (Fig. 2F). Meanwhile, the polyubiquitination levels of c-MYC were reduced by coexpression with MAX, suggesting that dimerization of c-MYC with MAX may provide the resistance to proteasomal degradation by reducing its ubiquitination (Fig. 2G). These results suggest that Smac mimetics alter the balance of MYC/MAX and MYC/MAD heterodimers by promoting the E3 ubiquitin ligase activity of cIAP1.

Identification of a Small-Molecule D19 as an Inhibitor of cIAP1 E3 Ligase. To directly test the possibility of inhibiting cIAP1 as a strategy to antagonize c-MYC, we developed a colorimetric assay using malachite green to quantitatively measure the levels of cIAP1 ubiquitination in vitro. This assay uses ATP consumption by E1 as a surrogate marker for E3 ubiquitin ligase activity. The pyrophosphate produced by E1-mediated ATP hydrolysis is hydrolyzed into phosphate by a pyrophosphatase that can form a colorimetric complex with malachite green (SI Appendix, Fig. S2 A–C). Using this assay, we screened a chemical library of ~50,000 compounds and identified a compound named D19 that inhibited cIAP1 autoubiquitination with IC50 of 14.1 μM (Fig. 3 A

Fig. 1. Smac mimetics stabilize c-MYC by promoting the E3 ubiquitin ligase activity of cIAP1. (A and B) The effects of Smac mimetics on protein levels of cIAP1 and c-MYC. H299 and MCF7 cells were treated with LCL161 (A) or Birinapant (B) at the indicated concentrations for 4 h. (C and D) The up-regulation of c-MYC protein levels by Smac mimetics is mediated by cIAP1. H299 cIAP1 knockdown cells (G) or cIAP1/2 double-knockout MEFs (D) were treated with LCL161 at the indicated concentrations for 4 h. (E and F) H299 cells expressing nontarget siRNA, cIAP1 siRNAs, or cIAP1/2 siRNAs were harvested for Western blotting using the indicated antibodies. (G) HEK293T cells were transfected with Flag-cIAP1 or Flag-cIAP1 H588A. After 48 h, the cells were harvested for Western blotting using the indicated antibodies.
D19 Changes the Dynamics of cIAP1 and E2 Interaction. To elucidate the molecular basis for the inhibition of cIAP1 ligase activity by D19, we first investigated whether the initial E1 activation and the concomitant step of E2-ubiquitin thioester formation were adversely affected by D19. We found that D19 had no effect on the formation of UbcH5b-ubiquitin thioester catalyzed by E1 (SI Appendix, Fig. S3A). We next assessed the role of cIAP1’s caspase-activating recruitment domain (CARD) as it is involved in promoting autoinhibition by preventing RING dimerization and binding to E2 (26). Autoubiquitination of the ΔBIR1/2CARD cIAP1 mutant was also significantly impaired by D19 (SI Appendix, Fig. S3B), indicating that the inhibitory effect of D19 was independent of the BIR1/2 or CARD.

The activation of cIAP1 involves dimerization (2). However, we found that the treatment of D19 could not promote the dimerization of Flag-tagged cIAP1 with GST-tagged cIAP1 (SI Appendix, Fig. S2D). In contrast, an inactive analog of D19, D19-Cl, which is unable to inhibit c-IAP1 autoubiquitination (SI Appendix, Fig. S3C), was also significantly impaired by D19 (SI Appendix, Fig. S3B), indicating that the inhibitory effect of D19 was independent of the BIR1/2 or CARD.

The catalytic transfer of ubiquitin from E2 to substrate involves the rapid assembly and disassembly of the E2–E3 complex, which is critical for progressive ubiquitination reactions (27). We next asked if D19 could affect the association of cIAP1 with E2. Under normal conditions, purified Flag-cIAP1 protein bound weakly to GST-UbcH5b. Interestingly, this interaction was significantly stabilized in the presence of D19 (Fig. 4A). In contrast, an inactive analog of D19, D19-Cl, which is unable to inhibit c-IAP1 autoubiquitination (SI Appendix, Fig. S3C and F), had no impact on cIAP1/UbcH5b binding (SI Appendix, Fig. S3G).
Moreover, we found that D19 did not inhibit clcAP1-Ub conjugate formation when preloaded E2-Ub was incubated with clcAP1 (SI Appendix, Fig. S3H). Taken together, we conclude that D19 may impair clcAP1 ligase activity by stabilizing the dynamic interaction of clcAP1 and the E2 complex to prevent successive rounds of clcAP1 and E2-Ub interaction in ubiquitination reaction.

To identify the protein-targeting mechanism of D19, we performed a differential scanning fluorimetry (DSF) assay to measure the protein melting temperature (Tm) change induced by D19. The presence of D19 dose-dependently increased the Tm of GST-clcAP1-RING domain protein (SI Appendix, Fig. S3), suggesting that D19 may directly bind to RING domain. Furthermore, using a BioLayer Interferometry assay, we found a clear dose-dependent interaction of D19 with the RING domain protein immobilized on the biosensor tip (Fig. 4C). Taken together, these results suggest that D19 inhibits clcAP1 ubiquitination activity by binding to the RING domain to interfere its dynamic interaction with E2.

Since Smac mimetics are known to promote the E3 ubiquitin ligase activity of clcAP1, we next characterized the effect of D19 on ubiquitination of clcAP1 with Smac. We found that D19 was highly effective in blocking clcAP1 autoubiquitination induced by Smac mimetics (Fig. 4D). Thus, the ability of D19 to block the dynamics of the E2-E3 complex is dominant over the activity of Smac mimetics in promoting clcAP1 E3 ligase activity.

**D19 Promotes the Proteasomal Degradation of c-MYC.** In contrast to Smac mimetics, which promoted increases in the levels of c-MYC (Fig. 1A and B), we found that treatment with D19 led to a dose- and time-dependent reduction of c-MYC and an increase of MAD1 protein levels with no significant effect on their mRNA levels (Fig. 5A and B and SI Appendix, Fig. S4A). As a result, D19 treatment reduced the prevalence of bound dimeric c-MYC/MAX while increasing that of MAD1/MAX (Fig. 5C). Consistent with the role of clcAP1 as an E3 ubiquitin ligase of MAD1 (14), we found that treatment with D19 inhibited the ubiquitination of MAD1 by clcAP1 (Fig. 5D). In contrast, the inactive analog D19-Cl had no effect on the levels of MAD1 protein in cells, confirming that D19 acts directly on the ubiquitination functions of clcAP1 (SI Appendix, Fig. S4B). In addition, D19 had no effect on p53 ubiquitination by MDM2 or on total protein ubiquitination (SI Appendix, Fig. S4 C and D). Treatment with D19 slowed the decay of MAD1 protein levels in the presence of the protein synthesis inhibitor cycloheximide (CHX) (Fig. 5E). The effect of D19 was clcAP1-dependent as D19 was unable to increase MAD1 protein in clcAP1 knockdown H1299 cells or clcAP1/2 double-knockout mouse embryonic fibroblasts (MEFs) (SI Appendix, Fig. S4 E and F). These data suggest that D19 is a selective inhibitor of clcAP1 ubiquitination activity in vivo.

We subsequently investigated the mechanism by which D19 regulates c-MYC protein levels. Preincubation with MG132 before D19 treatment blocked the down-regulation of c-MYC (Fig. 5F). Correspondingly, the polyubiquitination of endogenous c-MYC, barely detectable under control conditions even in the presence of MG132 due to its rapid degradation, was markedly enhanced by D19 treatment in the presence of MG132 (Fig. 5F), suggesting that D19 promotes the ubiquitination and proteasomal degradation of c-MYC protein. The accelerated turnover of c-MYC by D19 treatment was mediated by the clcAP1/MAD1 axis because D19 failed to decrease c-MYC protein levels in cells lacking either clcAP1 or MAD1 (Fig. 5G and H).

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**Fig. 3.** Identification of a small-molecule D19 as an inhibitor of clcAP1 E3 ligase. (A) The chemical structure of D19. (B) D19 inhibits clcAP1 autoubiquitination with an IC₅₀ of 14.1 μM. The autoubiquitination assay of clcAP1 was performed in the presence of the indicated concentrations of D19 for 2 h and then monitored by pyrophosphate production. Values are the mean of three independent experiments. (C) D19 inhibits clcAP1 autoubiquitination in a dose-dependent manner. In vitro clcAP1 autoubiquitination assay utilizing 35S-labeled clcAP1 in the presence of DMSO or indicated concentrations of D19 for 1 h. (D) The ubiquitination of MAD1 by clcAP1 is inhibited by D19 in a dose-dependent manner. 35S-labeled MAD1 was subjected to an in vitro ubiquitination assay with purified GST-clcAP1 for 1 h in the presence of DMSO or indicated concentrations of D19.

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**Fig. 4.** D19 changes the dynamics of clcAP1 and E2 interaction. (A) D19 enhances binding of clcAP1 and E2. Flag-tagged clcAP1 was coexpressed with recombinant GST-tagged UbcH5b or GST alone immobilized on glutathione agarose in the presence or absence of D19 (100 μM). (B) D19 inhibits clcAP1 autoubiquitination induced by Smac mimetics. A GST-clcAP1 autoubiquitination assay was performed for 30 min with the indicated concentrations of Birinapant or LCL161 with or without D19 (100 μM).
As we found that the effects of D19 are dominant over those of Smac mimetics, we next examined if D19 could abolish the upregulation of c-MYC protein driven by Smac mimetics. Interestingly, treatment with D19 blocked increases in the levels of c-MYC protein in cells treated by Smac mimetics (Fig. 5J). Thus, inhibition of cIAP1 E3-ubiquitinating activity by D19 can abrogate the activation of c-MYC induced by Smac mimetics.

We next compared D19 with JQ1, a BET bromodomain inhibitor, which has been shown to reduce the levels of c-MYC by repressing transcription (28, 29). We profiled the activities of D19 and JQ1 on c-MYC expression in eight cancer cell lines. We found that, while JQ1 treatment reduced levels of c-MYC in only four of eight cell lines, D19 showed efficacy in all eight cancer cell lines tested (Fig. 5J). Thus, targeting cIAP1 may provide a broad strategy for inhibiting c-MYC across a variety of tumor types.

**Developing cIAP1 E3 Ligase Inhibitors to Antagonize c-MYC in Cancer Treatment.** We next evaluated inhibition of cIAP1 E3 ligase activity with D19 as a potential anti-c-MYC strategy in cancer treatment. We first asked if D19 could antagonize the downstream oncogenic functions of c-MYC in cancer cells. Transcription of *ODC, CDC47, BRCA1*, and *MSH2* is promoted by c-MYC while *p21* is repressed (30–33). We found that treating cells with either D19 or JQ1 diminished the mRNA levels of *ODC, CDC47,*
and BRCA1, and MSH2, although D19 did not change MYC transcription (Fig. 6A). Interestingly, the mRNA levels of p21 were profoundly elevated by D19 treatment in multiple cell lines (Fig. 6B). Consistent with induction of p21, treatment with D19 led to inhibition of DNA synthesis and substantial G1 arrest (Fig. 6C and SI Appendix, Fig. S5 A and B).

Consistent with the ability of cIAP1 to synergistically cooperate with c-MYC to induce tumorigenesis (14, 34), coexpression of cIAP1 and c-MYC readily transformed NIH 3T3 cells, leading to the rapid formation of massive colonies in soft agar, which were effectively eliminated by the treatment of D19 (Fig. 6D). The formation of foci in MCF7 cells was also dose-dependently attenuated by D19 treatment (SI Appendix, Fig. S5C). Moreover, treatment with D19 resulted in pronounced cellular senescence as determined by β-galactosidase staining in treated H1299 cells (SI Appendix, Fig. S5D).

We next examined if the antitumor activity of D19 is dependent on c-MYC expression. We profiled the sensitivity of 25 cancer cell lines treated with D19 (SI Appendix, Table S1). The expression levels of c-MYC in these 25 cell lines obtained from the Cancer Cell Line Encyclopedia (CCLE) were compared with the logarithm of their respective IC50 values. We found that cell lines with high
c-MYC expression were significantly more sensitive to D19 than those with low c-MYC expression (Fig. 6E), suggesting that the antimtumor activity of D19 is dependent on c-MYC overexpression.

Our data suggest that inhibition of cIAP1 E3 ligase activity is a potential strategy to target c-MYC–dependent cancers. We next tested this hypothesis in animal models. Since the pharmacokinetic properties of D19 limited its application for in vivo treatment, we conducted medicinal chemistry optimization of D19 and identified an improved D19 analog, known as D19-14 (Fig. 6F), with a significantly increased ability to inhibit cIAP1 autoubiquitination and reduce protein levels of c-MYC in vitro compared with D19 (Fig. 6 G and H). Consistently, D19-14 was more potent in inhibiting the proliferation of cancer cells than D19 (SI Appendix, Fig. S5E). Patient-derived tumor organoids (PDTOs) have shown the advantage of mimicking the biological characteristics of the human tumor both phenotypically and genetically (35). We therefore tested D19-14 in three PDTOs from breast cancer patients. D19-14 effectively inhibited PDTO proliferation (Fig. 6I and SI Appendix, Fig. S5F) and decreased c-MYC protein levels (Fig. 6J).

In addition, D19-14 exhibited acceptable pharmacokinetics (PK) properties in vivo (area under curve (AUC) 4,075 mM-h and T1/2 4.9 h) and improved bioavailability in tumors (SI Appendix, Fig. S5 G and H). Pharmacodynamic studies indicated that D19-14 effectively decreased the levels of c-MYC protein in tumors (SI Appendix, Fig. S5I). We further evaluated the antitumor activity of D19-14 in a human xenograft model of acute myeloid leukemia using EOL1 cells with a high c-MYC mRNA level (CCLE data). Randomized cohorts of mice with established tumors were treated with D19-14 (50 mg/kg) or vehicle. Daily i.p. administration of D19-14 was well tolerated by mice (SI Appendix, Fig. S5J). The tumor growth was remarkably reduced by 12 d of D19-14 treatment (Fig. 6K and SI Appendix, Fig. S5K). Consistently, treatment with D19-14 led to a clear decrease in the protein levels of c-MYC in tumors compared with that of the vehicle-treated group when examined following the final day of treatment (Fig. 6L). Thus, inhibiting E3 ubiquitin ligase activity of cIAP1 reduces c-MYC levels and cancer growth in vivo.

Discussion

In this article, we describe a study using chemical biological approaches to differentiate the distinct biological consequences of inhibiting E3 ubiquitin ligase activity of cIAP1 by D19 vs. activating the E3 ubiquitin ligase activity of cIAP1 by Smac mimetics. Since the turnover of MAD1 is highly regulated by cIAP1–mediated ubiquitination and proteasomal turnover, rapid activation of cIAP1 E3 activity before cIAP1’s degradation in the presence of Smac mimetics is sufficient to promote the turnover of MAD1 to lead to stabilization of c-MYC, whereas stabilizing the interaction of cIAP1 with its E2 by D19 and D19-14 block successive rounds of ubiquitination reaction, which leads to stabilization of MAD1 and inhibition of c-MYC. Our study provides an interesting example of using chemical biological approaches to determine distinct biological consequences from inhibiting vs. activating an E3 ubiquitin ligase. D19-14 may serve as a lead compound for further development to improve the potency and pharmacokinetic properties as a treatment for cancers with c-MYC dysregulation.

Our study explored the possibility of targeting IAP proteins using pharmacological strategy to block the oncogenic activity of c-MYC. We show that inhibition of cIAP1 by D19 and its analog D19-14 can reduce the levels of c-MYC protein in a broad range of cancer cell lines and in xenograft tumor models in vivo. While ubiquitination modification is known to be critical in controlling key biochemical reactions regulating diverse cellular processes, developing small-molecule inhibitors for E3 ubiquitin ligases has been challenging to date. We show that D19 inhibits cIAP1 ubiquitination activity by binding to the RING domain to interfere with its dynamic interaction with E2. The mechanism by which the RING domain promotes ubiquitin transfer is not fully understood because the RING-binding site on the E2s is distant from the active site. It has been proposed that the RING domain brings the E2-Ub conjugate into close proximity with the substrate (36, 37). Recharging utilized E2 requires the residues within the H1 α-helix to be exposed to engage with E1; however, these residues are normally buried at the E2–E3 interface (36, 38). Therefore, the interactions between RING domains and E2s are generally transient with modest affinity, facilitating E2 “hit-and-run” from E3 before participation in the next cycle of E2-Ub conjugation (39). Meanwhile, E3 is released from E2 and accepts a new E2-Ub molecule for ubiquitin transfer. Thus, the ubiquitin modification of proteins relies on the rapid assembly and disassembly of the E2–E3 complex, which allows the catalytic transfer of ubiquitin from E2 to substrate and the initiation of subsequent rounds of ubiquitination (27).

In this regard, the enhanced interaction between cIAP1 and UbcH5b driven by D19 treatment may disturb the dynamics of the ubiquitin transfer process and result in the termination of ubiquitination. Our data suggest that stabilization of the weak interactions between ubiquitin and ubiquitination enzymes by small molecules may provide a useful strategy to selectively inhibit E3 ubiquitin ligase activities. Thus, D19 provides a prototypic example of stabilizing E2/RING–E3 interaction as an approach to block E3 ubiquitin ligase activity. This strategy may be extended to develop small-molecule inhibitors for other RING finger-E3 ubiquitin ligases, the largest ubiquitin ligase family in the human genome that includes many important E3s such as the anaphase-promoting complex.

While a subset of cancer cell lines can die by TNFα-mediated apoptosis when treated with Smac mimetics, many cancer cells are resistant to Smac mimetics (14, 40, 41). Our study uncovered the ability of Smacs to induce the protein levels of c-MYC in these resistance cancer cell lines. We found that the protein levels of c-MYC were elevated following treatment with Smac mimetics in such Smac nonresponsive cell lines, suggesting that increased levels of c-MYC may provide an underlying mechanism for this resistance. We confirm that Smac mimetics function as cIAP1 agonists to promote cIAP1 E3 ligase activity that triggers ubiquitination and degradation of its substrates, such as MAD1, with close proximity and high affinity. The ubiquitination of MAD1 by cIAP1 is important for the control of MAD1 protein levels in cancer cells; therefore, activating E3 ligase activity of cIAP1 by the treatment with Smac mimetics can boost ubiquitination and degradation of MAD1, resulting in the stabilization of c-MYC as MAD1 is no longer able to antagonize c-MYC’s oncogenic functions. Compared with Smac mimetics, the endogenous mitochondrial protein Smac is released into cytosol, but does not enter into the nucleus (42), which may explain why cellular Smac protein is not able to stabilize c-MYC and instead triggers apoptosis.

The MYC/MAX/MAD network model proposed by Eisenman and colleagues centers on MAX forming transactivating complexes when associated with MYC or repressive complexes when bound to MAD (15, 19). The MYC/MAX/MAD transcription network is tightly controlled through UPS during the cell cycle, proliferation, and differentiation. Both c-MYC and MAD1 are short-lived proteins that dynamically respond to, or guide, cell-cycle determination following external stimuli (15). However, how exactly protein levels of c-MYC and MAD1 are intrinsically balanced and regulated during cell-fate transitions is not fully understood. We found that MAD1 promotes c-MYC ubiquitination and degradation, revealing a mechanism of c-MYC/MAX/MAD network regulation during cell-fate transition. We propose inhibiting cIAP1-mediated ubiquitination of MAD1 as an anti-MYC strategy for the treatment of cancers.
The cDNAs coding Flag-c-MYC, c-MYC, HA-MAX, MAX, Flag-clAP1, clAP1, and Flag-clAP1-H588A were cloned into pM-CS vector. The cDNAs coding Flag-pH3, MDM2, Flag-MAD1, MAD1, Myc-clAP1-H588A, and Myc-clAP1 were cloned into the pMSCV vector (#88469; Addgene). The cDNAs coding BRC1A1, BARD1, Ubch5b, clAP1, clAP1ΔBIR1/2, clAP1ΔRING/ΔCARD, and clAP1-RING were subcloned into pGEX-4T-1 vectors (28-9545-49; GE Healthcare). The cDNAs coding E1 were subcloned into the pMAL vector. Other plasmids were prepared as described previously (14).

The shRNAs targeting human MAD1 and clAP1 were obtained from Sigma-Aldrich (C104450), and the sequences of the siRNA oligonucleotides are shown in S1 Appendix, Table S2. They were annealed and cloned into linearized pLKO.1-puro. All plasmids were verified by DNA sequencing. The other plasmids were prepared as described previously (14).

Materials and Methods

Constructs and Reagents. The cDNAs coding Flag-c-MYC, c-MYC, HA-MAX, MAX, Flag-clAP1, clAP1, and Flag-clAP1-H588A were cloned into pM-CS vector. The cDNAs coding Flag-pH3, MDM2, Flag-MAD1, MAD1, Myc-clAP1-H588A, and Myc-clAP1 were cloned into the pMSCV vector (#88469; Addgene). The cDNAs coding BRC1A1, BARD1, Ubch5b, clAP1, clAP1ΔBIR1/2, clAP1ΔRING/ΔCARD, and clAP1-RING were subcloned into pGEX-4T-1 vectors (28-9545-49; GE Healthcare). The cDNAs coding E1 were subcloned into the pMAL vector. Other plasmids were prepared as described previously (14).

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Statistical Analysis. All data are presented as the mean ± SEM or SD from at least three independent determinations, and statistical analyses were done using the software GraphPad Prism version 6.0 (GraphPad Software). Differences of means were tested for statistical significance with the two-tailed Student’s t-test. The animal protocol (IACUCIRB-2017-003) was approved by The Institutional Animal Care and Use Committee of Interdisciplinary Research Center on Biology and Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences.

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