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ORIGINAL ARTICLE

# Targeting oncogenic K-RAS mutants with small-molecule degrader XMU-MP-9 through NEDD4-1

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Degradation;
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**Abstract** *K-RAS* mutations represent a most prevalent oncogenic alteration in human cancers. Despite tremendous efforts, it remains a big challenge to develop strategies that specifically target the oncogenic K-RAS mutants. Here, taking advantage of our previous finding that NEDD4-1 is an E3 ubiquitin ligase for wild-type RAS proteins, we developed a compound XMU-MP-9 that can promote ubiquitination and degradation of various K-RAS mutants including K-RAS$^{G12V}$, and significantly inhibit proliferation and tumor development of K-RAS mutant harboring cells. Mechanistically, XMU-MP-9 acts as a bifunctional compound to bind the C2 domain of NEDD4-1 and an allosteric site of K-RAS to enhance NEDD4-1 and K-RAS interaction, and to induce a conformational change of NEDD4-1/K-RAS complex to allow NEDD4-1 targeting K128 of K-RAS for ubiquitination. Hence, our study presents an effective way to degrade K-RAS mutants to prevent tumor development.



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## 1. Introduction

Oncogenic mutations of *RAS* are associated with about 30% of all human cancers[1]. Mutations in RAS predominantly occur at G12, G13, and Q61, resulting in a persistence of the guanosine triphosphate (GTP)-bound state of RAS by impairing the intrinsic and GTPase-activating proteins (GAPs)-stimulated GTP hydrolysis reaction[2-4]. Oncogenic RAS mutants can trigger constitutive activation of downstream signaling pathways such as RAF/MEK/ERK signal transduction cascade, leading to uncontrolled cell proliferation, transformation, differentiation, and enhanced cell survival[5,6]. Although mutations of different RAS isoforms (K-RAS, H-RAS, and N-RAS ) are associated with different types of tumors in a non-random distribution pattern, K-RAS is mutated most frequently among the three human RAS proteins[7]. Indeed, targeting K-RAS mutants for cancer therapy has garnered most attention from the researchers.

Despite decades of extensive endeavors, targeting RAS mutants for cancer therapy remains intractable[1,8-10]. Because RAS proteins bind GTP with picomolar affinity, early approach on developing competitive antagonists of GTP binding achieved no success[1,10]. Meanwhile, the challenge of directly targeting RAS is also raised by no deep hydrophobic pockets on the surface of RAS for small-molecule compound binding[11], which led to a perception that RAS proteins are undruggable[1,12]. However, given the prevalence of RAS mutations in cancers, targeting mutated RAS proteins is still drawing very much attention[1,8-10,12,13]. Recent studies reported designs of pan-RAS inhibitors that can simultaneously bind to two or more adjacent sites on RAS proteins, therefore prevent effector protein binding[14,15]. Kim et al.[16] developed a pan-K-RAS inhibitor that can block nucleotide exchange of wild-type K-RAS and a broad range of K-RAS mutants. Another exciting approach was to selectively target $RAS^{G12C}$ mutant by covalently linking an inhibitor to the Cys12[17-20]. Hallin et al.[21] developed a non-covalent K-$RAS^{G12D}$-selective inhibitor taking advantage of the acidic side chain of Asp12, obtaining a 700-fold selectivity for binding to K-$RAS^{G12D}$ *vs.* wild-type K-RAS. Recent studies also developed tri-complex RAS inhibitors, which may induce tri-complex formation of immunophilin protein cyclophilin A, compound, and RAS, therefore blocks interactions of downstream effectors with RAS[22-25]. However, targeting the active state of K-RAS mutants lacking specific active or charged side groups like K-$RAS^{G12V}$ with small-molecule drugs still poses challenges.

Recent advances in the development of targeted protein degradation *via* ubiquitination machinery provide an exciting strategy for targeting undruggable proteins[26,27]. The proximity-inducing agents that can induce a close proximity between an E3 ubiquitin ligase and its non-native target protein include molecular glues and bifunctional compounds[28]. Proteolysis targeting chimeras (PROTACs) are the most advanced type of bifunctional small-molecule degraders, composed by a ligand for binding of protein of interest, another ligand specific for an E3 ubiquitin ligase, and a linker to connect the two ligands[29]. Using this technique, Popow et al.[30] developed von Hippel-Lindau (VHL)-based PROTACs to target various oncogenic K-RAS mutants for degradation. Our previous study showed that NEDD4-1 acts as a general E3 ubiquitin ligase for all three major forms of RAS (K-RAS, H-RAS, and N-RAS ) to control their abundance in normal cells[31]. NEDD4-1 preferably targets guanosine diphosphate (GDP)-bound RAS but not GTP-bound form for degradation because binding of GTP causes a conformational change of ubiquitination site K5 to evade NEDD4-1-mediated ubiquitination. Therefore, oncogenic RAS mutants that constitutively bind GTP are resistant to the ubiquitination mediated by NEDD4-1. Interestingly, even though NEDD4-1 does not promote ubiquitination of oncogenic RAS mutants, it binds these RAS mutants with a similar affinity to that of wild-type RAS[31]. In this study, taking advantage of the pre-existing interaction of K-RAS mutants and NEDD4-1, we successfully identified a small-molecule compound XMU-MP-9 that can act as a bifunctional agent to facilitate NEDD4-1-mediated ubiquitination and degradation of various oncogenic K-RAS mutants, thereby inhibits the proliferation and tumor development of K-RAS mutant harboring cancer cells.

## 2. Results and discussion

### 2.1. XMU-MP-9 promotes NEDD4-1-mediated K-$RAS^{G12V}$ degradation

Our previous study demonstrated that NEDD4-1 is a bona fide E3 ubiquitin ligase for wild-type RAS proteins, and although oncogenic RAS mutants are resistant to NEDD4-1-mediated ubiquitination, they still can bind to NEDD4-1[31]. We speculated that it might be possible to enable NEDD4-1 to ubiquitinate oncogenic RAS mutants using a small-molecule compound. This compound is expected to promote degradation of oncogenic RAS mutants in the presence of NEDD4-1, thereby potentially inhibiting the proliferation of wild-type cells but not NEDD4-1 knockout cells. Therefore, in attempting to identify small-molecule compound(s) that can induce NEDD4-1-mediated degradation of oncogenic K-RAS mutants, we utilized SW620, a human colorectal cancer cell line that harbors K-$RAS^{G12V}$, and performed a high-throughput screen to look for compounds having more growth inhibitory potency to wild-type (*NEDD4-1*$^{+/+}$) than to *NEDD4-1* knockout (*NEDD4-1*$^{-/-}$) SW620 cells. For this screen, cells seeded in 96-well plates were treated with chemical compounds for 4 days and then subjected to cell viability examination. Compounds with at least 20% more inhibitory effect on *NEDD4-1*$^{+/+}$ cells than that on *NEDD4-1*$^{-/-}$ cells were selected for further characterization (Fig. 1A). We used an in-house compound library containing a total of 4480 compounds and divided them into 1120 groups by mixing 4 different compounds into each group. In the first-round screening, 17 groups (68 compounds in total) met our selection criteria. The 68 compounds were then applied for a second-round screen and 13 compounds were identified with a preference to inhibit proliferation of wild-type SW620 cells (Fig. 1B and C). Among them, one compound showed a specific effect on inducing decrease of endogenous K-$RAS^{G12V}$ protein levels in *NEDD4-1*$^{+/+}$ but not *NEDD4-1*$^{-/-}$ cells (Supporting Information Fig. S1A), and was named XMU-MP-9 (Fig. 1D) in our following studies. XMU-MP-9 could induce decrease of endogenous K-$RAS^{G12V}$ protein levels in SW620 cells as early as 12 h treatment and reached a maximum effect at 36 to 48 h (Fig. S1B). In contrast, analogues of XMU-MP-9 shown in Fig. S1C failed to induce degradation of K-$RAS^{G12V}$ in SW620 cells. XMU-MP-9 not only presented a more potent effect on inhibiting growth of *NEDD4-1*$^{+/+}$ SW620 cells in 2-D culture and 3-D soft agar in comparison to *NEDD4-1*$^{-/-}$ SW620 cells (Fig. 1E and F), but also showed a NEDD4-1-dependent decrease of endogenous K-$RAS^{G12V}$ protein levels in a dose-dependent manner (Fig. 1G).

We confirmed that the decrease of K-$RAS^{G12V}$ was not through change of transcription as treatment of XMU-MP-9 did not affect

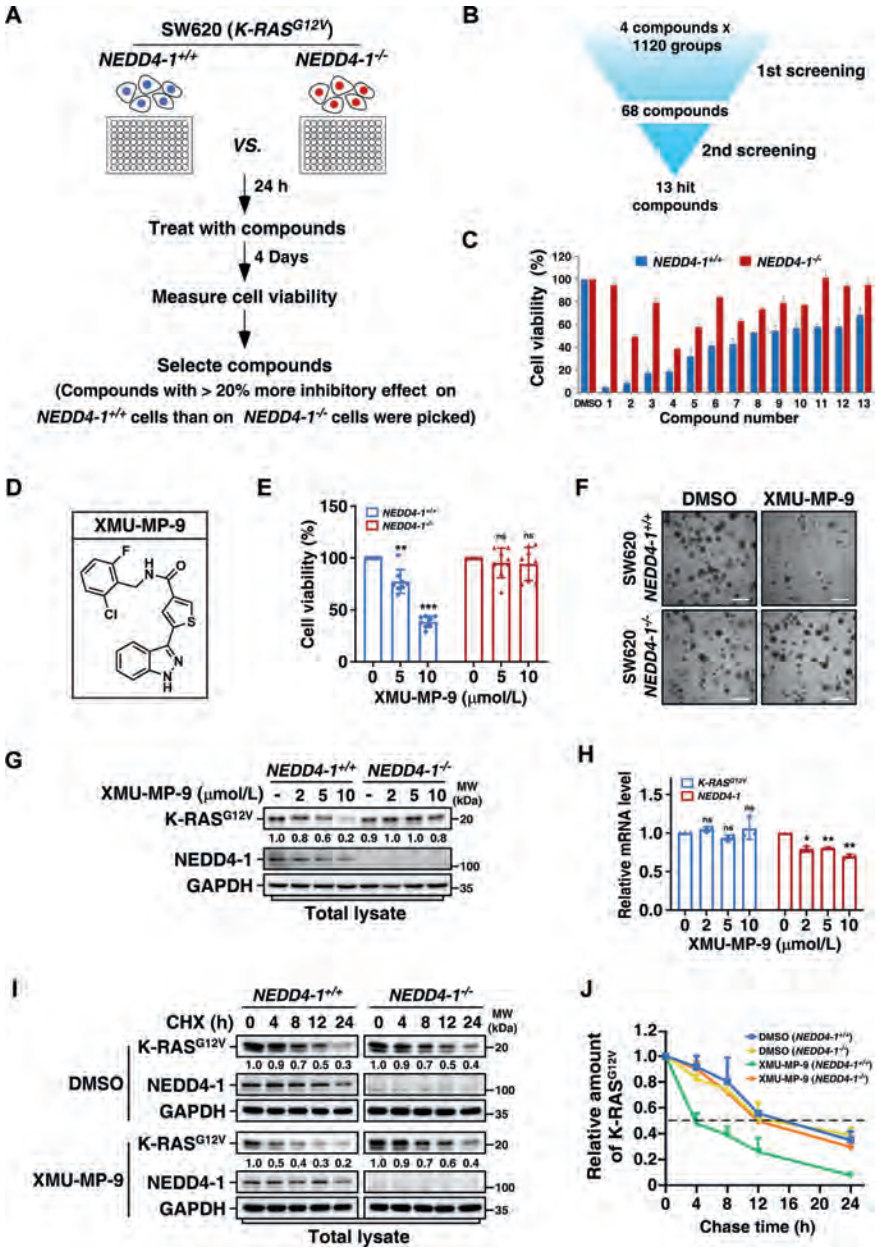


**Figure 1** XMU-MP-9 induces NEDD4-1-dependent K-RAS$^{G12V}$ degradation. (A) Schematic of high-throughput screening for compounds preferably inhibiting growth of wild-type (*NEDD4-1*$^{+/+}$) but not *NEDD4-1* knockout (*NEDD4-1*$^{-/-}$) SW620 cells. (B) Screening of the compound library for the hits. (C) Identification of individual compounds with preference for inhibiting growth of wild-type SW620 cells. *NEDD4-1*$^{+/+}$ and *NEDD4-1*$^{-/-}$ SW620 cells seeded in 96-well plates were treated 4 days with 10 μmol/L of each compound selected from the screen and then subjected to cell viability assay. Data were presented as mean ± SD of three individual experiments. (D) Chemical structure of XMU-MP-9. (E, F) XMU-MP-9 specifically inhibits growth of wild-type but not *NEDD4-1* knockout SW620 cells. *NEDD4-1*$^{+/+}$ and *NEDD4-1*$^{-/-}$ SW620 cells were seeded in 2-D culture and treated with indicated concentrations of XMU-MP-9 for 4 days before subjected to cell viability assay (E), or seeded in 3-D soft agar and treated 10 μmol/L XMU-MP-9 or DMSO as control for 2 weeks (F). Cell viabilities were determined and plotted as mean ± SD ($n = 8$). *P* values were determined by one-way ANOVA followed by Tukey's test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant. Scale bar indicates 100 μm. (G) XMU-MP-9 induces decrease of steady-states levels of K-RAS$^{G12V}$ in *NEDD4-1*$^{+/+}$ but not *NEDD4-1*$^{-/-}$ SW620 cells. *NEDD4-1*$^{+/+}$ and *NEDD4-1*$^{-/-}$ SW620 cells treated 48 h with XMU-MP-9 as indicated were subjected to immunoblotting assay. (H) Effects of XMU-MP-9 on transcription of *K-RAS*$^{G12V}$ and *NEDD4-1*. SW620 cells treated 48 h with XMU-MP-9 as indicated were subjected to qRT-PCR assay to determine the mRNA levels of *K-RAS*$^{G12V}$ and *NEDD4-1*. The results are presented as mean ± SD of 3 independent experiments. *P* values were determined by one-way ANOVA followed by Tukey's test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant. (I, J) XMU-MP-9 promotes NEDD4-1-dependent degradation of *K-RAS*$^{G12V}$. *NEDD4-1*$^{+/+}$ and *NEDD4-1*$^{-/-}$ SW620 cells treated with 10 μmol/L XMU-MP-9 and 100 μg/mL cycloheximide (CHX) were collected at indicated time points and subjected to immunoblotting assay to determine the protein levels (I). Relative amounts of K-RAS$^{G12V}$ at each time point to the level at time 0 were plotted. The results are presented as mean ± SD of 3 independent experiments (J).

the mRNA levels of *K-RAS*$^{G12V}$ (Fig. 1H). It is noteworthy that treatment of XMU-MP-9 induced decrease of mRNA levels of *NEDD4-1* (Fig. 1H), which agrees with our previous report that RAS signaling is required for *NEDD4-1* transcription[31]. Co-treatment with lysosome inhibitor chloroquine but not proteasome inhibitor MG-132 significantly blocked the decrease of endogenous K-RAS$^{G12V}$ induced by XMU-MP-9 (Fig. S1D), indicating a lysosomal degradation of K-RAS$^{G12V}$. This was further confirmed by using another 2 lysosome inhibitors, bafilomycin A1 (Baf-A1) and ammonium chloride (Fig. S1E). Moreover, in agreement with our previous study that K-RAS oncogenic mutants are resistant to NEDD4-1-mediated degradation, knockout of *NEDD4-1* in SW620 cells did not significantly affect protein half-life of K-RAS$^{G12V}$; however, treatment of XMU-MP-9 significantly decreased the K-RAS$^{G12V}$ protein half-life only in the presence of NEDD4-1 (Fig. 1I and J), indicating that XMU-MP-9-promoted K-RAS$^{G12V}$ degradation is indeed dependent on NEDD4-1.

### 2.2. *XMU-MP-9 promotes NEDD4-1-mediated ubiquitination of K-RAS$^{G12V}$ on K128*

We next conducted ubiquitination assays to verify the effect of XMU-MP-9 on promoting ubiquitination of K-RAS$^{G12V}$. Treatment of XMU-MP-9 significantly enhanced ubiquitination of endogenous K-RAS$^{G12V}$ in wild-type SW620 cells in a dose dependent manner, whereas knockout of *NEDD4-1* totally blocked the XMU-MP-9-promoted K-RAS$^{G12V}$ ubiquitination (Fig. 2A). To avoid potential contamination of K-RAS binding proteins, we performed 2 × anti-Flag immunoprecipitation (IP) to confirm the effect of XMU-MP-9 on promoting ubiquitination of K-RAS$^{G12V}$. Exogenously expressed Flag-tagged K-RAS$^{G12V}$ was subjected to anti-Flag immunoprecipitation, eluted by boiling in 1% SDS, then diluted in TNTE lysis buffer and re-immunoprecipitated with anti-Flag IP (2 × IP) (Fig. 2B). In addition, we performed an *in vitro* ubiquitination assay using bacterially expressed NEDD4-1 and Flag-tagged K-RAS$^{G12V}$, in which XMU-MP-9 drastically enhanced the NEDD4-1-mediated K-RAS$^{G12V}$ ubiquitination (Fig. 2C), demonstrating a clear evidence of XMU-MP-9 in directly assisting NEDD4-1-mediated ubiquitination of K-RAS$^{G12V}$. Notably, auto-ubiquitination of NEDD4-1 was not enhanced by treatment of XMU-MP-9 (Fig. 2D), suggesting that XMU-MP-9 does not function as an activator to increase the catalytic activity of NEDD4-1.

To identify the ubiquitination site(s) of K-RAS$^{G12V}$, we analyzed ubiquitinated K-RAS$^{G12V}$ obtained from the *in vitro* ubiquitination assay in the presence of NEDD4-1 and XMU-MP-9 by mass spectrometry. Only K128 of K-RAS$^{G12V}$ was detected with ubiquitin conjugation (Supporting Information Fig. S2A). To confirm this, we examined all the Lys-to-Arg mutations of K-RAS$^{G12V}$ and found that only K128R mutation totally abolished the XMU-MP-9-promoted K-RAS$^{G12V}$ degradation (Fig. 2E and Fig. S2B). Accordingly, K128R mutation substantially blocked the NEDD4-1-mediated ubiquitination of K-RAS$^{G12V}$ promoted by XMU-MP-9 both in cells and *in vitro* (Fig. 2F and G), indicating that XMU-MP-9 primarily assists NEDD4-1 in attaching ubiquitin to K128 of K-RAS$^{G12V}$.

### 2.3. *XMU-MP-9 functions as a bifunctional compound for NEDD4-1 and K-RAS$^{G12V}$*

Next, we sought to explore the mechanism of XMU-MP-9 in assisting the ubiquitination of K-RAS$^{G12V}$ mediated by NEDD4-1. Because XMU-MP-9 directly promotes NEDD4-1-mediated K-RAS$^{G12V}$ ubiquitination and degradation without increasing the catalytic activity of NEDD4-1, we speculated that it might act as a small-molecule degrader to help NEDD4-1 to ubiquitinate K-RAS$^{G12V}$. We therefore examined the bindings of XMU-MP-9 to NEDD4-1 and K-RAS$^{G12V}$ using microscale thermophoresis (MST)[32]. Interestingly, XMU-MP-9 interacted with both K-RAS$^{G12V}$ and NEDD4-1 with similar binding affinities ($K_d$ = 18.4 and 25.4 nmol/L, respectively) (Fig. 3A and B), and it could dose-dependently increase the binding affinity between K-RAS$^{G12V}$ and NEDD4-1 (Fig. 3C). We further carried out *in vitro* nickel (Ni) affinity pull-down experiment and found that XMU-MP-9 dose-dependently enhanced the interaction between His-K-RAS$^{G12V}$ and NEDD4-1 (Fig. 3D). Hence, XMU-MP-9 functions as a bifunctional compound to bind both NEDD4-1 and K-RAS$^{G12V}$, and might also induce a conformational change of the NEDD4-1/K-RAS$^{G12V}$ complex to allow NEDD4-1 to target K128 of K-RAS$^{G12V}$ for ubiquitination (Fig. 2F and G).

We next exerted photo-affinity labeling technology[33] to explore the binding pocket of XMU-MP-9 on K-RAS$^{G12V}$ and NEDD4-1. We designed and synthesized XMU-MP-9-PAL (photo-affinity labeling), a diazirine derivative of XMU-MP-9, as a photo-affinity probe (Supporting Information Fig. S3A). We performed mass spectrometry analysis to determine photo-affinity labeling sites in K-RAS$^{G12V}$ and NEDD4-1 after they were individually incubated with XMU-MP-9-PAL and irradiated with UV light. By examining peptides with a mass shift +413.0810 Da, we found that V108, P109, and L110 of NEDD4-1 and I93, V112, and L113 of K-RAS$^{G12V}$ could be labelled by XMU-MP-9-PAL (Fig. S3B and S3C). The V108, P109, and L110 are located at a potential binding cleft in the C2 domain of NEDD4-1 (Fig. 3E), whereas the I93, V112, and L113 are located inside K-RAS$^{G12V}$ (Fig. 3F). Interestingly, recent study suggested 8 novel allosteric sites in K-RAS[34], and the I93, V112, and L113 are very close to one of these allosteric sites.

We analyzed the structure of K-RAS$^{G12V}$ to look for potential amino acid residue(s) around this allosteric site critical for the binding of XMU-MP-9 and examined their effectiveness by site-directed mutagenesis. Among the mutations we have tested, F78A mutation significantly attenuated the effect of XMU-MP-9 on promoting NEDD4-1-mediated K-RAS$^{G12V}$ ubiquitination and degradation (Fig. 3G and Fig. S3D), and dramatically decreased the binding affinity of XMU-MP-9 to K-RAS$^{G12V}$ (Fig. 3H). The enhancement of interaction between NEDD4-1 and K-RAS$^{G12V}$ promoted by XMU-MP-9 was also abolished by F78A mutation of K-RAS$^{G12V}$ (Fig. S3E). Using the same strategy, we identified that K127 and F129 of NEDD4-1 were critical for its activity to target K-RAS$^{G12V}$ for ubiquitination and degradation upon XMU-MP-9 treatment (Fig. 3I and Fig. S3F). K127A/F129A mutations significantly decreased the binding affinity of NEDD4-1 with XMU-MP-9 (Fig. 3J) and attenuated XMU-MP-9-induced enhancement of interaction between NEDD4-1 and K-RAS$^{G12V}$ (Fig. S3G). Accordingly, F78A mutation of K-RAS$^{G12V}$ or K127A/F129A mutations of NEDD4-1 remarkably abolished the inhibitory effect of XMU-MP-9 on the growth of SW620 cells (Fig. S3H and S3I). We confirmed that F78A mutation did not affect K-RAS$^{G12V}$ activity in triggering downstream MAP kinase pathway activation (Fig. S3J), and K127A/F129A mutations did not affect NEDD4-1 to target wild-type K-RAS for degradation (Fig. S3K), indicating that these mutations unlikely caused misfolding and inactivation of K-RAS$^{G12V}$ and NEDD4-1.

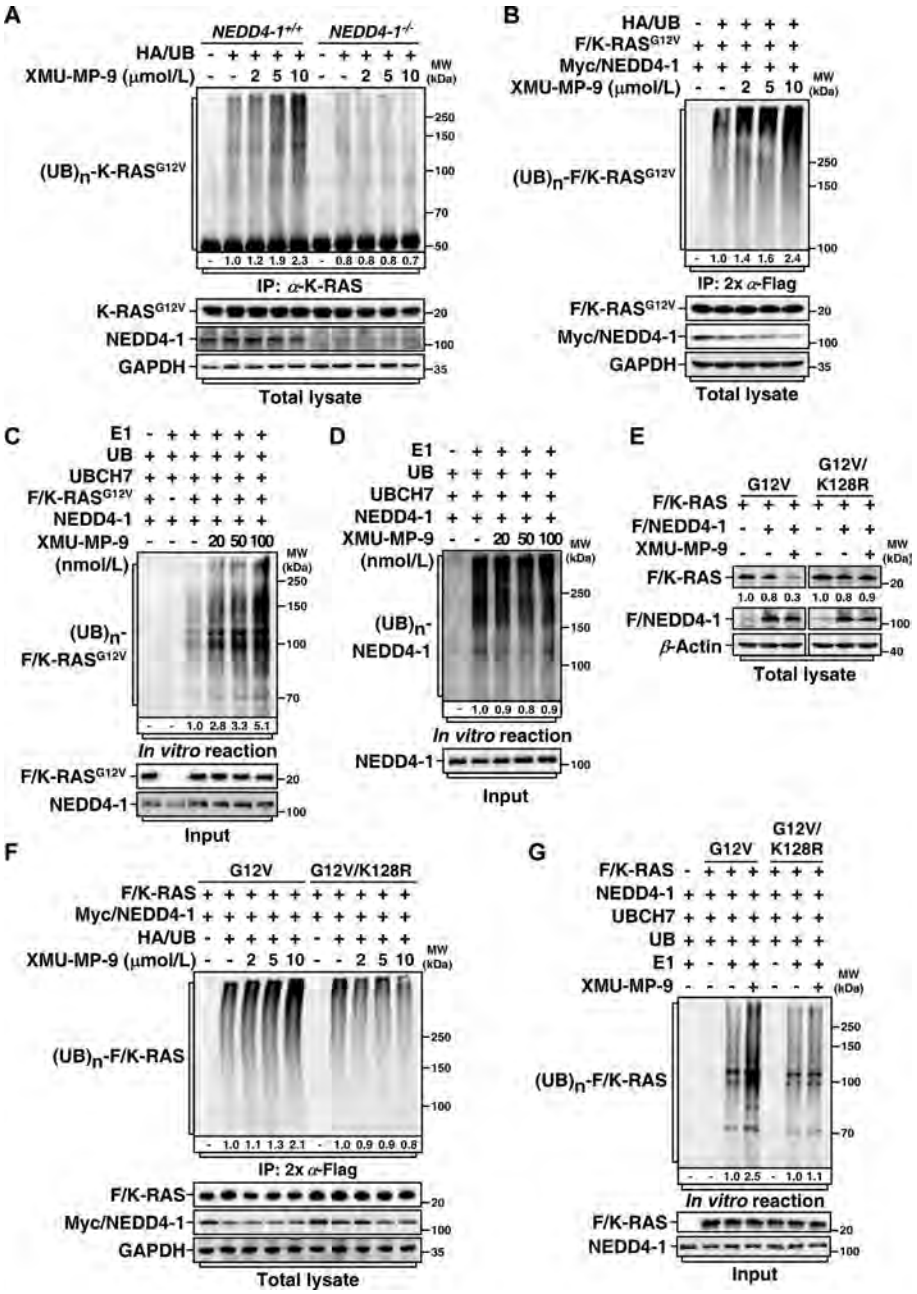


**Figure 2** XMU-MP-9 promotes NEDD4-1-mediated ubiquitination of K-RAS$^{G12V}$. (A) XMU-MP-9 promotes ubiquitination of endogenous K-RAS$^{G12V}$ in wild-type but not *NEDD4-1* knockout cells. *NEDD4-1*$^{+/+}$ and *NEDD4-1*$^{-/-}$ SW620 cells transduced with lentivirus encoding HA-tagged ubiquitin (HA/UB) were pre-treated 1 h with 120 μmol/L chloroquine and then 1 h with indicated concentrations of XMU-MP-9 before subjected to anti-K-RAS immunoprecipitation (IP). Ubiquitin-conjugated K-RAS$^{G12V}$ was detected by immunoblotting assay. (B) XMU-MP-9 promotes NEDD4-1-mediated ubiquitination of exogenously expressed K-RAS$^{G12V}$. HEK293T cells transfected with indicated combinations of HA/UB, Flag-tagged K-RAS$^{G12V}$ (F/K-RAS$^{G12V}$), and Myc-tagged NEDD4-1 (Myc/NEDD4-1) were treated same as in (A) and then subjected to 2 × anti-Flag IP. Ubiquitin-conjugated F/K-RAS$^{G12V}$ was detected by immunoblotting with anti-HA antibody. (C) XMU-MP-9 promotes NEDD4-1-mediated ubiquitination of K-RAS$^{G12V}$ *in vitro*. NEDD4-1 and F/K-RAS$^{G12V}$ purified from bacteria were subjected to an *in vitro* ubiquitination assay in the presence or absence of indicated concentrations of XMU-MP-9. Reactions were stopped by boiling in 1% SDS and ubiquitination products were subjected to immunoprecipitation with anti-Flag antibodies. Ubiquitinated F/K-RAS$^{G12V}$ was detected by immunoblotting assay with anti-UB antibody. (D) XMU-MP-9 does not enhance auto-ubiquitination of NEDD4-1. NEDD4-1 purified from bacteria was subjected to an *in vitro* ubiquitination assay with or without XMU-MP-9 as indicated. Ubiquitinated NEDD4-1 was detected by immunoblotting assay with anti-UB antibody. (E) K128R mutation blocks XMU-MP-9-induced degradation of F/K-RAS$^{G12V}$. HEK293T cells transfected with Flag-tagged NEDD4-1 (F/NEDD4-1) and F/K-RAS$^{G12V}$ or F/K-RAS$^{G12V/K128R}$ as indicated were treated with or without XMU-MP-9 (10 μmol/L, 48 h) and then subjected to immunoblotting assay. (F) K128R mutation blocks XMU-MP-9-induced ubiquitination of F/K-RAS$^{G12V}$ in cells. HEK293T cells transfected with indicated combinations of HA/UB, Myc/NEDD4-1, F/K-RAS$^{G12V}$, and F/K-RAS$^{G12V/K128R}$ were treated same as in (B). (G) K128R mutation blocks NEDD4-1-mediated ubiquitination of F/K-RAS$^{G12V}$ *in vitro*. NEDD4-1, F/K-RAS$^{G12V}$, and F/K-RAS$^{G12V/K128R}$ purified from bacteria were subjected to an *in vitro* ubiquitination assay as in (C).

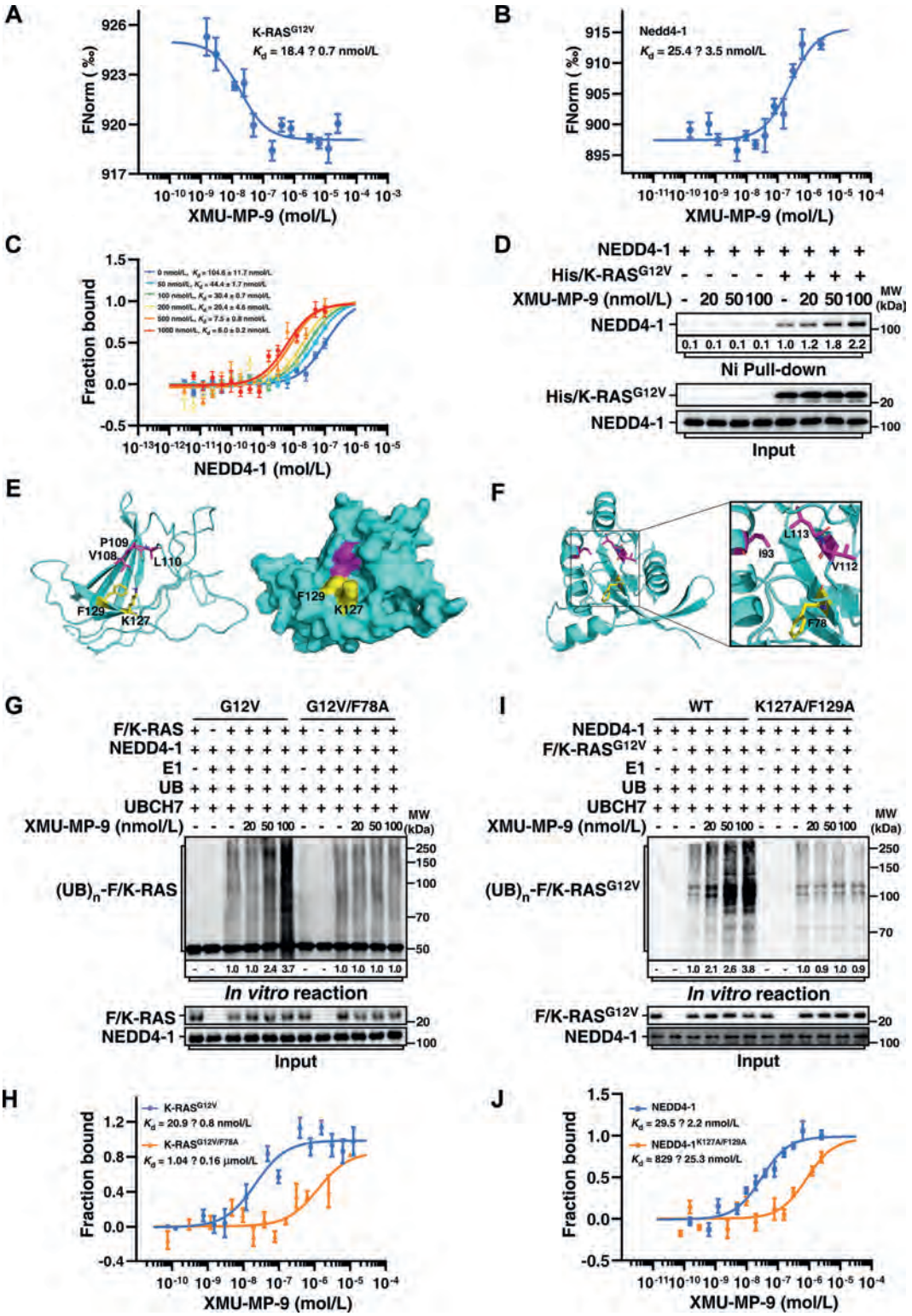


**Figure 3** XMU-MP-9 acts as a bifunctional compound for NEDD4-1 and K-RAS$^{G12V}$. (A) XMU-MP-9 binds to K-RAS$^{G12V}$. Red-NHS-labeled K-RAS$^{G12V}$ (10 nmol/L) with indicated concentrations of XMU-MP-9 were applied for microscale thermophoresis (MST) assays. Data of normalized fluorescence (FNorm in [‰]) were presented as mean ± SD of three individual experiments. The dissociation curve was fitted to the data to calculate the $K_d$ value. (B) XMU-MP-9 binds to NEDD4-1. Same as in (A) except Red-NHS-labeled NEDD4-1 instead of K-RAS$^{G12V}$ was used. (C) Presence of XMU-MP-9 increases binding affinity of NEDD4-1 to K-RAS$^{G12V}$. Red-NHS-labeled K-RAS$^{G12V}$ (10 nmol/L) was mixed with indicated concentrations of NEDD4-1 in the presence of different concentrations of XMU-MP-9 as indicated for MST assays. Data of fraction bound were presented as mean ± SD of three individual experiments. (D) XMU-MP-9 enhances interaction between K-RAS$^{G12V}$ and NEDD4-1. Bacterially expressed and purified His-tagged (His/K-RAS$^{G12V}$) and NEDD4-1 were subjected to nickel (Ni) affinity pull-down assay. Associated NEDD4-1

### 2.4. XMU-MP-9 promotes ubiquitination and degradation of various K-RAS mutants

Besides G12V mutation, various mutations at residues 12, 13, and 61 of *RAS* such as G12D, G12C, G12S, G13V, G13D, and Q61H also occur frequently in various human cancers[7]. In addition, because NEDD4-1 is a general E3 ubiquitin ligase for controlling all three major forms of RAS (K-RAS, H-RAS, and N-RAS ) protein levels[31], we further compared the effects of XMU-MP-9 on NEDD4-1-mediated degradation of different mutants of the three forms of RAS. In agreement with our previous report[31], overexpression of NEDD4-1 itself did not significantly affect the steady-state protein levels of these oncogenic mutants of K-RAS; however, treatment of XMU-MP-9 significantly induced decreases of the protein levels of all these K-RAS mutants. In contrast to its remarkable effects on inducing degradation of K-RAS mutants, XMU-MP-9 showed much less impact on degradation of H-RAS or N-RAS mutants (Supporting Information Fig. S4A), indicating that XMU-MP-9 is a selective degrader for K-RAS. We compared the amino acid sequence around the K128 in K-RAS with that in H-RAS and N-RAS. Interestingly, it is arginine (R) instead of lysine (K) at 128 in H-RAS, and the Q131 in K-RAS is replaced by histidine (H) in N-RAS (Fig. S4B), giving a good explanation why XMU-MP-9 had no effect on inducing H-RAS for degradation and much less effect on inducing N-RAS degradation.

To further examine the specificity of XMU-MP-9, we performed proteomic analysis to examine XMU-MP-9 treatment-induced global change of proteins. As expected, protein levels of K-RAS$^{G12V}$ were decreased, and a large number of proteins were even more dramatically down-regulated or up-regulated than K-RAS$^{G12V}$, which is understandable because the signaling cascade reactions are generally amplified in a stepwise manner. This is in agreement with previous report that knockout of *K-RAS* could result in hundreds of gene expression down-regulated and up-regulated[35]. In contrast, treatment of XMU-MP-9 on *NEDD4-1* knockout cells affected much less proteins compared with wild-type cells (Fig. S4C), indicating that majority of proteins affected by XMU-MP-9 treatment is dependent on NEDD4-1. In addition, we found that treatment of XMU-MP-9 had most significant effects on cell cycle and cell metabolism through KEGG clustering analysis (Fig. S4D), which is in line with that RAS-RAF-ERK signaling pathway predominantly regulates cell cycle and cell metabolism.

To compare the efficacies of XMU-MP-9 on promoting degradations of different K-RAS oncogenic mutants, we took advantage of endogenous NEDD4-1 in HEK293T cells to avoid potential variation of the protein levels of NEDD4-1 caused by overexpression. Meanwhile, we also generated *NEDD4-1* knockout HEK293T cells for controls. XMU-MP-9 showed similar 50% effective concentrations ($EC_{50}$ = 1.9 to 3.6 μmol/L) to these K-RAS oncogenic mutants in wild-type HEK293T cells, whereas knockout of *NEDD4-1* blocked the degradations of these K-RAS mutants induced by XMU-MP-9 (Fig. 4A and Fig. S4E). Moreover, we confirmed that the XMU-MP-9-induced degradations of these K-RAS mutants were all *via* lysosomal pathway (Fig. 4B).

We further verified the effects of XMU-MP-9 on promoting NEDD4-1-mediated ubiquitination of different K-RAS oncogenic mutants by executing ubiquitination assays in cells and *in vitro*. Similar as it did to K-RAS$^{G12V}$, XMU-MP-9 dramatically promoted NEDD4-1-mediated ubiquitination of all the other K-RAS oncogenic mutants exogenously expressed in HEK293T cells (Fig. 4C). Additionally, *in vitro* ubiquitination assays using bacterially expressed Flag-tagged K-RAS mutants also confirmed that XMU-MP-9 could directly assist NEDD4-1 to ubiquitinate all these K-RAS mutants (Fig. 4D), demonstrating a general effect of XMU-MP-9 on promoting NEDD4-1-mediated ubiquitination of different K-RAS mutants. Hence, our study identified a general small-molecule degrader for different K-RAS oncogenic mutants by leveraging NEDD4-1, the natural E3 ubiquitin ligase for RAS proteins.

### 2.5. XMU-MP-9 inhibits proliferation and anchorage-independent growth of K-RAS mutant harboring cancer cells

We next examined the effects of XMU-MP-9 on protein levels of endogenous K-RAS mutants and the downstream signaling. For this end, we treated various cancer cell lines harboring different oncogenic K-RAS mutations, including SW620 (colorectal, K-RAS$^{G12V}$), AsPC-1 (pancreatic, K-RAS$^{G12D}$), MIA PaCa-2 (pancreatic, K-RAS$^{G12C}$), A549 (lung, K-RAS$^{G12S}$), HCT 116 (colorectal, K-RAS$^{G13D}$), and NCI-H460 (lung, K-RAS$^{Q61H}$), with different doses of XMU-MP-9 and determined the endogenous K-RAS protein levels. In agreement with that XMU-MP-9 could promote NEDD4-1 to target various K-RAS mutants for ubiquitination (Fig. 4C and D), treatment of XMU-MP-9 dose-dependently induced decrease of endogenous K-RAS mutant protein levels in these cells. Accordingly, along with the decrease of K-RAS protein levels induced by treatment of XMU-MP-9, phosphorylation levels of B-RAF and MEK were markedly decreased (Fig. 5A).

To probe the potential of XMU-MP-9 in *K-RAS*-driven cancer therapy, we first examined the growth inhibitory effects of XMU-MP-9 on these human cancer cells. As expected, proliferation of these cells in 2-D tissue culture were all substantially inhibited by treatment of XMU-MP-9 in a dose dependent manner (Fig. 5B). Meanwhile, growth of these cancer cells in 3-D soft agar culture

was detected by immunoblotting assay. (E) Ribbon (left) and surface (right) structure of the C2 domain of NEDD4-1 with representation of XMU-MP-9-PAL-labelled residues (magenta) and residues critical for XMU-MP-9 binding (yellow). The structure of the C2 domain of NEDD4-1 is derived from PDB code 3B7Y. (F) Ribbon structure of K-RAS$^{G12V}$ (PDB: 8AZZ) with representation of XMU-MP-9-PAL-labelled residues (magenta) and residue critical for XMU-MP-9 binding (yellow). The magnified area shows a close-up view of these sites. (G) F78A mutation of K-RAS$^{G12V}$ prevents XMU-MP-9-promoted ubiquitination of K-RAS$^{G12V}$. NEDD4-1, F/K-RAS$^{G12V}$, and F/K-RAS$^{G12V/F78A}$ purified from bacteria were subjected to an *in vitro* ubiquitination assay in the absence or presence of indicated concentrations of XMU-MP-9. (H) F78A mutation decreases binding affinity of K-RAS$^{G12V}$ to XMU-MP-9. Binding affinities of XMU-MP-9 to K-RAS$^{G12V}$ and K-RAS$^{G12V/F78A}$ were determined by MST assays and compared as fraction bound. Data are presented as the mean ± SD from three repeated experiments. (I) K127A/F129A mutation of NEDD4-1 blocks XMU-MP-9-promoted ubiquitination of K-RAS$^{G12V}$. F/K-RAS$^{G12V}$, NEDD4-1, and NEDD4-1$^{K127A/F129A}$ purified from bacteria were subjected to an *in vitro* ubiquitination assay in the absence or presence of indicated concentrations of XMU-MP-9. (J) K127A/F129A mutation decreases binding affinity of NEDD4-1 to XMU-MP-9. Same as in (H) except NEDD4-1 and NEDD4-1$^{K127A/F129A}$ were used.

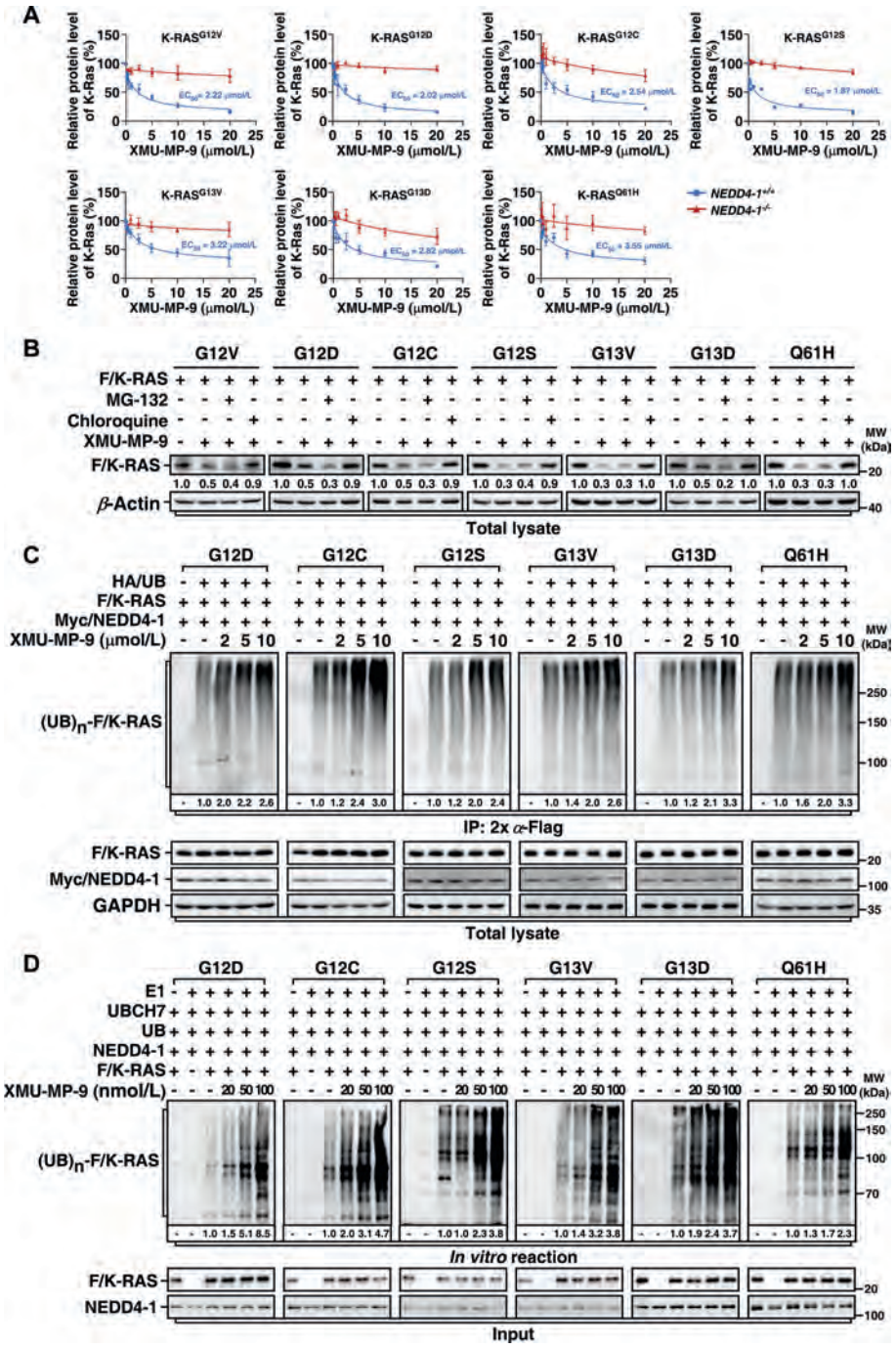


**Figure 4** XMU-MP-9 has a general effect on promoting ubiquitination and degradation of various K-RAS oncogenic mutants. (A) Quantification of relative protein levels of indicated K-RAS mutants treated 48 h with different concentrations of XMU-MP-9. Data were represented as mean ± SEM of 3 independent experiments. (B) Degradation of K-RAS mutants induced by XMU-MP-9 is *via* lysosomal pathway. HEK293T cells transfected with indicated K-RAS mutants were treated with combinations of XMU-MP-9 (10 μmol/L, 48 h) and proteasome inhibitor MG-132 (20 μmol/L, 3 h) or lysosome inhibitor chloroquine (120 μmol/L, 12 h) as indicated, and then subjected to immunoblotting assay. (C) XMU-MP-9 promotes ubiquitination of various K-RAS mutants in cells. HEK293T cells transfected with indicated combinations of HA/UB, Myc/NEDD4-1, and Flag-tagged K-RAS (F/K-RAS ) mutants as indicated were pre-treated with chloroquine (120 μmol/L, 1 h) and then treated 1 h with indicated concentrations of XMU-MP-9 before subjected to 2 × anti-Flag IP followed by immunoblotting with anti-HA antibody to examine ubiquitin-conjugated F/K-RAS. Expression of Myc/NEDD4-1 and F/K-RAS were confirmed by immunoblotting the total cell lysates. (D) XMU-MP-9 promotes ubiquitination of various K-RAS mutants *in vitro*. Bacterially expressed and purified NEDD4-1 and indicated F/K-RAS mutants were subjected to an *in vitro* ubiquitination assay in the presence or absence of indicated concentrations of XMU-MP-9.

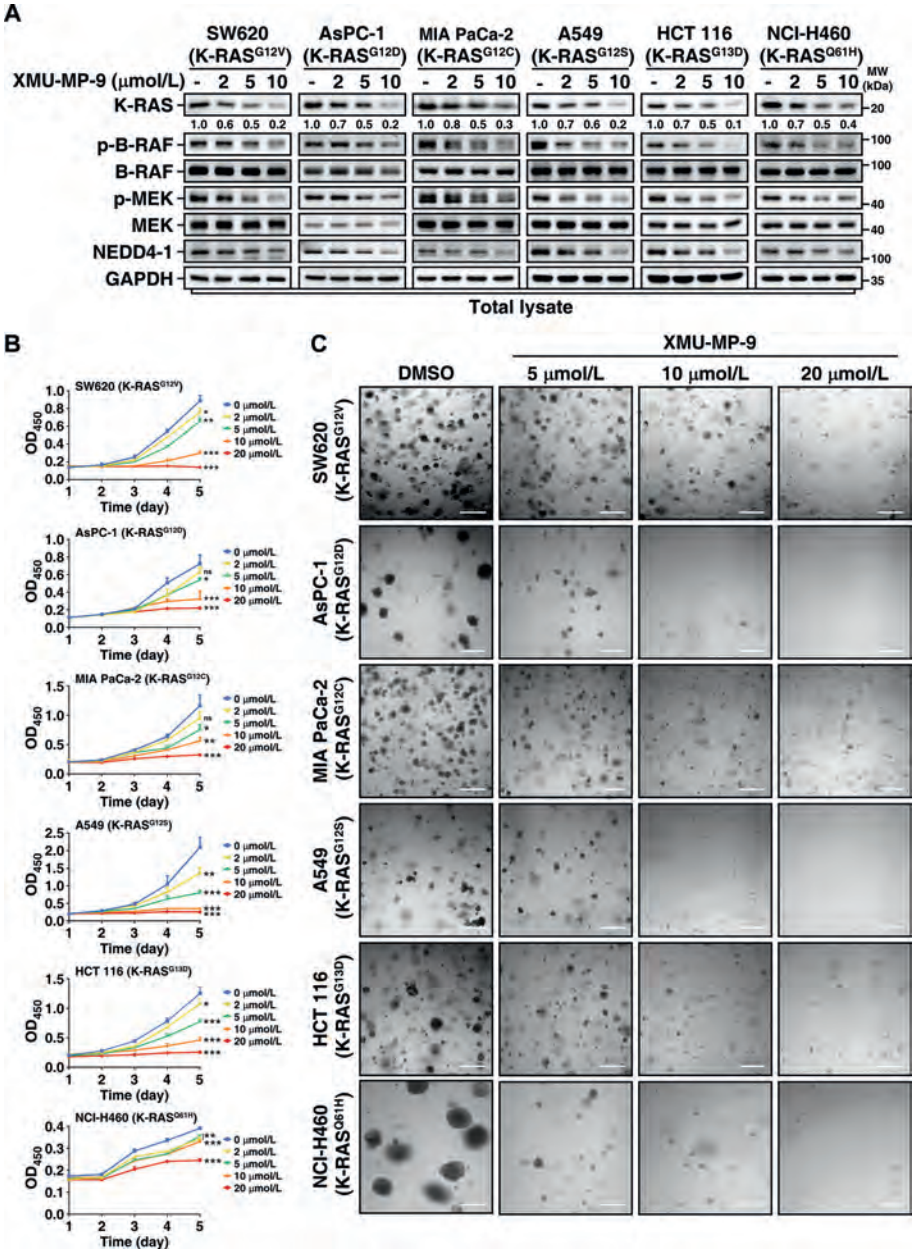


**Figure 5** XMU-MP-9 induces degradation of endogenous K-RAS mutants and inhibits growth of K-RAS mutant harboring cancer cells. (A) XMU-MP-9 promotes degradation of endogenous K-RAS mutants and inhibits its downstream signaling in K-RAS mutant harboring cancer cells. Different cancer cells harboring indicated K-RAS mutants were treated 48 h with indicated concentrations of XMU-MP-9 and then subjected to immunoblotting assay. (B) XMU-MP-9 inhibits proliferations of K-RAS mutant harboring cancer cells in 2-D culture. Cancer cells harboring indicated K-RAS mutants were cultured with indicated concentrations of XMU-MP-9. Cell viabilities were determined and plotted as mean ± SD ($n = 6$). *P* values were determined by one-way ANOVA followed by Tukey's test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, not significant. (C) XMU-MP-9 suppresses anchorage-independent growth of K-RAS mutant harboring cancer cells. Cancer cells harboring indicated K-RAS mutants were seeded in 3-D soft agar and treated with indicated concentrations of XMU-MP-9 or DMSO as control for 2 weeks. Scale bar indicates 100 μm.

were also dramatically inhibited by XMU-MP-9 (Fig. 5C), in line with the key role of RAS/RAF/MAPK signaling pathway in cancer cell proliferation and anchorage-independent growth[1].

To confirm that XMU-MP-9 treatment-induced growth inhibition was through targeting RAS proteins, we treated HT-29, a human colorectal cancer cell line harboring constitutively active B-RAF$^{V600E}$ mutant, with XMU-MP-9. As predicted, although the K-RAS protein levels were decreased by treatment of XMU-MP-9, phosphorylation levels of B-RAF$^{V600E}$ and MEK were not changed (Supporting Information Fig. S5A). In contrast to the remarkable inhibitory effects on both 2-D and 3-D growth of K-RAS mutant harboring cancer cells, XMU-MP-9 treatment showed a much less effect on inhibiting growth of HT-29 cells, especially in the 3-D soft agar assay (Fig. S5B and S5C), suggesting that RAS/RAF/MAPK signaling pathway has a more pivotal role in promoting anchorage-independent growth than in

promoting cell proliferation in 2-D culture. Thus, it is plausible that XMU-MP-9 indeed induces degradation of oncogenic K-RAS proteins to inhibit proliferation and anchorage-independent growth of cancer cells harboring oncogenic K-RAS mutations.

### *2.6. XMU-MP-9 suppresses tumor development of K-RAS mutant harboring cancer cells in vivo*

We next assessed the efficacy of XMU-MP-9 in suppressing tumor growth of *K-RAS* harboring cancer cells *in vivo*. To this end, the pharmacokinetic properties of XMU-MP-9 were evaluated in mice. XMU-MP-9 was found to have limited oral exposure with oral bioavailability (*F%*) of 3.8% (Supporting Information Table S1). XMU-MP-9 was then administered at a dose of 40 mg/kg once or twice daily *via* tail vein injection. Remarkably, treatment of XMU-MP-9 dose-dependently suppressed growth of xenograft tumors formed by SW620 cells (Fig. 6A−C). Accordingly, the protein levels of K-RAS$^{G12V}$ were decreased in tumors treated with XMU-MP-9, and the phosphorylation levels of B-RAF and MEK were consequently attenuated, as examined by immunoblotting and immunohistochemistry assays (Fig. 6D and E). In line with the effects of XMU-MP-9 on growth of HT-29 cells (Fig. S5B and S5C), treatment of XMU-MP-9 did not suppress growth of xenograft tumors formed by subcutaneously injected HT-29 cells into nude mice (Supporting Information Fig. S6A−S6C). Accordingly, although the protein levels of K-RAS were decreased in XMU-MP-9 treated tumors, phosphorylation levels of B-RAF$^{V600E}$ and MEK were not significantly changed (Fig. S6D and S6E).

Emerging evidences indicate that oncogenic mutations of K-RAS may lead to immune evasion in tumor microenvironment[36]. Therefore, we further evaluated the anti-tumor efficacy of XMU-MP-9 in immunocompetent mice. We subcutaneously injected CT-26, a mouse colorectal cancer cell line harboring *K-RAS*$^{G12D}$, into BALB/c mice to generate transplanted tumors. Treatment of XMU-MP-9 even presented a more robust inhibitory effect on tumors formed by CT-26 cells in BALB/c mice than tumors formed by SW620 cells in nude mice (Fig. 6F−H). Similarly, the protein levels of K-RAS$^{G12D}$ and the phosphorylation levels of B-RAF and MEK in CT-26 tumors treated with XMU-MP-9 were dramatically decreased (Fig. 6I and J). In addition, treatment of XMU-MP-9 did not show significant weight loss and obvious toxicity to heart, liver, spleen, lung, or kidney tissues of mice (Fig. S6F and S6G). Moreover, we compared the effects of XMU-MP-9 on inducing degradation of K-RAS$^{G12V}$ in SW620 colon cancer cells with that of wild-type *K-RAS* in some normal cell lines. XMU-MP-9 showed much less potencies to promote wild-type K-RAS degradation in NCM460 (normal human colon mucosal epithelial), BEAS-2B (normal human bronchial epithelial), MCF10A (normal human mammary epithelial), THLE-2 (normal human liver epithelial), LO2 (normal human hepatocyte), NIH3T3 (normal mouse embryonic fibroblast), and MEF (mouse embryonic fibroblast) cells than to promote K-RAS$^{G12V}$ degradation in SW620 cells (Fig. S6H). Hence, XMU-MP-9 demonstrated a good potential in treatment of *K-RAS*-driven cancers, which may lead to a new strategy for development of anticancer drugs.

### *2.7. Discussion*

Given the fact that RAS mutations occur so frequently in human cancers, directly targeting RAS mutants has always been an appealing approach for clinical therapy of *RAS*-driven cancers. While there have been significant breakthroughs in the development of allele-specific covalent inhibitors for K-RAS$^{G12C}$ and the advancements in non-covalent inhibitor research for K-RAS$^{G12D}$, designing small-molecule inhibitors for K-RAS mutants like K-RAS$^{G12V}$, which lack specific active or charged side chains, remains a big challenge because there is no suitable binding pocket on the surface of these mutants[21,37]. Thus, it is highly desirable to develop innovative strategies for targeting these RAS mutants commonly found in human cancers. Recent studies developed PROTACs that can target various K-RAS mutants[30]; however, due to their relatively larger sizes, PROTACs generally have poorer drug-like properties compared with molecular glue degraders[26,38]. Although functions as a bifunctional compound, XMU-MP-9 does not contain a flexible linker and has a relatively small molecular weight. Rather than induces a non-native interaction, XMU-MP-9 stabilizes the pre-existing interaction between NEDD4-1 and K-RAS mutants. More interestingly, it looks like that XMU-MP-9 not only enhances the interaction between NEDD4-1 and K-RAS$^{G12V}$ (Fig. 3C and D), but also induces a conformational change of K-RAS$^{G12V}$ to expose its K128 to NEDD4-1. In line with this, the binding site of XMU-MP-9 identified in K-RAS$^{G12V}$ is close to an allosteric site that was recently reported by Weng et al.[34], and K128 is also around this allosteric site. Binding of XMU-MP-9 might stabilize this allosteric conformation that allows NEDD4-1 to access to K128 of K-RAS$^{G12V}$ (Fig. 2F and G). Therefore, in addition to its binding property, XMU-MP-9 may also act as a molecular wedge for stabilizing the transit conformation of K-RAS$^{G12V}$.

Because there is no specific active or charged side group available on RAS mutants like RAS$^{G12V}$, it is extremely hard to specifically target these RAS mutants without affecting wild-type RAS in normal tissues. This raises safety concerns for using inhibitors to target these RAS mutants in patients. Our previous study indicated that RAS signaling is required for transcription of *NEDD4-1*. The negative feedback regulatory loop between NEDD4-1-mediated RAS degradation and RAS signaling-regulated *NEDD4-1* transcription plays a key role in maintaining homeostasis of RAS signaling in normal cells. Oncogenic mutations of RAS block NEDD4-1-mediated *RAS* degradation, resulting in uncontrolled RAS signaling and, as a consequence, up-regulated NEDD4-1 levels[31]. In fact, *NEDD4-1* was found frequently up-regulated in various types of human cancers and showed a positive correlation with *RAS* levels[31,39], thus providing a therapeutic window for treatment of *RAS*-driven cancers with NEDD4-1-dependent RAS degrader. Indeed, treatment of XMU-MP-9 promoted degradation of K-RAS mutants and consequently decreased endogenous levels of NEDD4-1 (Figs. 1H, 5A and 6D and I), suggesting that NEDD4-1-dependent RAS degradation will be restrained when RAS signaling is low, which may considerably lessen its potential toxic effects to normal tissues. As a support, our experiment revealed that treatment of XMU-MP-9 did not show obvious toxicity to the treated mice (Fig. S6F and S6G).

Our previous study demonstrated that NEDD4-1 is a natural E3 ubiquitin ligase for all three RAS (K-RAS, H-RAS, and N-RAS ) proteins. Although oncogenic RAS mutants are resistant to NEDD4-1-mediated ubiquitination, they still can bind NEDD4-1[31]. Based on this finding, we utilized NEDD4-1, instead of hijacking an irrelevant E3 ubiquitin ligase that is usually used in PROTAC or molecular glue degrader design, successfully identified a small-molecule degrader for K-RAS mutants. This strategy allowed compounds to work on the pre-

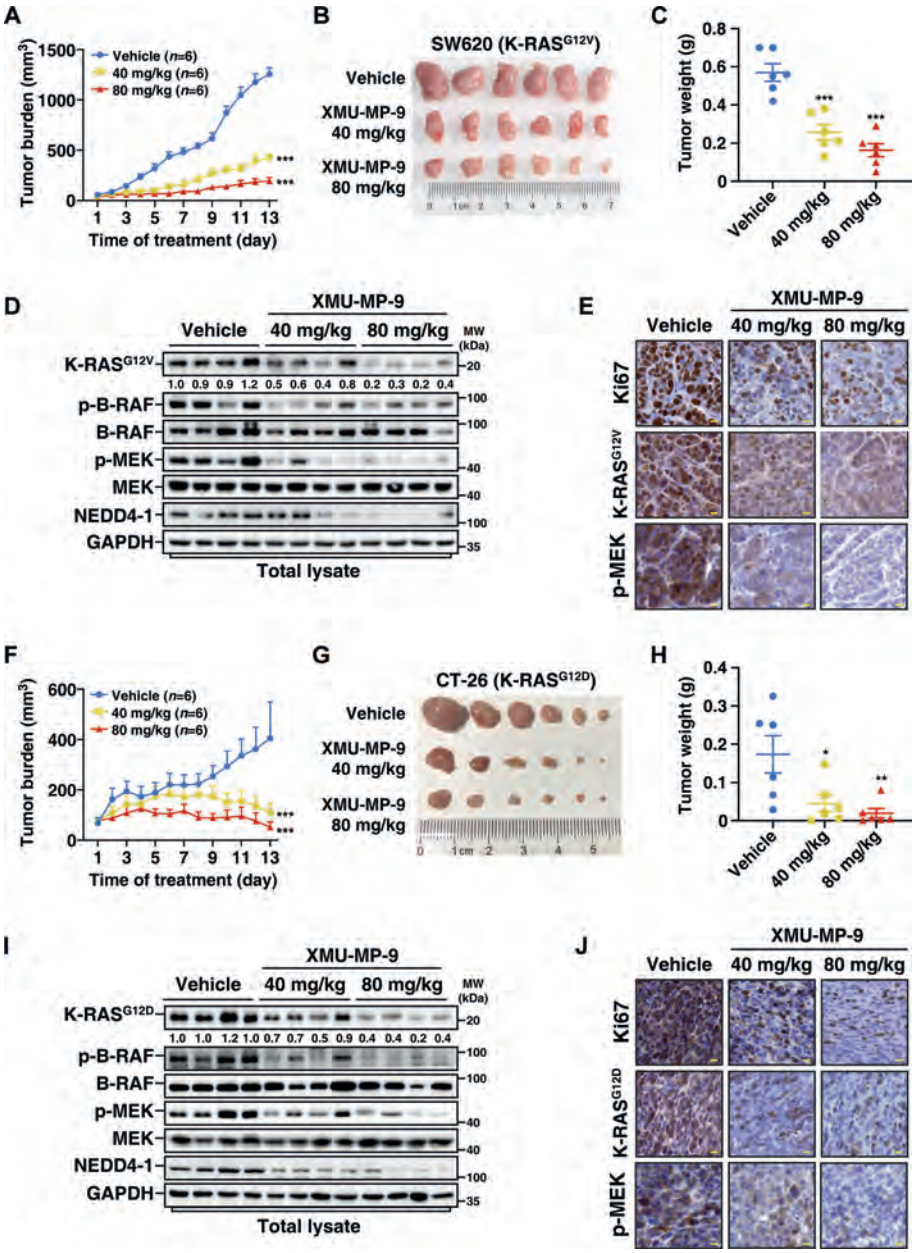


**Figure 6** XMU-MP-9 suppresses growth of *K-RAS* mutant harboring cancer cells formed tumor in mice. (A−C) XMU-MP-9 inhibits xenograft tumor growth of SW620 cells in nude mice. SW620 cells were subcutaneously injected into nude mice to form xenograft tumors. The tumor-bearing mice were given a daily dose of 40 or 80 mg/kg of XMU-MP-9 (injected with a dose of 40 mg/kg once or twice a day *via* tail vein) or vehicle as control. Tumor volumes were measured and plotted as mean ± SEM ($n = 6$ animals per group). *P* values were determined by two-way ANOVA followed by Tukey's test. ***$P < 0.001$ (A). The tumors were obtained 13 days after drug treatment by sacrificing the mice (B), weighted and plotted as mean ± SEM ($n = 6$ animals per group). *P* values were determined by one-way ANOVA followed by Tukey's test. ***$P < 0.001$ (C). (D, E) Treatment of XMU-MP-9 decreases K-RAS$^{G12V}$ levels and downstream signaling in SW620 cells formed tumors. Representative tumors were subjected to immunoblotting (D) or IHC assay (E). Scale bar indicates 10 μm. (F−H) XMU-MP-9 inhibits transplant tumor growth of CT-26 cells in BALB/c mice. CT-26 cells were subcutaneously injected into BALB/c mice to form transplant tumors. The tumor-bearing mice were treated and analyzed same as in (A−C). (I, J) Treatment of XMU-MP-9 decreases K-RAS$^{G12D}$ levels and downstream signaling in CT-26 cells formed tumors. Representative tumors were subjected to immunoblotting (I) or IHC assay (J). Scale bar indicates 10 μm.

existing NEDD4-1 and K-RAS complex and led to identification of small-molecule degraders with relative small sizes. Because NEDD4-1 is also a natural E3 ubiquitin ligase for H-RAS and N-RAS[31], it is possible to obtain small-molecule degraders for H-RAS or N-RAS mutants as well using the same strategy. Hence, our study presents a promising approach to achieve small-molecule degraders for all kinds of RAS mutants.

We had put great efforts to get structural information of complex of XMU-MP-9 and K-RAS mutants, but unfortunately we could not obtain crystal for X-ray study, likely due to the

conformational change of K-RAS induced by the binding of compound to the allosteric site recently reported on K-RAS[34]. Meanwhile, due to lack of structural information for this allosteric modulation of K-RAS, we were unable to perform computational modeling study to provide more insights about the interaction between XMU-MP-9 and K-RAS. In addition, because of the poor solubility of XMU-MP-9, it is also difficult to solve the complex structure using nuclear magnetic resonance (NMR) technique. Cryo−electron microscopy (cryo-EM) has been successfully used to determine structures of numerous biomacromolecules[40]. However, due to the sphere-like shape of NEDD4-1/K-RAS/XMU-MP-9 complex, the size of this complex is not big enough for the analysis using current cryo-EM technology. Nevertheless, we believe that the fast development of cryo-EM and other technologies may be able to provide detailed structural information that will guide us to improve our compound or help us to design new K-RAS degraders based on this study in the near future. The PK data of XMU-MP-9 revealed that further medicinal efforts are need to improve its drug-like properties. But XMU-MP-9 would serve as a starting point to develop K-RAS targeted therapy through NEDD4-1 mediated degradation.

## 3. Conclusions

In summary, our study successfully identified a small-molecule degrader XMU-MP-9 that may target all major oncogenic K-RAS mutants for degradation through NEDD4-1, the bona fide E3 ubiquitin ligase of RAS proteins. Functioning as a bifunctional reagent, XMU-MP-9 can bind to an allosteric site of K-RAS and C2 domain of NEDD4-1 to enhance the interaction between NEDD4-1 and K-RAS mutants, therefore remarkably promotes ubiquitination of K-RAS mutants *in vivo* and *in vitro*. XMU-MP-9 strongly inhibits proliferation and tumor development of cancer cells bearing oncogenic K-RAS mutants, and showed no obvious toxicity to the treated mice, presenting a promising approach for developing anticancer drugs using NEDD4-1-based small-molecule degraders of K-RAS mutants.

## 4. Experimental

### *4.1. Chemical synthesis of XMU-MP-9 and XMU-MP-9-PAL*

Chemical synthesis processes of XMU-MP-9 and XMU-MP-9-PAL are illustrated as Schemes S1 and S2, respectively. All reagents and solvents were purchased from commercial sources and used without further purification unless otherwise noted. All intermediates or target products were purified by silica gel chromatography. Silica gel chromatography was performed on Teledyne ISCO CombiFlash system (RF200) eluting with dichloromethane/methanol (DCM/MeOH) or petroleum ether/ethyl acetate (PE/EA). $^1$H NMR and $^{13}$C NMR spectra were recorded with a Bruker Ultrashield Plus-600 ($^1$H: 600 MHz; $^{13}$C: 150 MHz), spectrometer instrument in dimethyl sulfoxide (DMSO)-$d_6$, with tetramethylsilane (TMS) as the internal standard. Chemical shifts ($\delta$) of NMR were reported in parts per million (ppm) relative to the internal standard, and coupling constants ($J$) were reported in hertz (Hz). Mass spectra were recorded on QDa II Detector with UV detection at 210−400 nm (Waters LC/MS system, SunFire C18 column, 5 μm, 4.6 mm × 50 mm). A gradient elution was performed using solvent A (0.035% TFA in water) and solvent B (0.035% TFA in methanol) at a flow rate of 1.0 mL/min as follows: 0−0.5 min, 95% A; 0.5−4.5 min, linear gradient to 5% A; 4.5−5.5 min, further gradient to 0% A; 5.5−9.0 min, isocratic at 0% A; 9.0−10.0 min, re-equilibration at 5% A. High resolution mass spectra were obtained on Thermo Fisher Scientific Q-Exactive orbitrap Mass Spectrometer.

### *4.2. Antibodies and chemical reagents*

Mouse anti-GAPDH (sc-32233), anti-$\beta$-Actin (sc-47778), anti-UB (sc-8017), and anti-Myc (SC-40) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA); rabbit anti-Ki67 (12202T), anti-NEDD4-1 (3607), anti-phospho-B-RAF (2696), anti-B-RAF (14814), anti-phospho-MEK1/2 (9154), and anti-MEK1/2 (9122) antibodies were purchased from Cell Signaling Technology (Boston, MA, USA); rabbit anti-K-RAS (BS6231) antibody was purchased from Bioworld (St. Louis Park, MN, USA); rabbit anti-K-RAS (A1190) antibody was purchased from ABclonal (Wuhan, China); mouse anti-FLAG M2 antibody (F1804) was from Sigma−Aldrich (St Louis, MO, USA); and rat anti-HA antibody (11867431001) was from Roche (Basel, Switzerland). Cycloheximide (239764) and lysosome inhibitor chloroquine (C6628) were from Sigma−Aldrich (St Louis, MO, USA). Proteasome inhibitor MG-132 (HY-13259) and Cell Counting Kit-8 (HY-K0301) were from MedChemExpress (Shanghai, China).

### *4.3. DNA constructs*

The complementary DNA (cDNA) of human *NEDD4-1* was a generous gift from Dr. W. Sundquist. Human cDNAs of *K-RAS*, *H-RAS*, and *N-RAS* were cloned from HEK293T cells using RT-PCR, and various mutations of *RAS* were introduced by PCR-based site-directed mutagenesis. Cloning for protein expression in mammalian cells was carried out using a modified pCMV5 vector for transfection, pBOBI vector for lentivirus infection. pGEX-4T-1 and pProEX were used for bacterial expression of proteins.

### *4.4. Cell culture, transfection, and lentivirus infection*

Human embryonic kidney HEK293T (CRL-3216), human colon cancer SW620 (CCL-227), HCT 116 (CCL-247), human pancreas cancer AsPC-1 (CRL-1682), MIA PaCa-2 (CRM-CRL-1420), human lung cancer NCI-H460 (HTB-177), A549 (CRM-CCL-185), mouse colon cancer CT-26 (CRL-2638), mouse fibroblast NIH3T3 (CRL-1658), mouse embryonic fibroblast MEF (CRL-2991), and human liver epithelial THLE-2 (CRL-2706) cell lines were obtained from ATCC. Human colon cancer HT-29 (SCSP-5032), human bronchial epithelial BEAS-2B (SCSP-5067), and human mammary epithelial MCF10A (SCSP-575) cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai). Human colon mucosal epithelial NCM460 (H1-3701) and human hepatocyte LO2 (H1-3701) cell lines were purchased from Cyagen (Guangzhou). SW620 cells were cultured in Leibovitz's L-15 medium (Basal Media); HEK293T, A549, NCM460, NIH3T3, and MEF cells were cultured in high-glucose Dulbecco's modified Eagle medium (Gibco); MIA PaCa-2 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Gibco) added with 1 mmol/L sodium pyruvate; AsPC-1, NCI-H460, CT-26, NCM460, and LO2 cells were cultured in RPMI medium 1640 (Gibco); HCT 116 and HT-29 cells were

cultured in McCoy's 5A medium (Sigma−Aldrich), BEAS-2B and THLE-2 cells were cultured in BEGM Bullet Kit medium (Lonza/Clonetics, CC-3170), MCF10A cells were cultured in MEGM Kit medium (Lonza/Clonetics, CC-3150), all supplemented with 10% (*v*/*v*) fetal bovine serum (Gibco) and 100 U/mL streptomycin and penicillin (Millipore). All cells were cultured at 37 °C in a humidified incubator with 5% $CO_2$ except SW620 with 0% $CO_2$. The cell lines were routinely tested and confirmed to be negative for mycoplasma.

Polyethylenimine (PEI) was used for transient transfection of HEK293T cells as previously described[41]. Briefly, mixtures of PEI and plasmids (3:1, *w*/*w*) were added to cell cultures and incubated for 6 h before replaced with fresh medium. Recombinant lentivirus used to infect SW620 cells for protein expression was generated by the ViraPower Lentiviral Expression System (Invitrogen) according to the manufacturer's instruction.

### 4.5. Generation of *NEDD4-1*$^{-/-}$ cell lines

For gene silencing of *NEDD4-1* in SW620 and HEK293T cells, design of sgRNAs and off-target sites prediction were performed by using the Zhang laboratory CRISPR design tool (https://zlab.bio/guide-design-resources). The sequences for human *NEDD4-1* gRNA-1 and gRNA-2 are 5′-TCAATCATGGGTAGGCAGAC-3′ and 5′-TCCAAGTTACTTGACGGTGG-3′, respectively. pLentiCRISPRv2 vector was used to express sgRNAs and the pool of *NEDD4-1*$^{-/-}$ cells were selected by treatment with puromycin (2 μg/mL) for 7 days. The efficiency of *NEDD4-1* knockout was assessed by quantitative reverse transcription-PCR (qRT-PCR) and immunoblotting assays.

### 4.6. Cell viability and 3-D soft agar assays

Cell growth rate was determined by counting the cell numbers in triplicate daily after seeding into 96-well plate using a CCK-8 kit following the manufacturer's instruction. Briefly, the culture plates were incubated with CCK-8 solution (10 μL/well) for 1 h, and then measured the absorbance at 450 nm using a microplate reader (Tecan Spark, Männedorf, Switzerland).

For 3-D soft agar assay, cells in 500 μL of 0.25% (*w*/*v*) Noble agar (214220, BD, Franklin Lakes, NJ, USA) with complete medium were seeded to each well of 24-well plates precoated with equal volume of 1% (*w*/*v*) Noble agar. Additional 300 μL of overlay medium was added after cell plating and the overlay medium was changed every 3 days. The cells were cultured for 2 weeks and then subjected to photographing using a Nikon eclipse Ti-U inverted microscope to examine colony formation in soft agar.

### 4.7. Microscale thermophoresis (MST) assay

Target proteins were fluorescently labeled using the protein labeling kit RED-NHS (NanoTemper Technologies). Labeling reaction was performed following the manufacturer's instructions with 10 μmol/L protein. Labeled protein was adjusted to 20 nmol/L with PBS buffer supplemented with 0.02% Tween 20. The ligand (XMU-MP-9 or NEDD4-1) was dissolved in PBS buffer supplemented with 10% DMSO and serially diluted using the same buffer. Additional XMU-MP-9 was supplied as required for examining NEDD4-1 and K-RAS$^{G12V}$ interaction. For the measurement, fluorescently labeled protein and ligand were mixed in 1:1 volume and then incubated for 5 min at room temperature before loaded into Monolith NT.115 Premium Capillaries (NanoTemper Technologies). MST was measured using a Monolith NT.115 instrument (NanoTemper Technologies) at 25 °C with parameters adjusted to auto-detected LED power and medium MST power. Data of three independently pipetted measurements were analyzed using the MO Affinity Analysis software (NanoTemper Technologies).

### 4.8. Quantitative proteomic assay

The quantitative proteomic analysis was performed as previous reported[42]. Briefly, SW620 *NEDD4-1*$^{+/+}$ and *NEDD4-1*$^{-/-}$ cells treated with DMSO or 10 μmol/L XMU-MP-9 for 24 h were lysed in urea buffer containing a concentration of 8 mol/L urea and EDTA-free protease inhibitor by scraping and sonication. The supernatants were collected by centrifugation at 21,000×*g* for 20 min at 4 °C before subjected to concentration determination and digestion. Liquid chromatography coupled online to a hybrid TIMS quadrupole time-of-flight mass spectrometer (Bruker timsTOF Pro) was performed. For data-independent acquisition, we adopted a 25 *m*/*z* precursor isolation width to cover 400−1200 mz. diaPASEF (.d) files were searched using DIA-NN (V.1.8.1) against a UniprotKB human protein database without decoys.

### 4.9. Transplant tumor formation assay

For generation of xenograft tumors of human colorectal SW620 and HT-29 cells in nude mice and transplant tumors of mouse colorectal CT-26 cells in BALB/c mice, 1−2 × $10^6$ cells suspended in 100 μL PBS were subcutaneously injected in nude mice or BALB/c mice. The growth of tumors was measured with digital calipers every day for 10−14 days after the tumors were visible. Nude mice and BALB/c mice were purchased from and housed in the Laboratory Animal Center of Xiamen University (China). All procedures involving mice for transplant tumor growth assays were approved by the Institutional Animal Care and Use Committee of Xiamen University.

### 4.10. Immunoprecipitation, immunoblotting, and nickel (Ni) pull-down assays

Immunoprecipitation and immunoblotting were carried out as previously described[43]. Briefly, cells were lysed on ice with TNTE lysis buffer (50 mmol/L tris−HCl pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5% Triton X-100, 10 μg/mL pepstatin A, 10 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride) and cell lysates were collected by centrifugation at 20,000×*g* at 4 °C for immunoprecipitation or immunoblotting assays with appropriate antibodies. For Ni pull-down assay, bacterially expressed GST/NEDD4-1 was purified using glutathione Sepharose beads in TNTE lysis buffer and free *NEDD4-1* protein was obtained by tobacco etch virus (TEV) protease cleavage. His-tagged K-RAS$^{G12V}$ was purified using $Ni^{2+}$-NTA-agarose beads for Ni pull-down assay.

### 4.11. Ubiquitination assay

For ubiquitination assay of Flag-tagged K-RAS in cells, cell lysates were subjected to anti-Flag immunoprecipitation, eluted by boiling in 1% SDS for 5 min, diluted 10 times in TNTE lysis buffer, and then re-immunoprecipitated with anti-Flag (2 × IP). The ubiquitin-conjugated proteins were detected by immunoblotting HA-tagged ubiquitin (HA/UB) with anti-HA antibody. For ubiquitination assay of endogenous K-RAS, cell lysates were subjected

to anti-K-RAS immunoprecipitation followed by immunoblotting. For *in vitro* ubiquitination assays, bacterially expressed and purified F/K-RAS and NEDD4-1 proteins were applied to *in vitro* ubiquitination reaction as previously described[43]. Reactions were stopped by boiling in 1% SDS for 5 min, and then diluted 10 times in TNTE lysis buffer before subjected to anti-Flag IP, followed by immunoblotting ubiquitin with anti-UB antibody to examine ubiquitin-conjugated F/K-RAS proteins.

### 4.12. Immunohistochemistry assay

For immunohistochemistry assay, tumors and tissues were fixed in 4% (*v*/*v*) paraformaldehyde (PFA), embedded in paraffin, sectioned at 3 μm, deparaffinized, and rehydrated. Sections were stained with hematoxylin and eosin (H&E) to examine the morphology. Immunohistochemical staining was performed using the UltrasensitiveTM SP kit (MXB, KIT-9720) with appropriate primary antibodies overnight at 4 °C. Chromogenic revelation was performed with DAB kit (MXB, DAB-2031). Images were obtained using Zeiss AxioScan7.

### 4.13. Photo-affinity labeling

Purified protein (K-RAS$^{G12V}$ or NEDD4-1) was incubated with 10 μmol/L XMU-MP-9-PAL for 30 min at 4 °C in PBS buffer and then photo-crosslinked by 365 nm UV irradiation for 20 min on ice. After photo-affinity labeling, protein sample was mixed with an equal volume of 2 × SDS sample loading buffer followed by SDS gel electrophoresis. Protein band was excised from the gel and subjected to LC−MS/MS analysis.

### 4.14. Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed using SuperReal PreMix Plus (SYBR Green) (FP205, TIANGEN, Beijing, China) according to the manufacturer's protocol on a Mastercycler EP gradient S RealPlex2 (Eppendorf, Hamburg, Germany). Total RNAs for qRT-PCR were extracted from cells using TRIzol (15596018CN, Invitrogen, CA, USA), and cDNA was synthesized with FastKing RT kit (KR116, TIANGEN, Beijing, China). The relative changes of gene expression were determined using the $2^{-\Delta\Delta Ct}$ method and normalized to *GAPDH*. The primers for human *K-RAS* are 5′-GAGTACAGTGCAA TGAGGGAC-3′ (forward) and 5′-CCTGAGCCTGTTTTGTG TCTAC-3′ (reverse); for human *NEDD4-1* are 5′-CAGGCCCTCA ATCACAAGC-3′ (forward) and 5′-AGGCCCTAGATCATT GGAAGT-3′ (reverse); for human *GAPDH* are 5′-CATGAGAAG TATGACAACAGCCT-3′ (forward) and 5′-AGTCCTTCCACGA TACCAAAGT-3′ (reverse).

### 4.15. Statistics and reproducibility

GraphPad Prism 8 software was used to analyze all quantitative data. The data are represented as mean ± standard error of mean (SEM) or mean ± standard deviation (SD) calculated using GraphPad. Significance was tested using unpaired two-tailed Student's *t* test, one-way ANOVA with Tukey's test and two-way ANOVA with Tukey's test. Protein bands in immunoblotting assay were visualized by Sagecreation MiniChemi system and analyzed with Lane 1D software. $P < 0.05$ was considered a statistically significant difference ($^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$). All data are representative of at least three independent experiments unless otherwise specified.

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## Author contributions

Taoling Zeng and Tingting Jiang conducted the experiments and analyzed the data. Baoding Zhang, Ting Zhang, and Xianming Deng conceived and performed small-molecule degrader design and chemical synthesis. Wanjun Dai and Yunzhan Li helped with the animal experiments. Xun Yin and Zhuoran Yu performed molecular biology experiments and protein purification. Caiming Wu performed MST assay. Yaying Wu performed mass spectrometry. Ximin Chi helped for binding site identification. Hong-Rui Wang conceived the idea, supervised the study, and wrote the manuscript.

## Conflicts of interest

The authors declare no conflicts of interest.

## Appendix A. Supporting information

Supporting information to this article can be found online at https://doi.org/10.1016/j.apsb.2025.07.031.

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