
Figures and figure supplements

Degradation of LMO2 in T cell leukaemia results in collateral breakdown of transcription complex partners and causes LMO2-dependent apoptosis

Naphannop Sereesongsaeng *et al.*

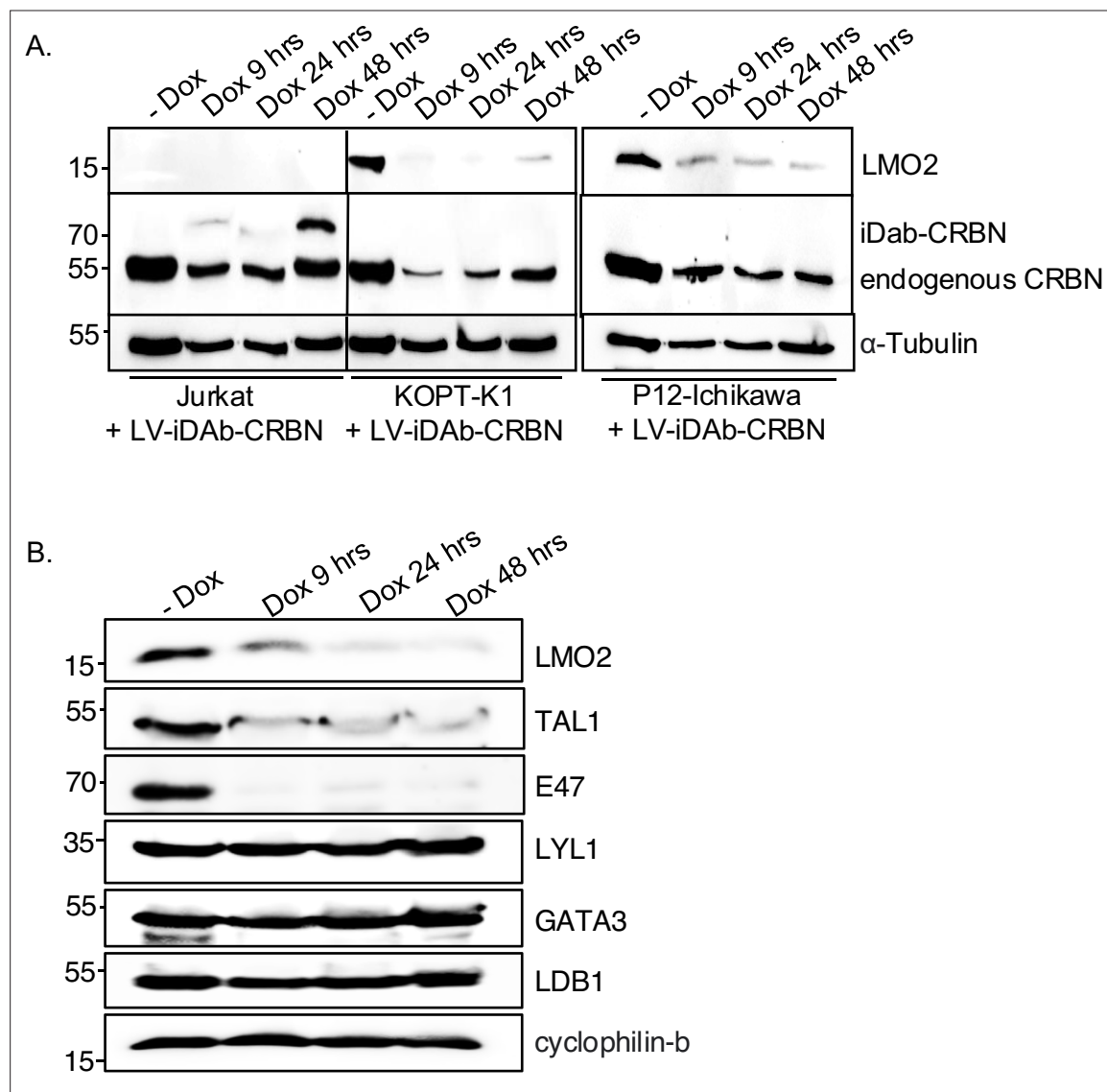


Figure 1. Lentivirally expressed biodegrader affects the LMO2 multi-protein complex in T cell lines. **(A)** The T cell lines Jurkat (LMO2-), KOPT-K1 (LMO2+), and P12-Ichikawa (LMO2+) cells were infected with lentivirus packaging plasmids and transfer vector (TLCV2-VH576-L10-CRBN) for 16 hr followed by 2 μ g/ml doxycycline induction for 9, 24, and 48 hr. Western blotting analysis was used to detect LMO2, endogenous CRBN, and VH576-L10-CRBN levels after lentivirus infection. α -Tubulin was used as an internal loading control for western blotting analysis. **(B)** The level of LMO2 and proteins associated with the LMO2 transcription complex (TAL1, E47, Lyl-1, GATA3, and LDB1) was determined after KOPT-K1 cells were infected with TLCV2-VH576-L10-CRBN followed by 2 μ g/ml doxycycline induction for 9, 24, and 48 hr. Cyclophilin-b was used as an internal loading control.

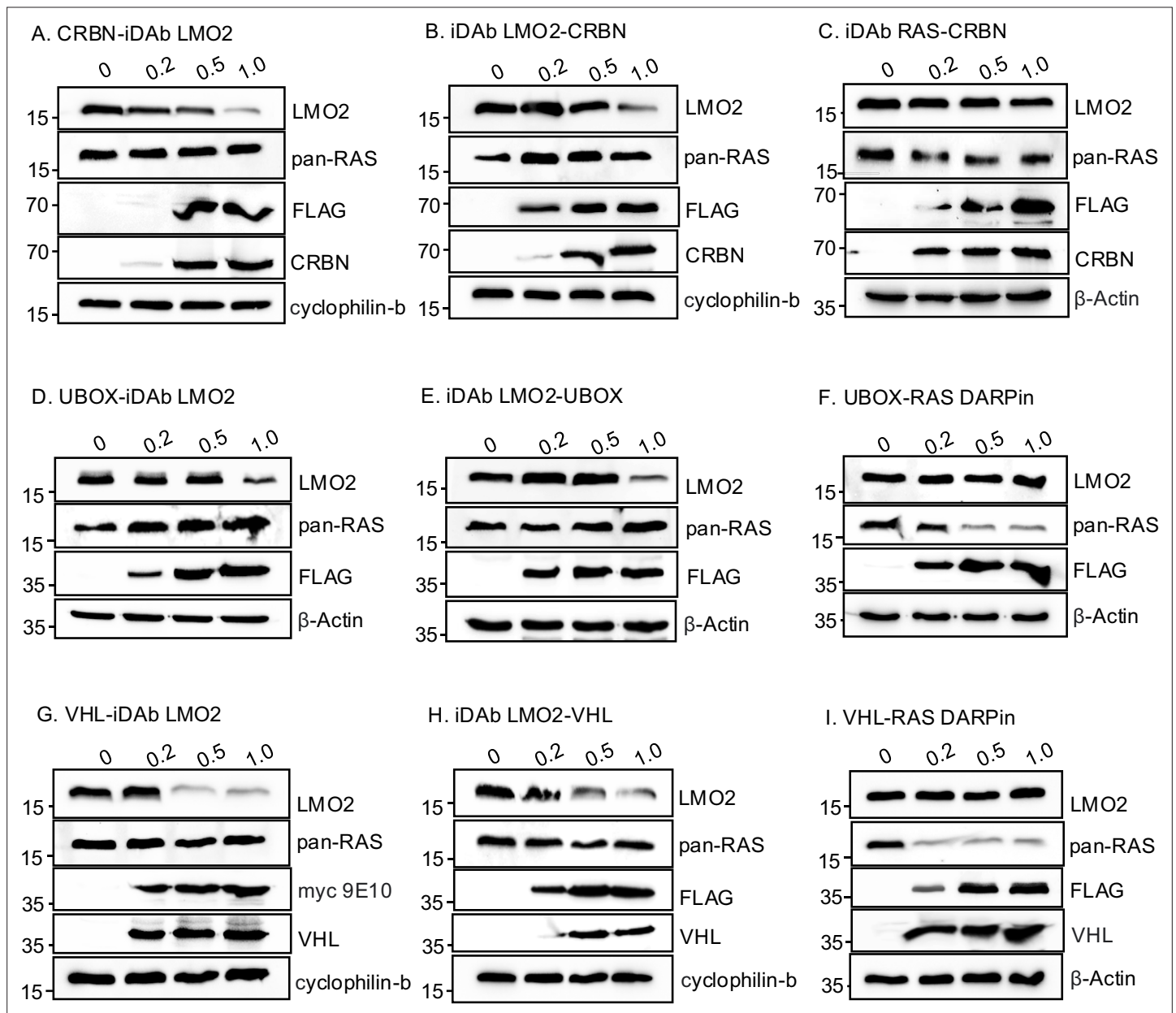


Figure 1—figure supplement 1. LMO2 protein degradation in HEK293T cells after transfection with biodegrader constructs. HEK293T cells were co-transfected with 1 µg pEF-BOS-LMO2 and plasmids expressing different intracellular domain antibody (iDAb) LMO2-E3 ligase fusions for 24 hr. The amount of the iDAb LMO2-E3 ligase plasmids was titrated from 0 to 1.0 µg. pEF-BOS (vector plasmid) was used to equalise the amount of plasmid transfection to 2 µg. Western blotting analysis showing the expression of LMO2 in cells transfected with CRBN-iDAb LMO2 (A), iDAb LMO2-CRBN (B), anti-RAS iDAb-CRBN (C), UBOX-iDAb LMO2 (D), iDAb LMO2-UBOX (E), UBOX-anti-RAS DARPIn (F), VHL-iDAb LMO2 (G), iDAb LMO2-VHL (H), and VHL-anti-RAS DARPIn (I). The antibodies used for protein detection are shown to the right of each panel. The anti-FLAG and antimyc 9E10 antibodies detect the FLAG and myc tag on each iDAb-E3 ligase, the anti-CRBN antibody detects the transfected iDAb-CRBN in (A–C) and anti-VHL detects the transfected iDAb-VHL in (G–I). Loading control proteins were detected with either anti-cyclophilin-b or anti-β-actin.

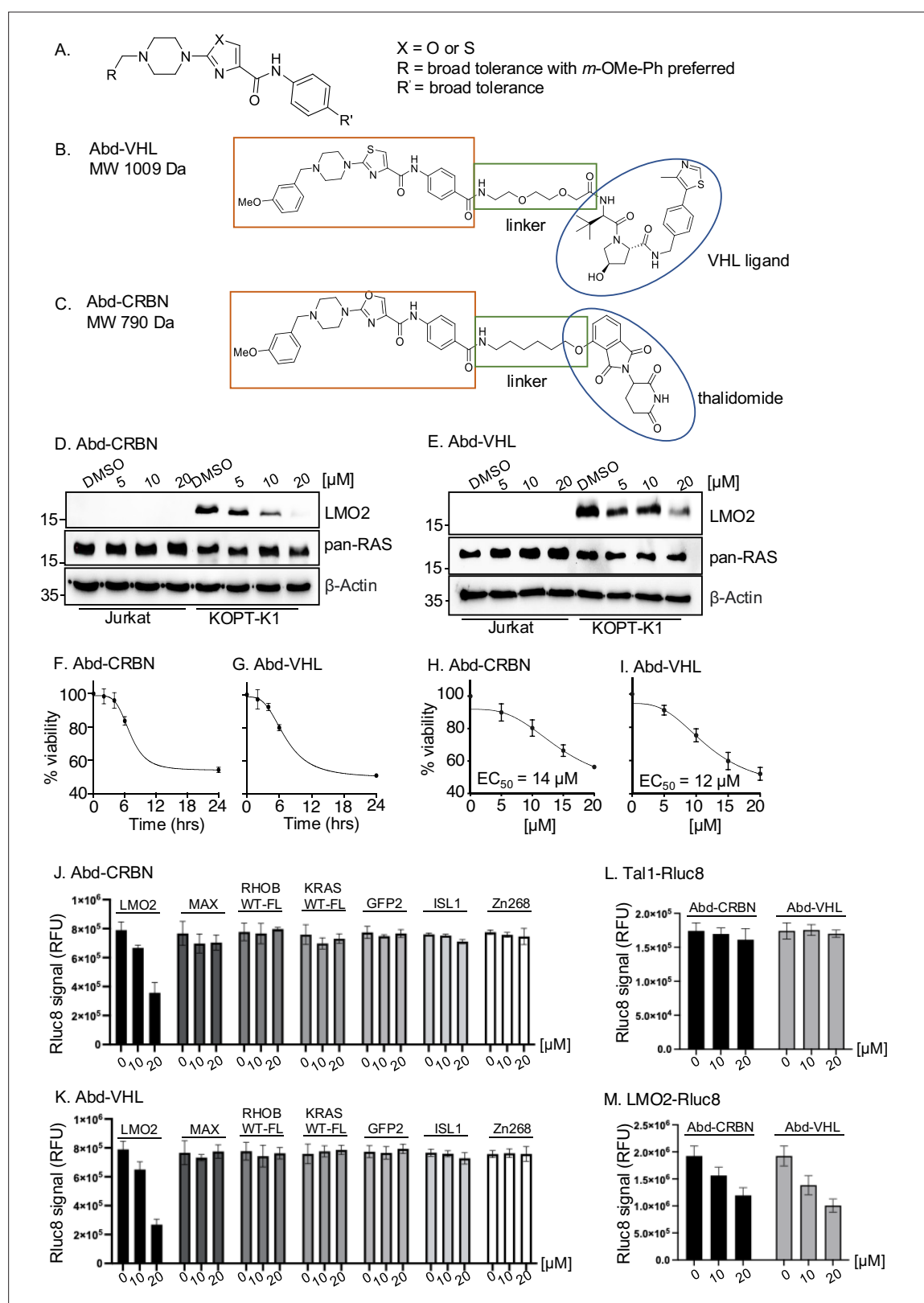


Figure 2. LMO2 antibody-derived (Abd) degraders and effects on cellular protein. **(A)** Structure-activity observations of LMO2-binding compounds derived using intracellular domain antibody (iDAb) VH576 in competitive, cell-based BRET (designated Abd compounds) contributed to a general structure of the LMO2-binding compounds. Based on broad tolerance to chemical modification at position R', two proteolysis targeting chimeras (PROTACs) were synthesised, one bearing the VHL ligand (Abd-VHL, **B**) and the other bearing thalidomide (the CRBN ligand) (Abd-CRBN, **C**). Each

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PROTAC thus comprises the LMO2-binding ligand, a linker and an E3 ligase ligand. **(D–E)** KOPT-K1 and Jurkat cells were treated with different concentrations of Abd degraders at 0, 5, 10, and 20 μM for 24 hr, protein extracts prepared and fractioned by SDS-PAGE followed by western blotting. Abd-CRBN was used on cells in **(D)** and Abd-VHL in **(E)**. Cells treated with 1% DMSO were used as a control and β -actin was used as an internal loading control for the western blotting analysis. The viability of KOPT-K1 cells was treated with 15 μM Abd-CRBN **(F)** or 15 μM Abd-VHL **(G)** for 0, 2, 4, 6, and 24 hr, using CellTiter-Glo assay. Data are presented as a relative to luminescence at 0 hr, normalised to 100%. Determination of DC_{50} values of Abd-CRBN **(H)** and Abd-VHL **(I)** after 24 hr treatment in KOPT-K1 was determined using CellTiter-Glo calculated by GraphPad Prism 9.0 software. All the values were presented as the average values relative to cell viability values in control (DMSO-treated cells) normalised to 100%. Data represent mean + SEM ($n=3$). Luciferase cell-based report assays were used to determine the degradation of the proteins after transfected HEK293T cells with different Renilla luciferase (Rluc8) reporter plasmids (pEF-LMO2-Rluc8, pEF-MAX-Rluc8, pEF-RHOBWT-FL, pEF-KRSWT-FI-Rluc8, pEF-GFP2-Rluc8, pEF-ISL1-Rluc8, and pEF-Zn268) followed by Abd-CRBN **(J)** and Abd-VHL **(K)** treatment at 0, 10, and 20 μM for 24 hr. The potential degradation of pEF-Tal-Rluc8 **(L)** and pEF-LMO2-Rluc8 **(M)** was determined by luciferase cell-based report assays after the treatment with Abd-CRBN or Abd-VHL at 0, 10, and 20 μM for 24 hr. Data represent mean + SEM ($n=3$).

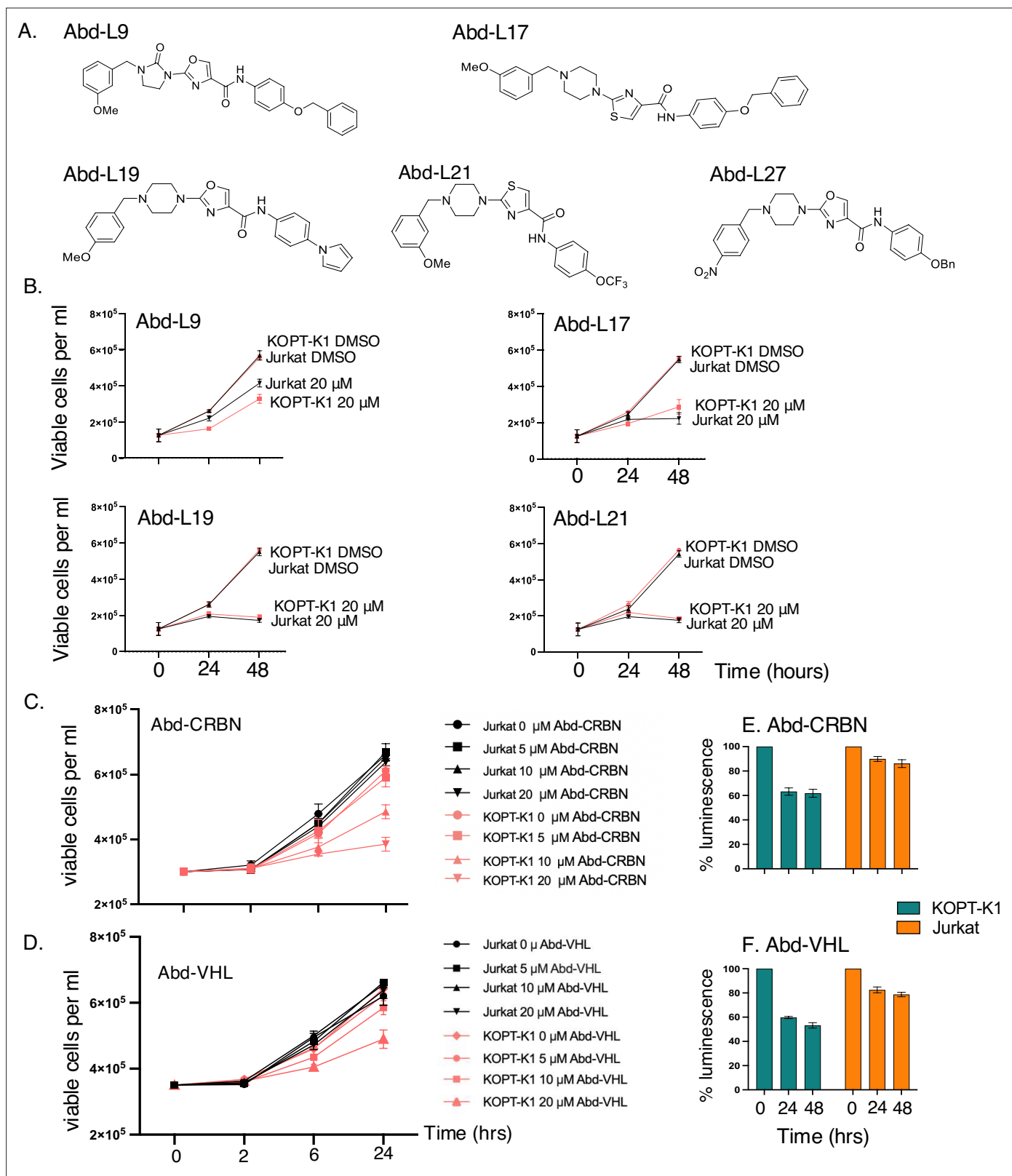


Figure 2—figure supplement 1. Viability of Jurkat and KOPT-K1 cells after treatment with antibody-derived (Abd) compounds and Abd degraders. (A) Chemical structures of LMO2 Abd compounds. (B) Jurkat and KOPT-K1 cells were treated at 20 μ M of Abd compounds from 0 to 48 hr. Cell viability after the treatment was measured by counting viable cells after staining cells with Trypan Blue. (C and D) Cell viability was also determined using Trypan Blue after the treatment with different concentrations of Abd degrader proteolysis targeting chimera (PROTAC) compounds (0, 5, 10, and 20 μ M) for 2, 6, 24, and 48 hr. (E and F) Cell viability was also determined using luminescence after the treatment with different concentrations of Abd degrader proteolysis targeting chimera (PROTAC) compounds (0, 5, 10, and 20 μ M) for 2, 6, 24, and 48 hr. Figure 2—figure supplement 1 continued on next page

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and 24 hr. (**E** and **F**) CellTiter-Glo viability assays of Jurkat and KOPT-K1 cells treated with a single 20 μ M dose of Abd-CRBN (**E**) or Abd-VHL (**F**) for up to 48 hr. All the values are presented as the average viable cells per ml. Data represent mean + SEM (n=3).

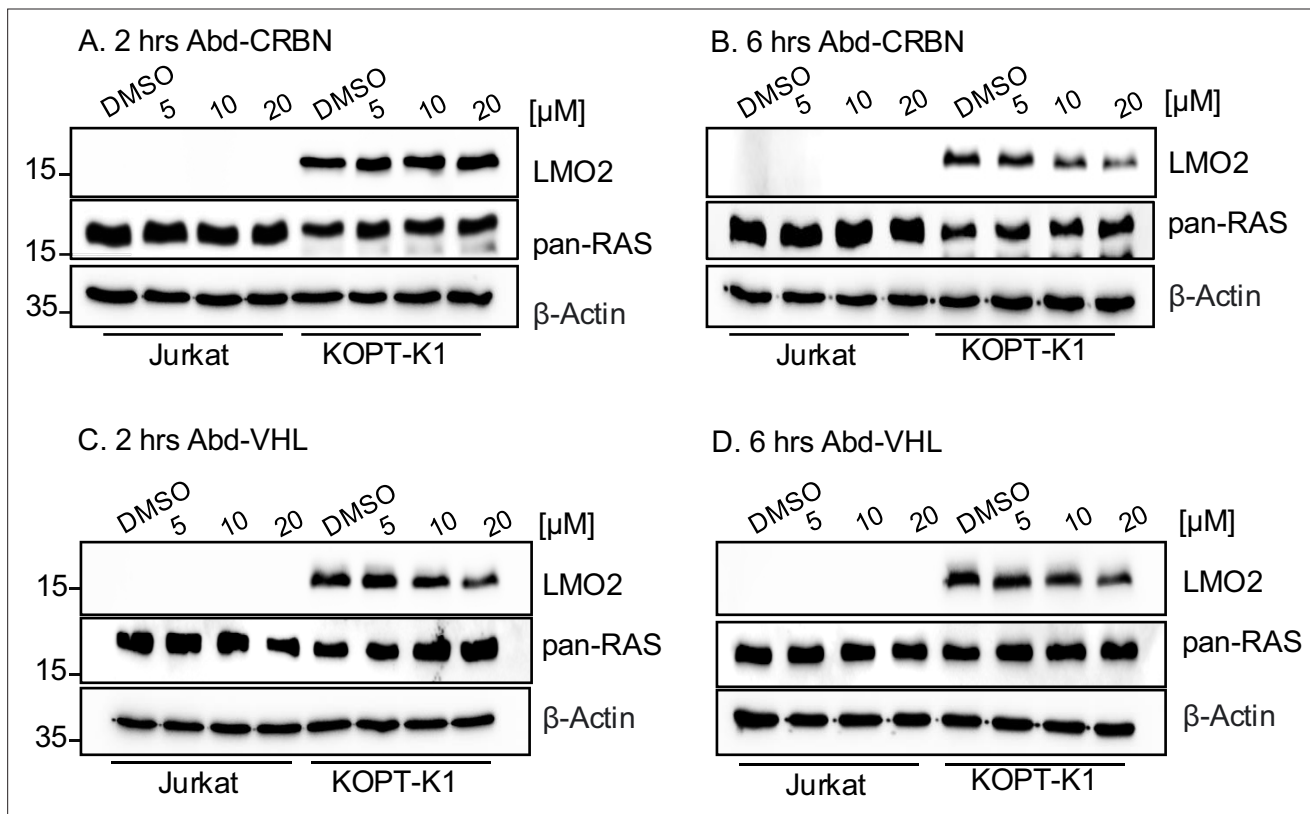


Figure 2—figure supplement 2. LMO2 antibody-derived (Abd) degraders and effects on cellular proteins. KOPT-K1 and Jurkat cells were treated with different concentrations of Abd-CRBN (A and B) or Abd-VHL (C and D) at 0, 5, 10, and 20 μ M for 2 and 6 hr, proteins extracted and fractionated by SDS-PAGE followed by western blotting. β -Actin was used as a loading control for analysis.

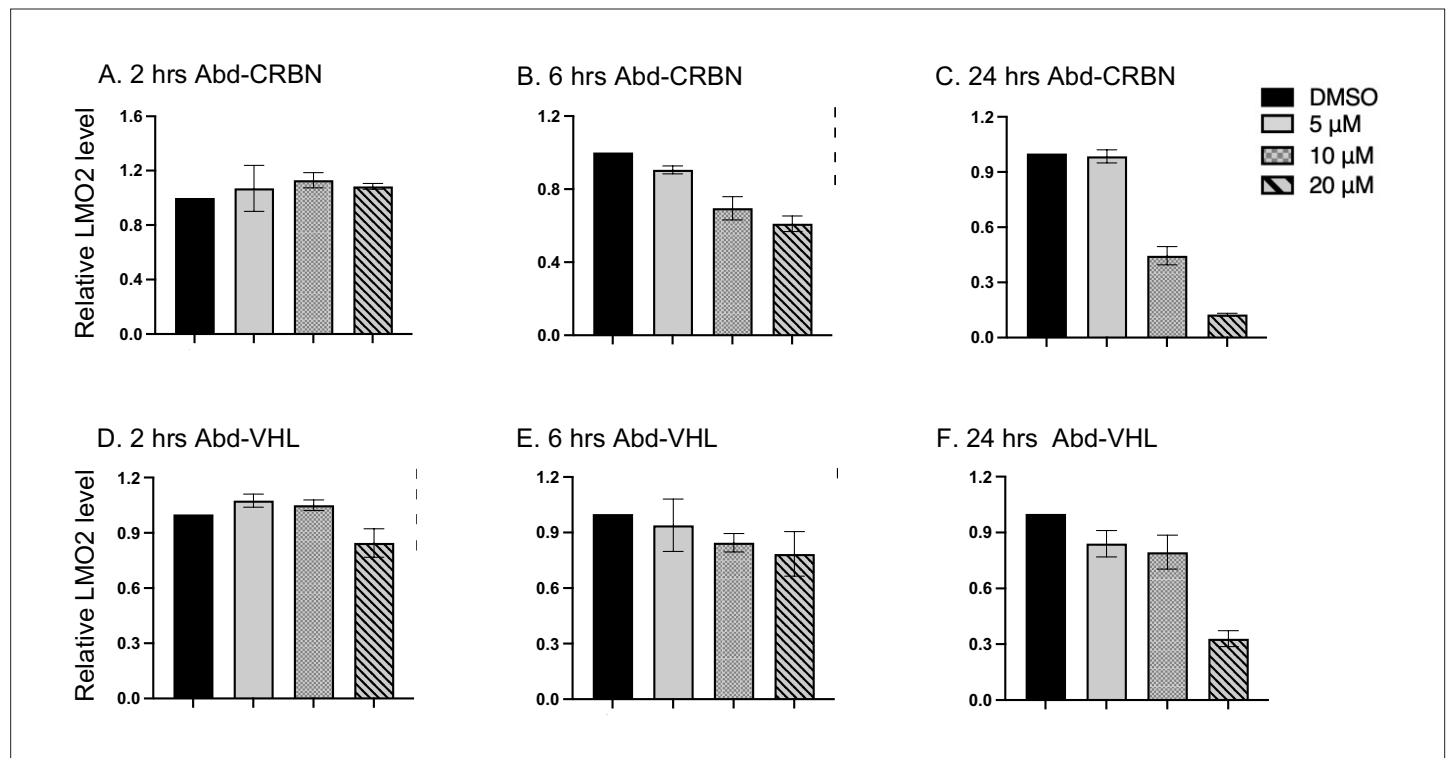


Figure 2—figure supplement 3. Densitometry analysis of LMO2 protein expression. Quantification of western blot data from **Figure 2D–I**. KOPT-K1 and Jurkat cells were treated with different concentrations of Abd-CRBN or Abs-VHL at 0, 5, 10, and 20 μ M for 2, 6, and 24 hr. The densitometry values were determined by using Image Lab software. Data represent mean + SEM (n=3).

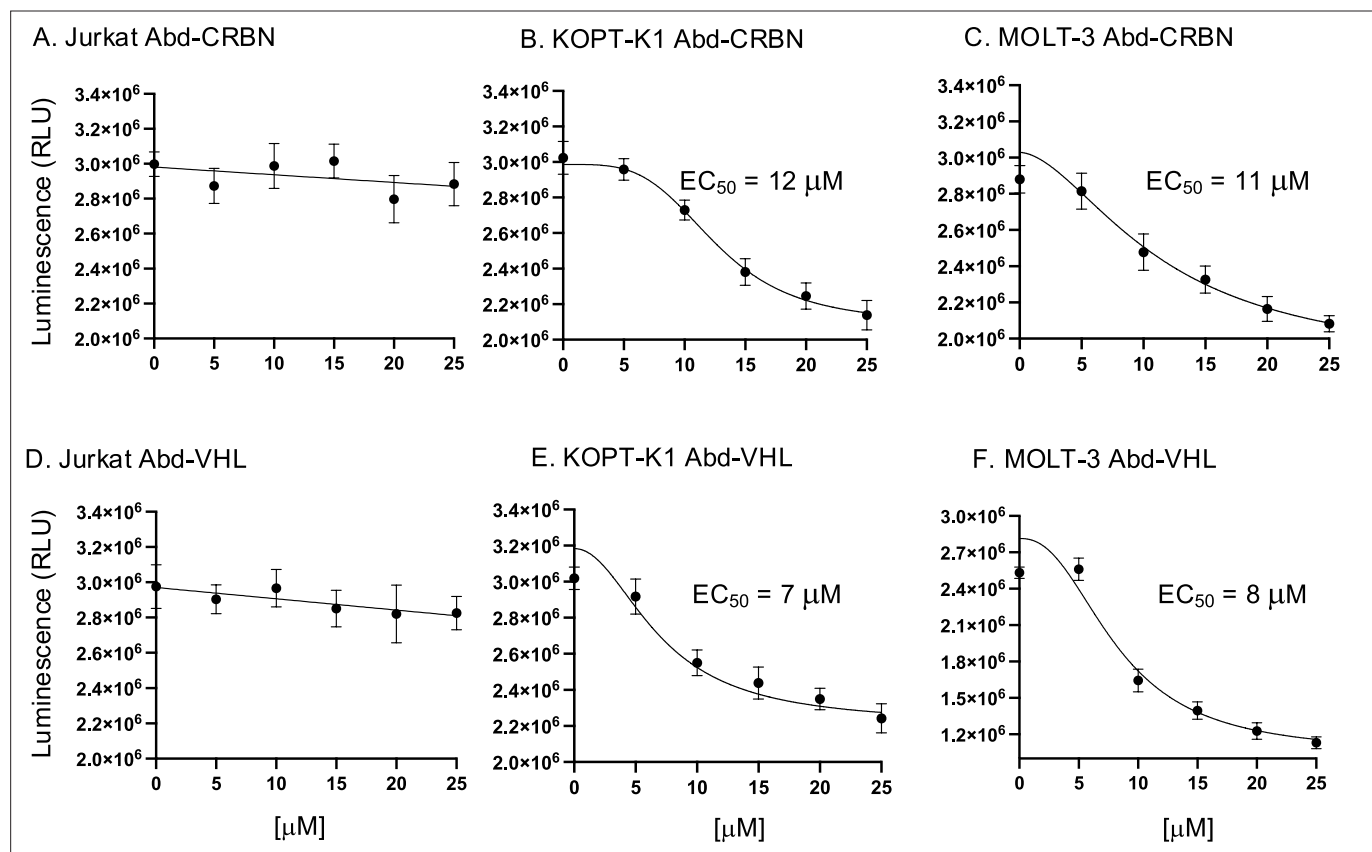


Figure 2—figure supplement 4. Dose response of T cell lines with antibody-derived (Abd) degraders. T cells were treated with Abd-CRBN or Abd-VHL in a dose range from 0 to 25 μM for 24 hr. The effect on viability was measured using CellTiter-Glo assays at 24 hr in Jurkat, KOPT-K1, and MOLT-3 with Abd-CRBN (A–C) or with Abd-VHL (D–F). Data represent mean \pm SEM (n=3). EC_{50} values were determined from dose-response curves generated using GraphPad Prism 9.0 software.

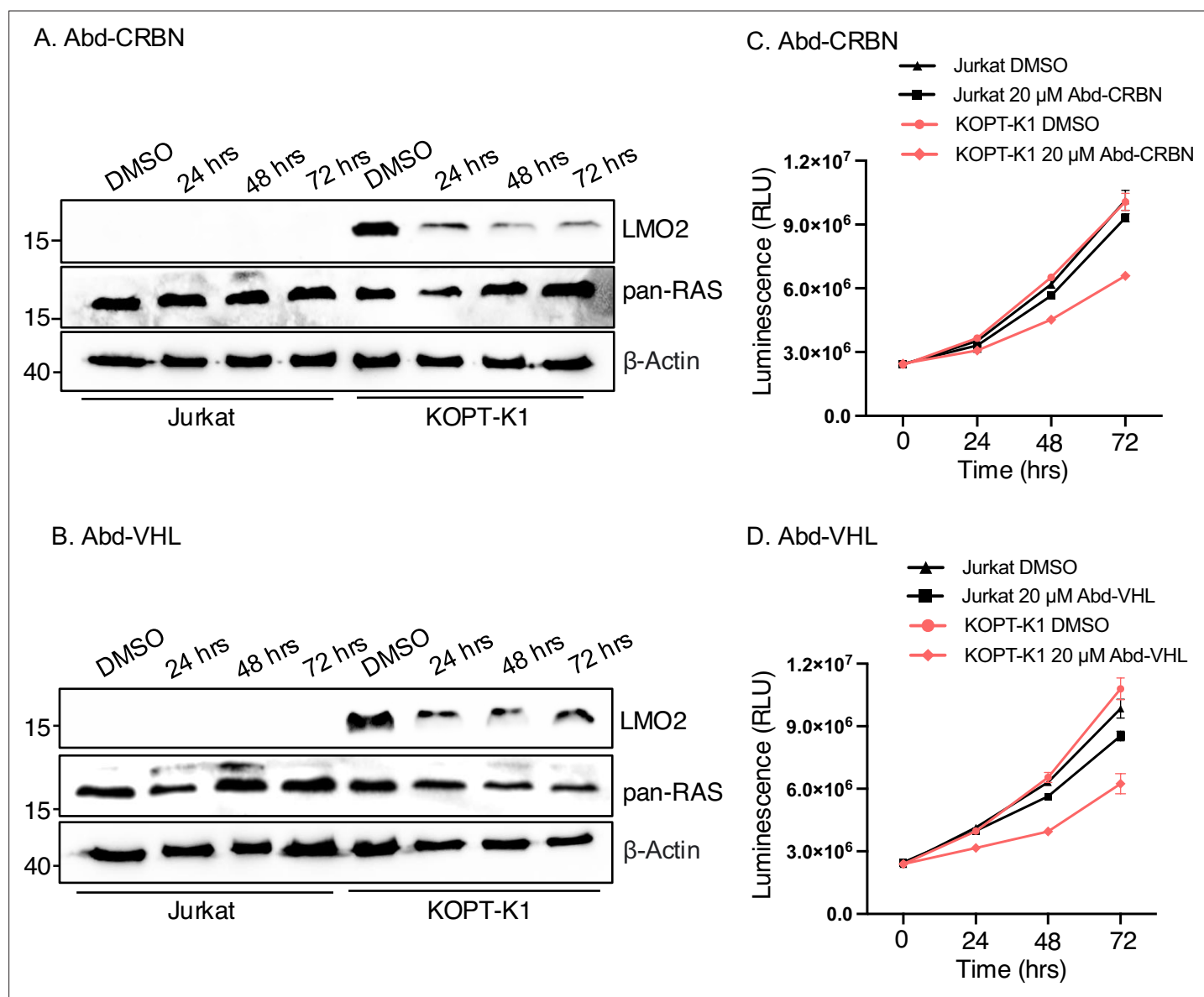


Figure 2—figure supplement 5. Longevity of responses of KOPT-K1 cells to single-dose treatment with antibody-derived (Abd) degraders. Jurkat and KOPT-K1 cells were treated with 20 μ M of Abd compounds and cultured from 0 to 72 hr. Western blot data of LMO2 and RAS expression in cells were treated with Abd-CRBN (A) and Abd-VHL (B). β -Actin was used as an internal loading control for western blotting analysis. Cell viability was determined using the CellTiter-Glo assay after the treatment with Abd-CRBN (C) and Abd-VHL (D) at points during the 72 hr. All the values are presented as the average luminescence values. Data represent mean + SEM (n=3).

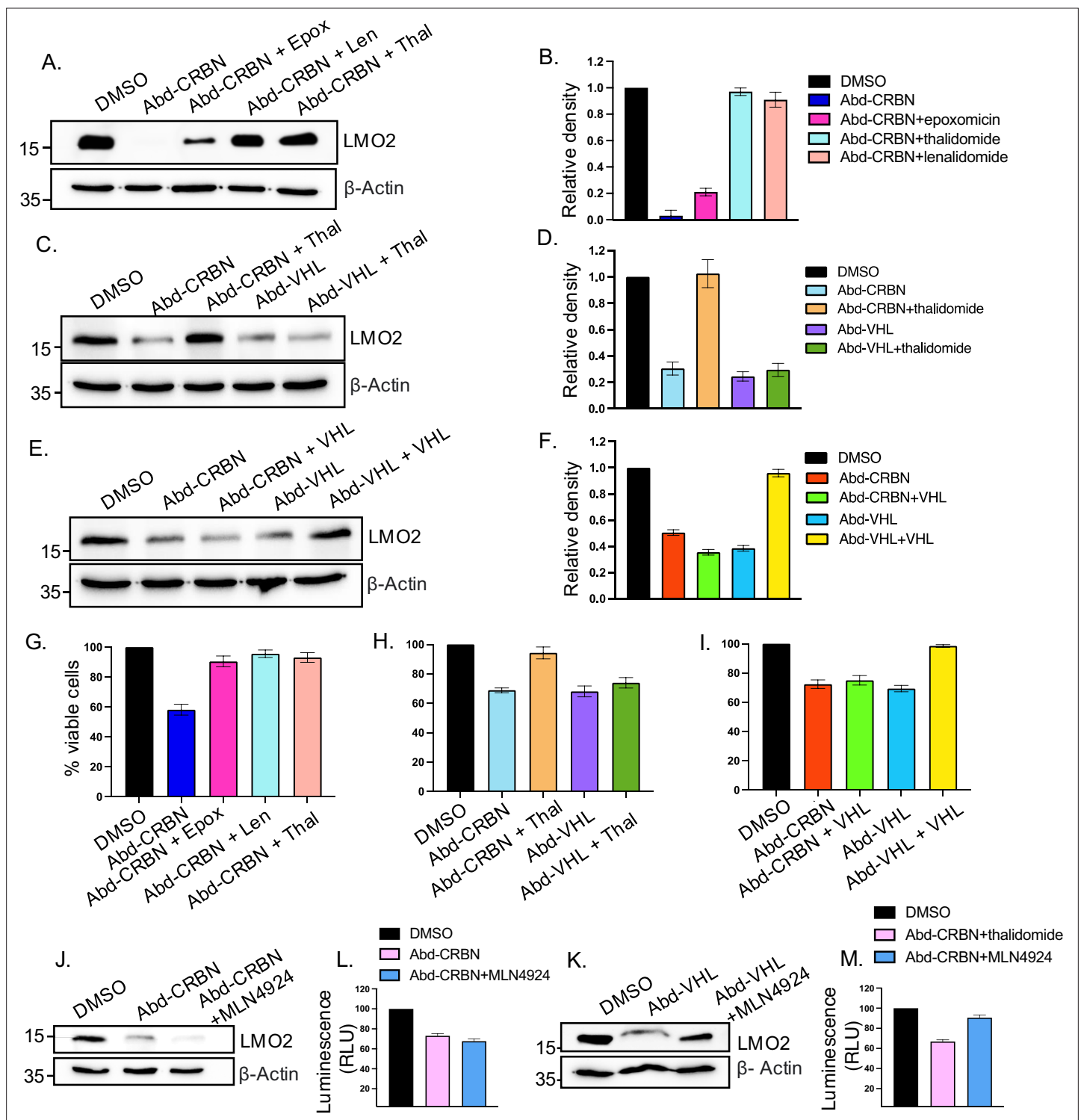


Figure 2—figure supplement 6. LMO2 levels and viability of KOPT-K1 cells treated with antibody-derived (Abd) degrader compounds and E3 ligase inhibitors. The involvement of proteasome machinery was investigated in KOPT-K1 with proteasome inhibitors or by competing with free E3 ligase ligands. Western blot data show LMO2 expression in KOPT-K1 cells after the treatment with or without inhibitors followed by Abd compounds (**A**, **C**, **E**). Inhibitors used were the proteasome inhibitor epoxomicin (Epox) (0.8 μ M), CRBN inhibitors (10 μ M thalidomide [thal] and 10 μ M lenalidomide [len]) (**A**, **C**), or free VHL ligand (**E**). Inhibitors were added for 2 hr prior to the treatment of 20 μ M Abd-CRBN or Abd-VHL and further cultured for 24 hr. β -Actin was used as a loading control for analysis. Densitometry data of LMO2 in KOPT-K1 are presented as mean relative densitometry units, with standard deviation (**B**, **D**, **F**). After the treatment, cell viability was measured with Trypan Blue (**G**, **H**, and **I**). Data represent mean + SEM (n=3). KOPT-K1 cells were also treated with or without protein NEDDylation inhibitor (10 μ M MLN4941) for 2 hr prior to the treatment of Abd-CRBN (**J**) or Abd-VHL (**K**)

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for 24 hr. CellTiter-Glo assays (**L** and **M**) were used to measure viability, presented as relative to luminescence in DMSO-treated cells, and normalised to 100%. Data represent mean + SEM (n=3).

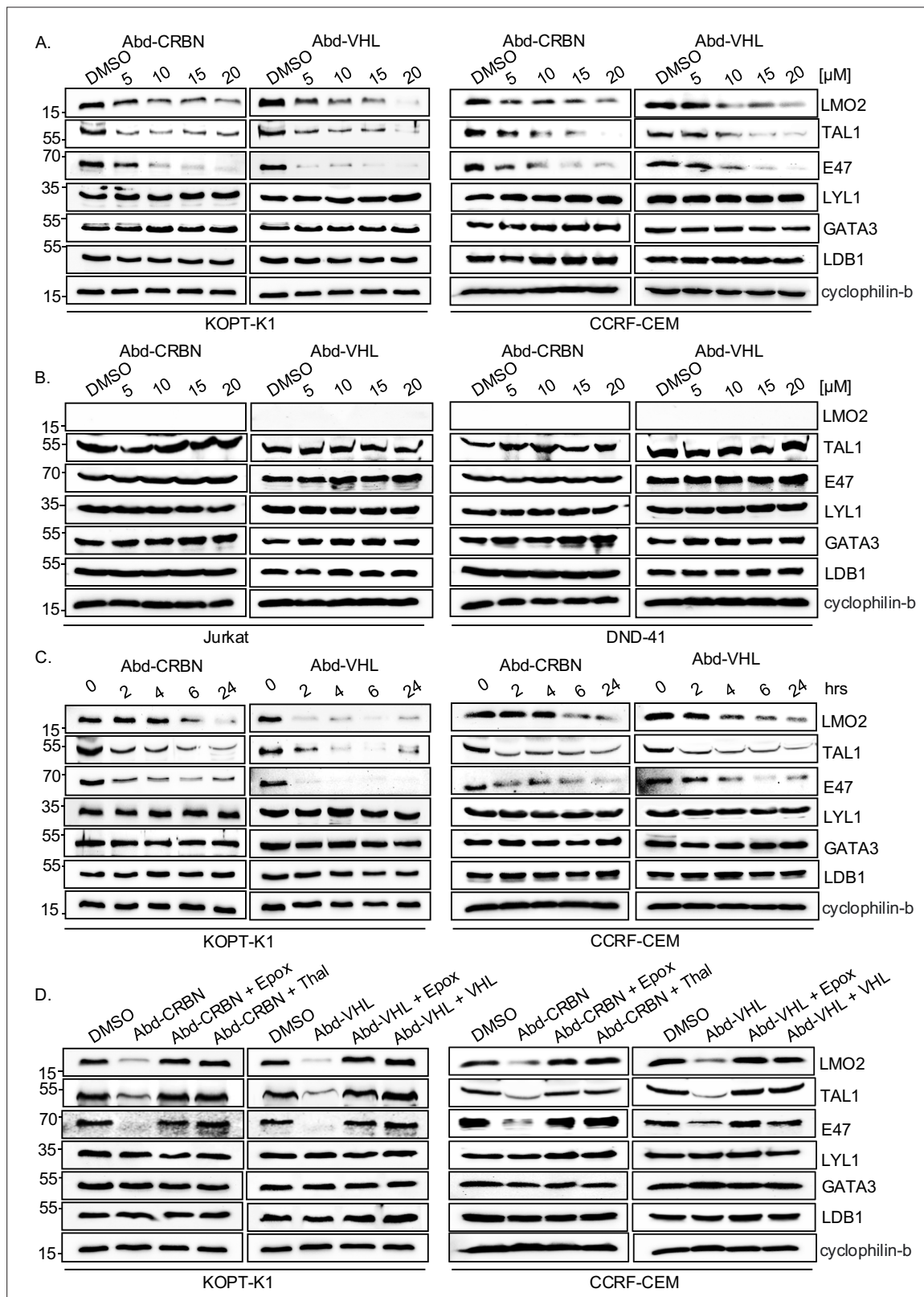


Figure 3. Proteins associated within the LMO2 transcription complex are co-degraded with the LMO2 proteolysis targeting chimeras (PROTACs). LMO2-expressing (KOPT-K1 and CCRF-CEM) and non-expressing T cells (Jurkat and DND-41) were treated with antibody-derived (Abd) degraders, and protein extracts were prepared for western blot, detecting LMO2, TAL1, E47, Lyl-1, GATA3, LDB1 proteins (cyclophilin-b was used as an internal loading control) after cells were treated with different concentrations of Abd degraders at 0, 5, 10, 15, and 20 μ M for 24 hr. (A) Western blotting analysis with

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KOPT-K1 and CCRF-CEM extracts and **(B)** western blotting analysis with Jurkat and DND-41 extracts. **(C)** KOPT-K1 and CCRF-CEM were treated with Abd degraders at 20 μ M for 2, 4, 6, and 24 hr. Western blotting analysis data show LMO2, TAL1, and E47 proteins expression in KOPT-K1 and CCRF-CEM that was affected by treatment. **(D)** The involvement of proteasome machinery in protein complex degradation was investigated in KOPT-K1 and CCRF-CEM with either proteasome inhibitors or by competing the potential of the Abd degraders with free E3 ligase ligand. Western blot data show LMO2, TAL1, and E47 expression in KOPT-K1 and CCRF-CEM after the treatment with or without inhibitors followed by Abd compounds. Inhibitors used were the proteasome inhibitor epoxomicin (0.8 μ M), or CRBN inhibitors (10 μ M thalidomide) or free VHL ligand. Cyclophilin-b was used as an internal loading control for western blotting analysis.

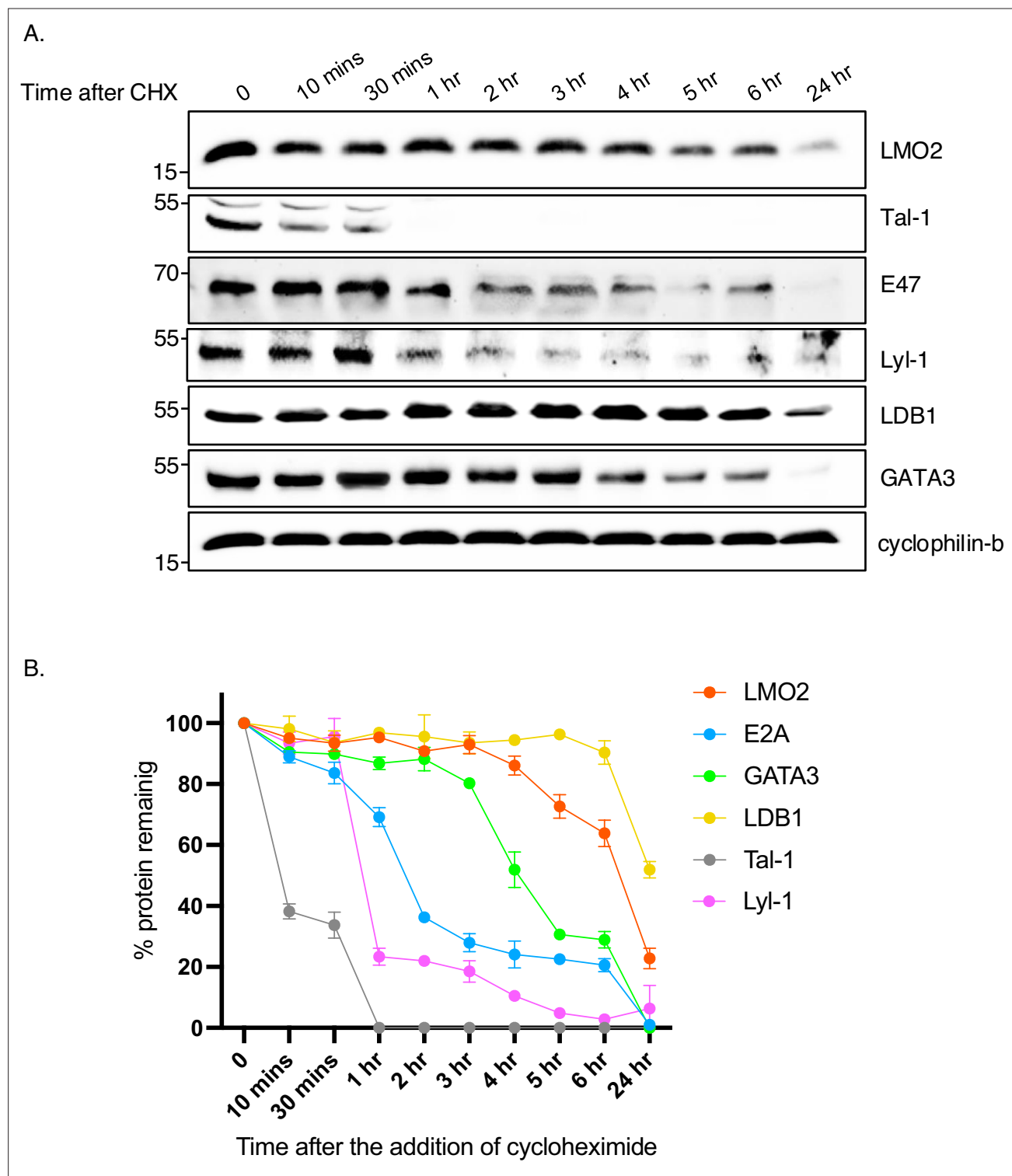


Figure 3—figure supplement 1. Half-lives of LMO2 and LMO2 protein complex in KOPT-K1 cells. Western blot data showed LMO2 and LMO2 protein complex (TAL1, E47, LYL1, LDB1, and GATA3) level after the treatment with 20 μ g/ml cycloheximide from 0 to 24 hr (A). The level of protein remaining is presented as a relative to protein level at 0 hr (B). Data represent mean + SEM (n=3).

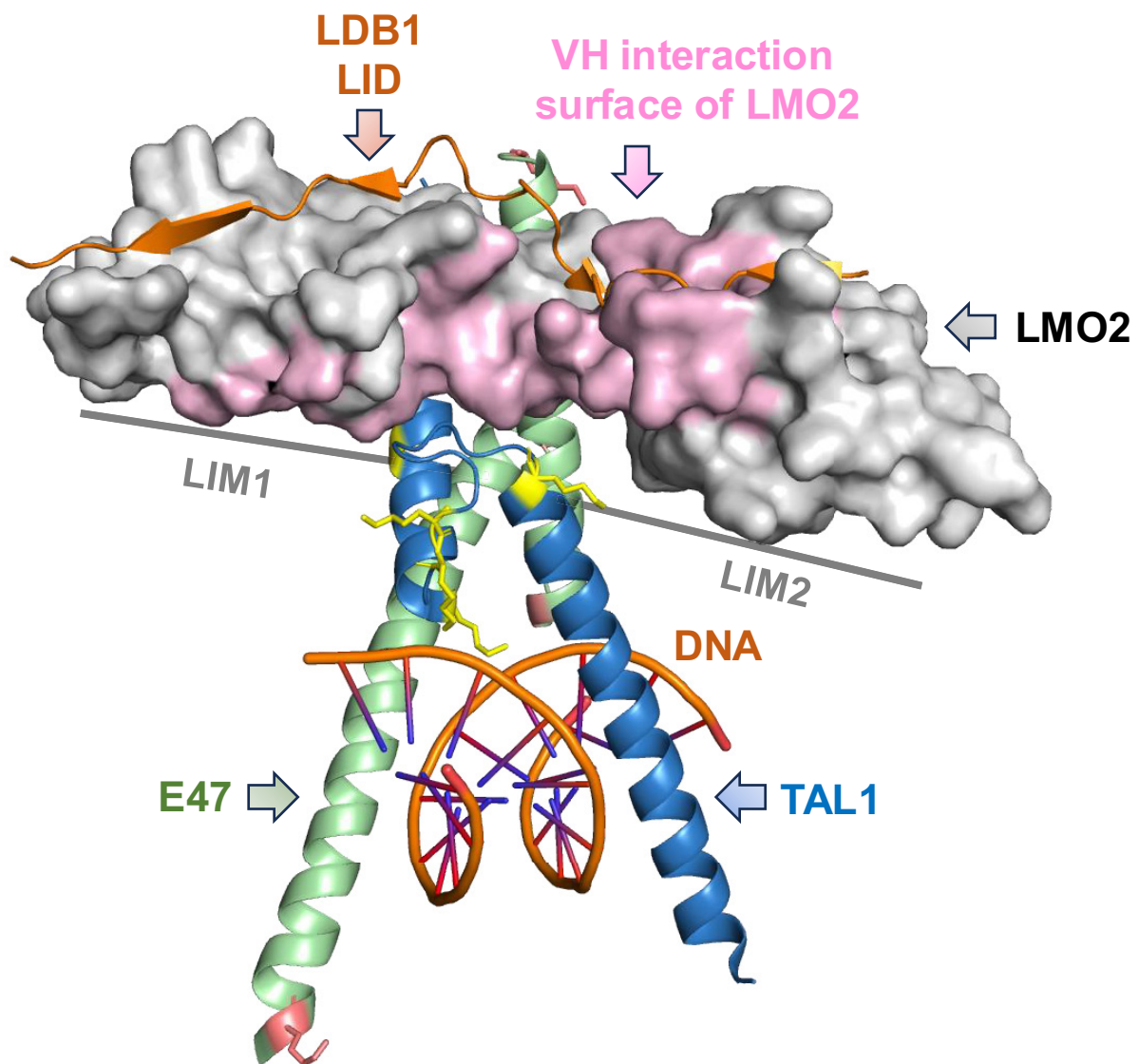


Figure 3—figure supplement 2. The LMO2 transcription complex indicating lysine residues in the basic-helix-loop-helix (bHLH) heterodimer for potential lysine ubiquitination. The crystal structure of the LMO2-TAL1-E47 complex, adapted from *El Omari et al., 2013*, is shown with LMO2 contacting the LID domain of LDB1, the TAL1 and E47 bHLH domains, and DNA. LMO2 is shown in grey; the VH576 interaction domain in pink (*Sewell et al., 2014*); LID in orange; TAL1 in blue, and E47 in green. Lysine residues in TAL1 and E47 are in yellow and salmon, respectively.

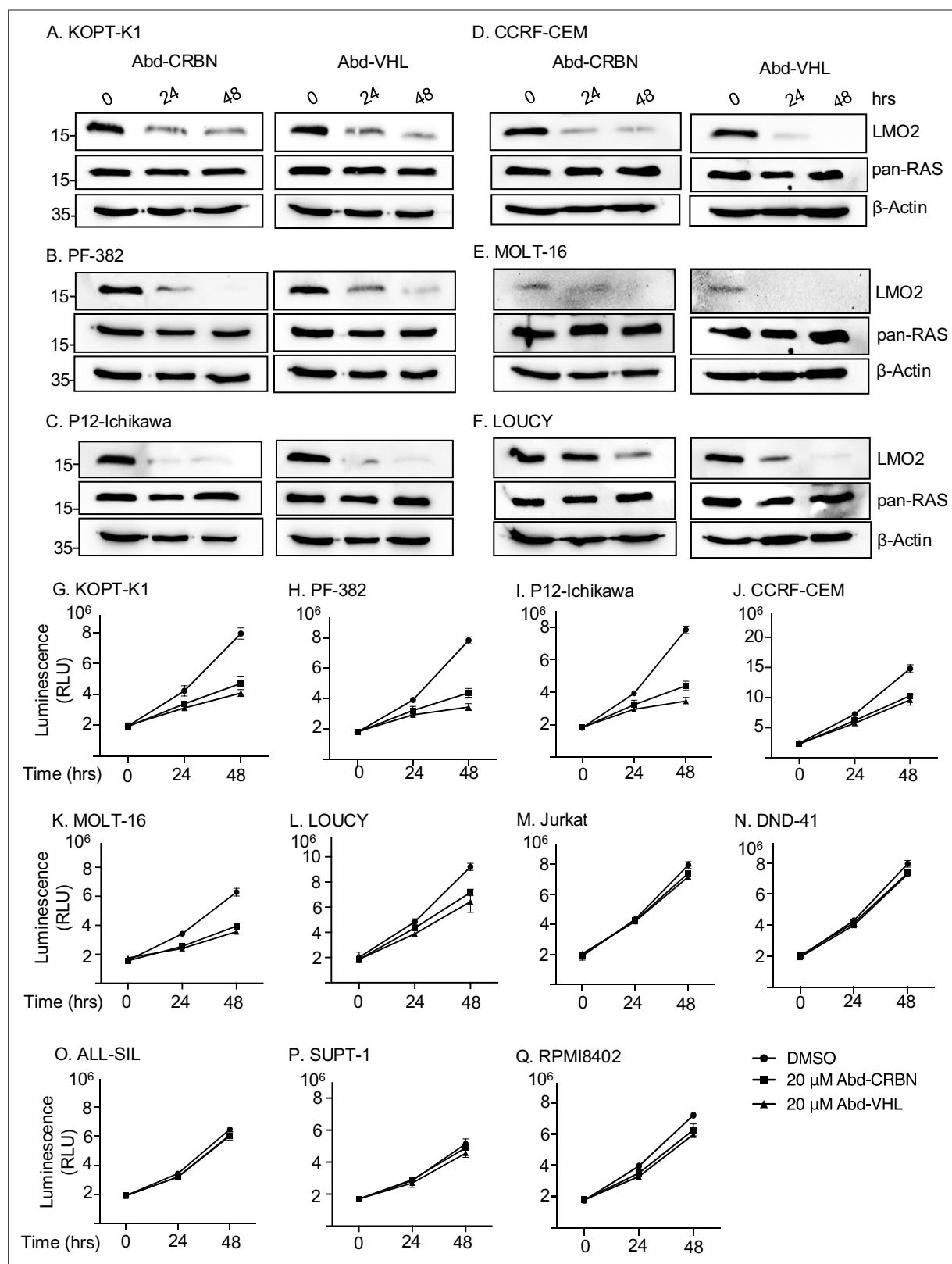


Figure 4. Loss of LMO2 protein inhibits T cell acute leukaemia (T-ALL) cell growth. LMO2-expressing T cells were treated with 20 μM antibody-derived (Abd)-CRBN or Abd-VHL for 24 and 48 hr, and protein extracts were prepared for western blot to detect LMO2 protein, RAS protein as negative control, and β-actin was used as an internal loading control. Western blotting analysis data show LMO2 expression in KOPT-K1 (A), PF-382 (B), P12-Ichikawa (C), CCRF-CEM (D), MOLT-16 (E), and LOUCY (F) that was affected by treatment with the LMO2 proteolysis targeting chimera (PROTAC) compounds.

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Comparative cell numbers were determined from increase in viability determined using the CellTiter-Glo assay. Data represent mean + SEM (n=3). LMO2-expressing cell lines tested were KOPT-K1 (**G**, t(11;14)(p13;q11)), PF-382 (**H**), P12-Ichikawa (**I**, t(11;14)(p13;q11)), CCRF-CEM (**J**), MOLT-16 (**K**), and LOUCY (**L**). LMO2 non-expressing T cells were also treated with 20 μ M Abd-CRBN or Abd-VHL for 24 and 48 hr after which relative luminescence was determined using the CellTiter-Glo assay. Cells tested were Jurkat (**M**), DND-41 (**N**), ALL-SIL (**O**), SUPT-1 (**P**), and RPMI8402 (**Q**). Data represent mean + SEM (n=3).

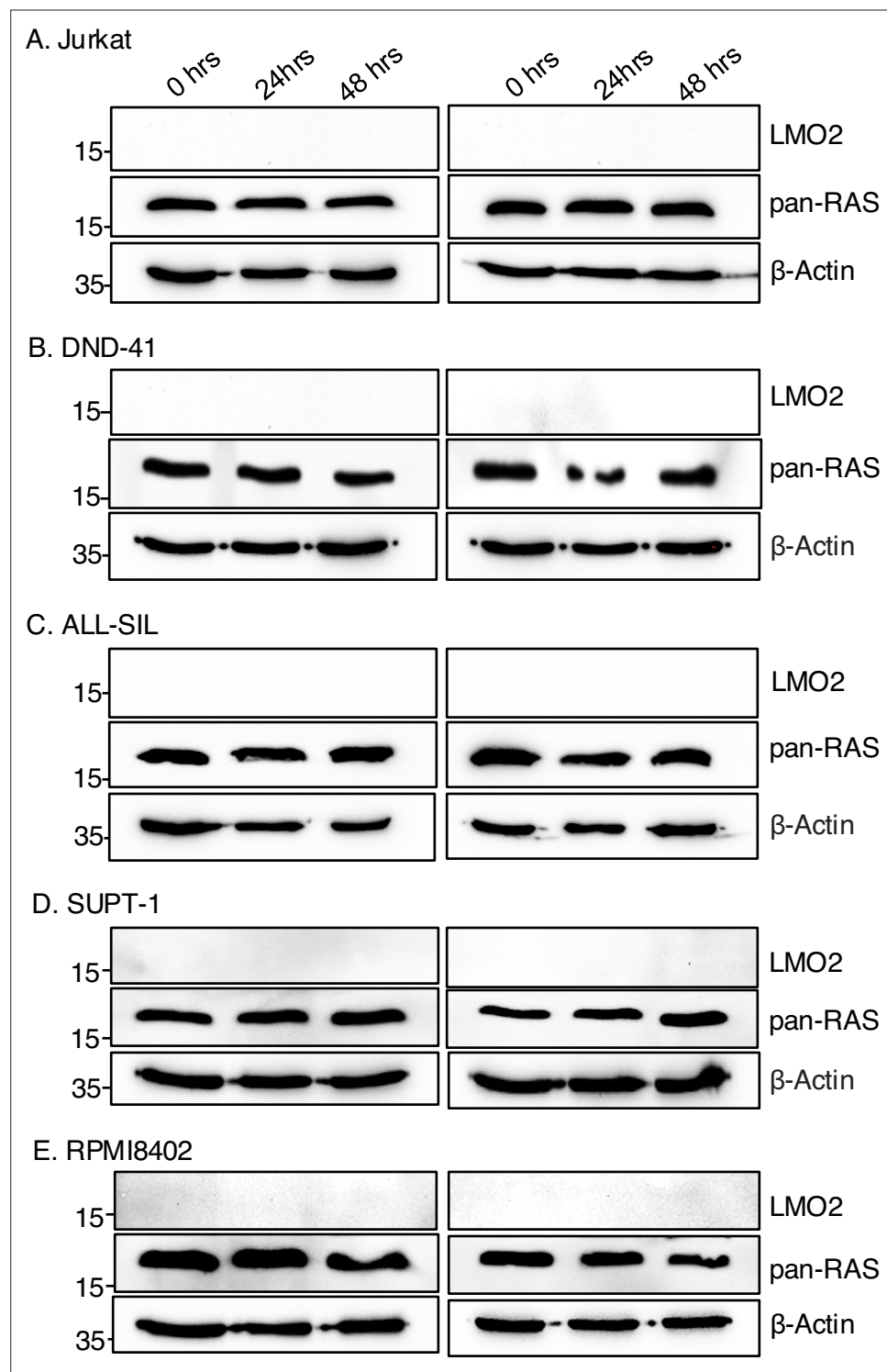


Figure 4—figure supplement 1. Verification of LMO2 non-expressing T cell lines. Five human T cell lines were verified by western analysis of total protein extracts and probed with anti-LMO2, anti-RAS, and β-actin as an internal loading control. No LMO2 was detectable. In addition, off-target effects, as determined by RAS protein levels, of the antibody-derived (Abd)-CRBN and Abd-VHL were tested by treating the cells with 20 μM Abd degraders for 24 and 48 hr followed by western blotting.

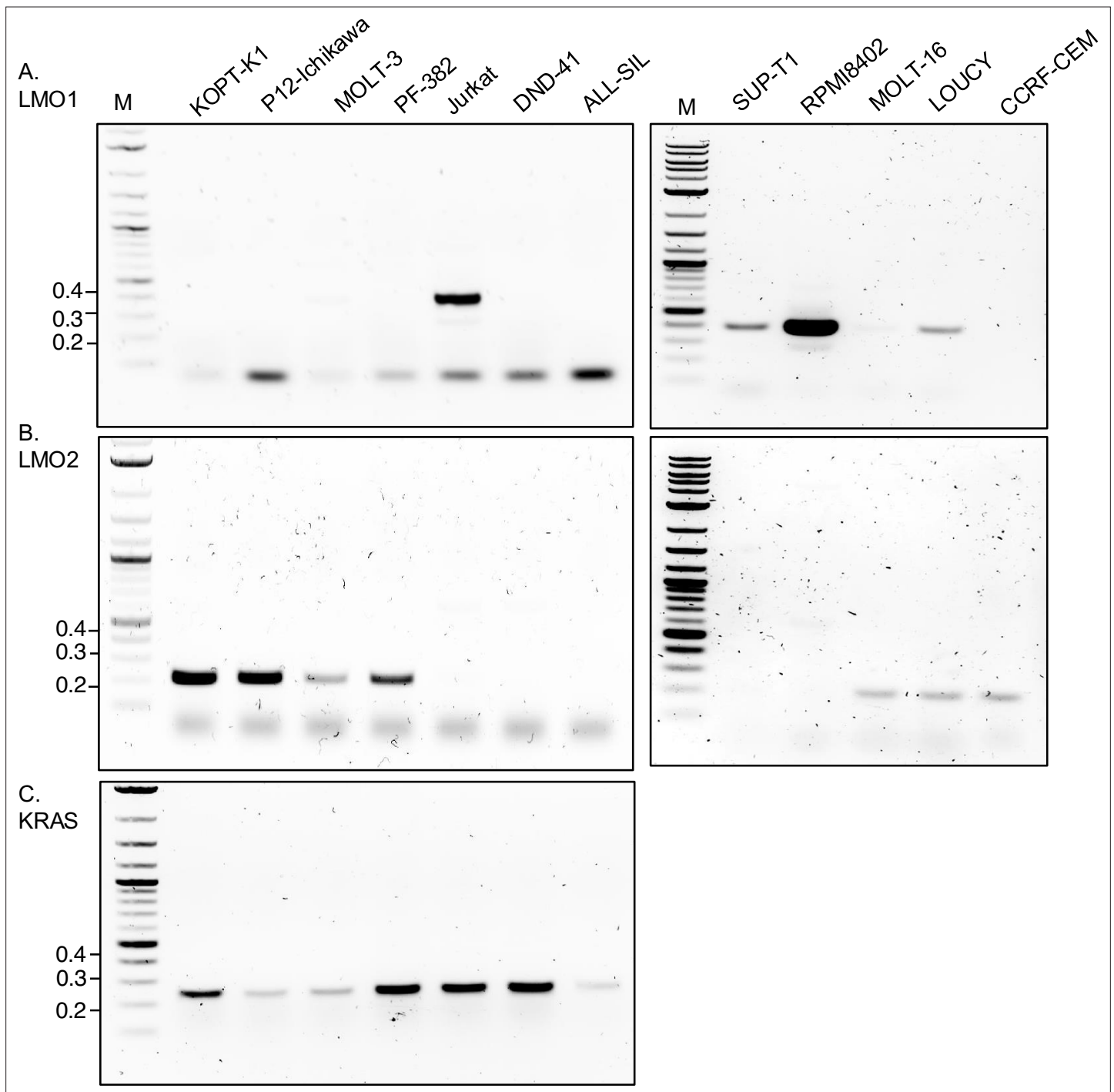


Figure 4—figure supplement 2. Reverse transcription-PCR (RT-PCR) analysis of the human T cell acute leukaemia (ALL) panel. Agarose gel electrophoresis of LMO1 (A), LMO2 (B), and KRAS (C) PCR products from the different human T cell lines (KOPT-K1, P12-Ichikawa, MOLT-3, PF-382, Jurkat, DND-41, and ALL-SIL). Lane M represents a 1 kb ladder. RT-PCR primer sequences. LMO1: PCR product 410 bp Forward GATCCAGCCCAA AGGGAAGCAG Reverse GATAAAGGTGCCATTGAGCTG LMO2: PCR product 220 bp Forward GATTCTCGGCCATCGAAAGG Reverse GATGTTTG TAGTAGAGGCGCCG KRAS: PCR product 249 bp Forward GATATGACTGAATATAAACTTGTGGTAG Reverse GATGGCAAATACACAAAGAAAGC.

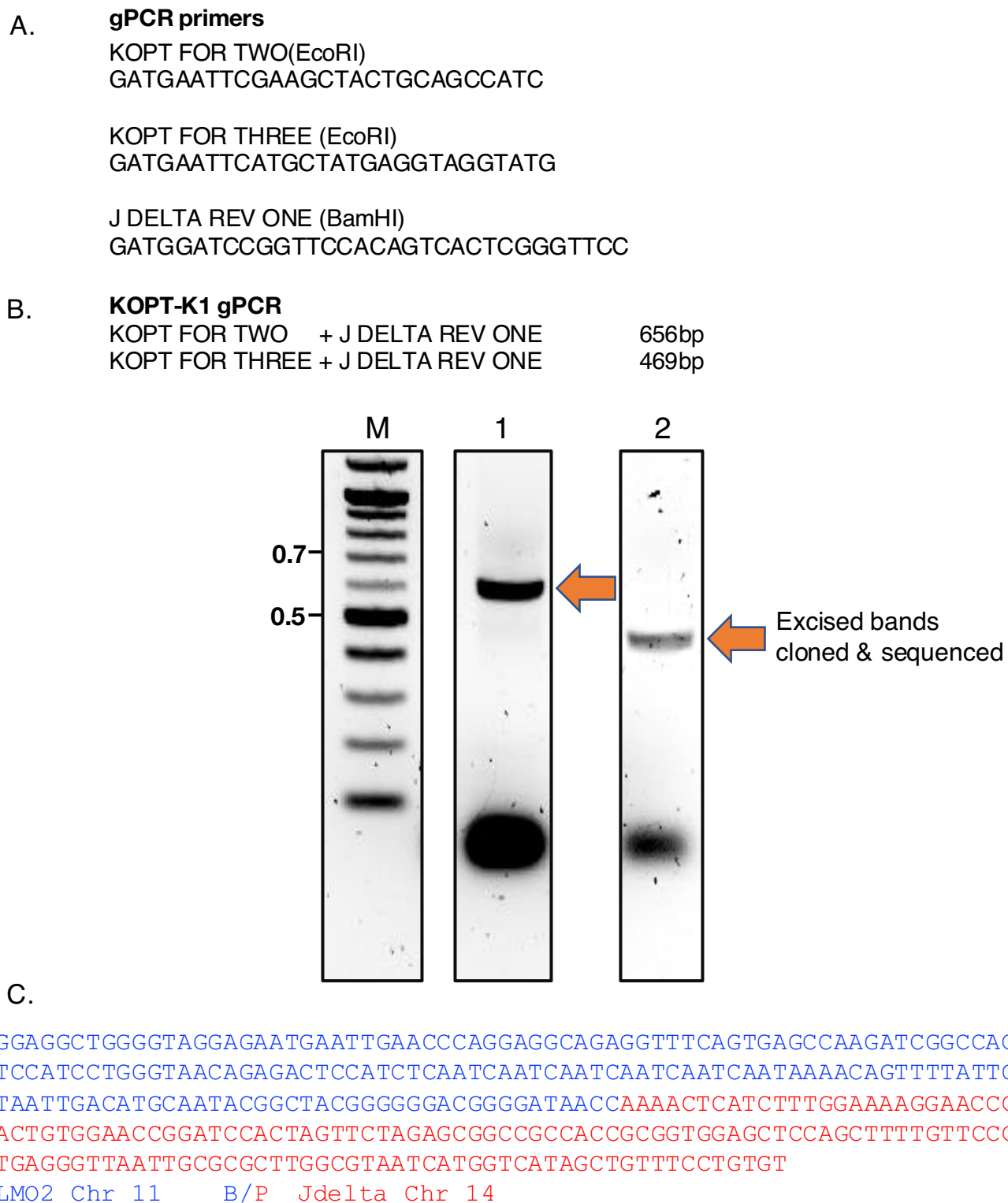


Figure 4—figure supplement 3. Confirmation of chromosomal translocation in KOPT-K1 tissue culture cells. The human T cell acute leukaemia (T-ALL) cells carry an LMO2 activating chromosomal translocation t(11;14)(p13;q11). The gPCR forward primers were designed to bind different 3'-ends of the breakpoint region, and the reverse primer was designed to bind at 5'-ends of the breakpoint region (**A**). Agarose gel electrophoresis was used to evaluate the PCR products (**B**), the different size of PCR products is shown in lanes 1 and 2. Lane M represents a 1 kb marker ladder. (**C**) The sequence of the genomic PCR primers was derived from the published breakpoint (*Dong et al., 1995*) and the relevant germline sequences of chromosomes 11 and 14.

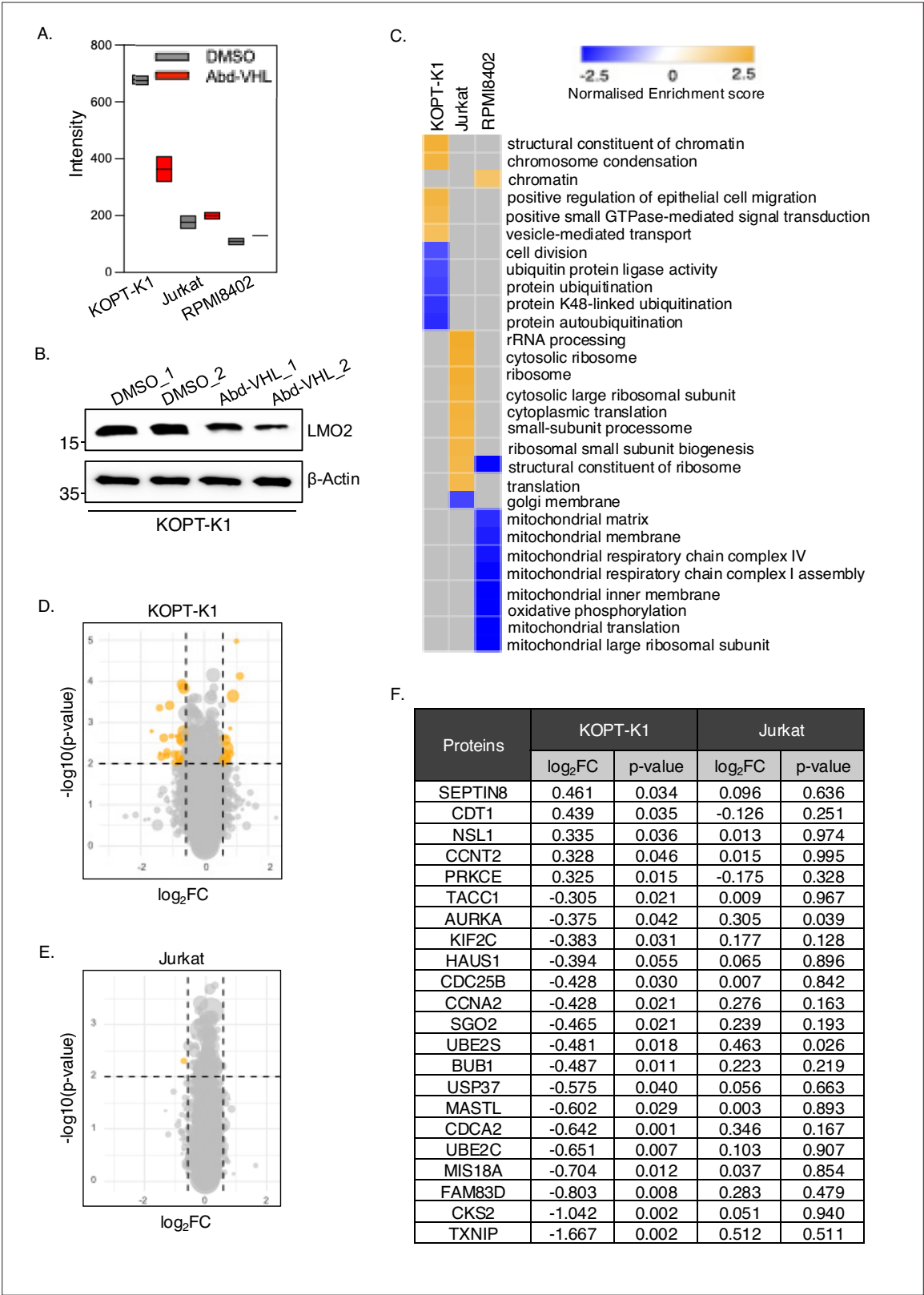


Figure 5. Proteomic profiling of T cell acute leukaemia (T-ALL) cells after treatment with antibody-derived (Abd) proteolysis targeting chimera (PROTAC) degrader. LMO2-expressing T cells (KOPT-K1) and LMO2 non-expressing T cells (Jurkat and RPMI8402) were treated in duplicate with 15 μ M Abd-VHL for 24 hrs. **(A)** Floating bar plots of LMO2 protein levels measured by targeted proteomics in control (DMSO treated) (grey bars) and Abd-VHL treated (red bars) in KOPT-K1, Jurkat, and RPMI8402 cells. **(B)** Western blotting analysis of LMO2 levels in KOPT-K1 cells when treated with DMSO only and Abd-

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VHL for 24 hr. β -Actin protein detection was used as an internal loading control for western blotting analysis. **(C)** Heatmap displaying enriched terms from Gene Set Enrichment Analysis (GSEA) in KOPT-K1, Jurkat, and RPMI8402 cells after Abd-VHL treatment compared to control treatment (DMSO), based on global proteomics analysis. The enrichment score ranges from -2.5 (blue) to 2.5 (yellow). **(D and E)** Volcano plot of proteomic changes following Abd-VHL treatment compared to control treatment (DMSO) in KOPT-K1 **(D)** and Jurkat **(E)**. The plots are based on the fold change (\log_2FC) and p-value ($-\log_{10}(p\text{-value})$). Respectively, the yellow or grey circles indicate proteins with statistically significant or non-significant up-regulation/down-regulation. The cut-off for the volcano plots is $\log_2FC = 0.585$, $p\text{-value} = 0.05$. **(F)** A tabulation of statistically significant proteins in KOPT-K1, represented as yellow circles **(D)** compared to Jurkat cells. The data are presented as fold change (\log_2FC) and p-value ($-\log_{10}(p\text{-value})$). The source data are available as **Figure 5—source data 3**.

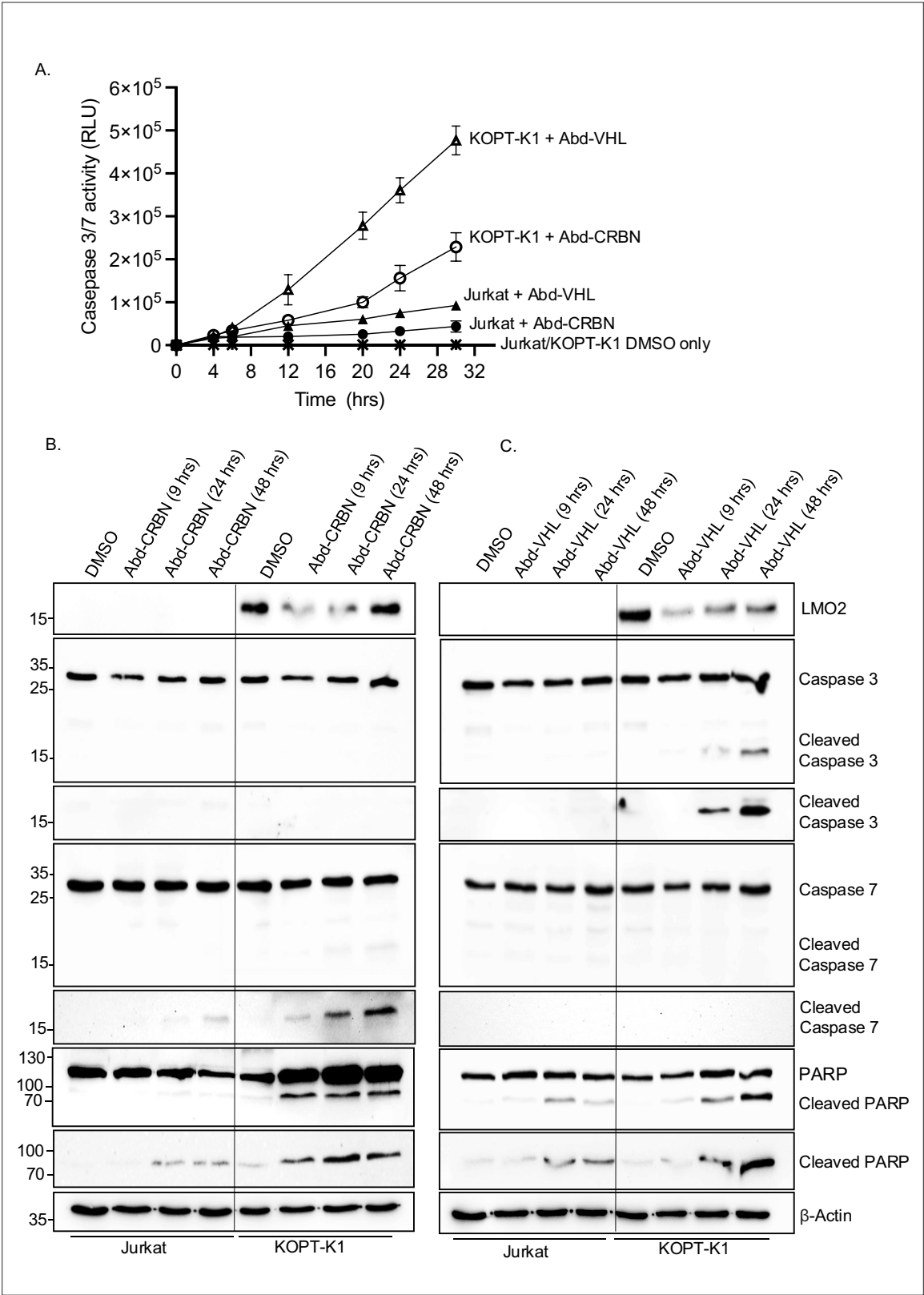


Figure 6. Caspase and poly(ADP-ribose) polymerase (PARP) cleavage in cells after treatment with antibody-derived (Abd) degraders indicative of apoptosis initiation. KOPT-K1 and Jurkat cells were treated with Abd-CRBN or Abd-VHL and effects on viability and programmed cell death were analysed at different times. **(A)** Time course of progressive expression of caspases after the treatment with compounds was assayed for caspase 3/7 levels using Caspase-Glo 3/7. Cells were treated with 20 μ M Abd-CRBN or Abd-VHL followed by cell culture up to 30 hr. Cells treated with 1% DMSO

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in culture medium were used as a control. Data represent mean + SEM (n=3). KOPT-K1 and Jurkat cells were treated with 20 μ M Abd-CRBN (**B**) or Abd-VHL (**C**) and cultured for 24 or 48 hr. Cell extracts were made and proteins subjected to western blotting analysis with specific antibodies for detection of LMO2, caspase 3, cleaved caspase 3, caspase 7, cleaved caspase 7, PARP, and cleaved PARP. β -Actin protein detection was used as an internal loading control for western blotting analysis.

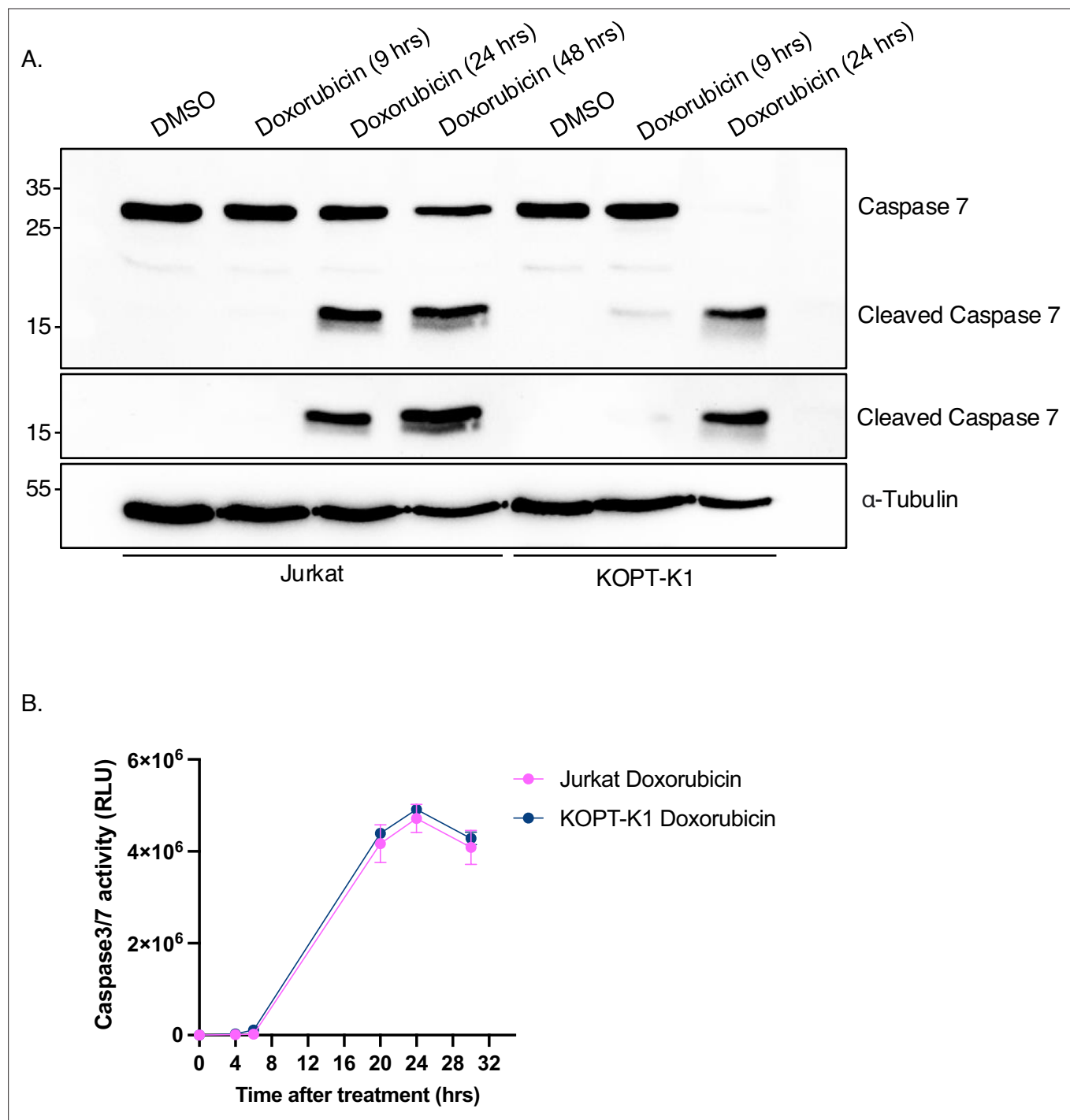
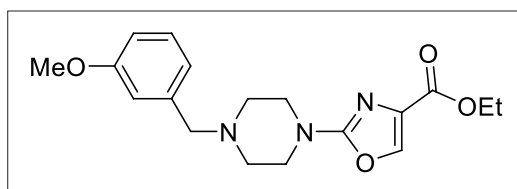
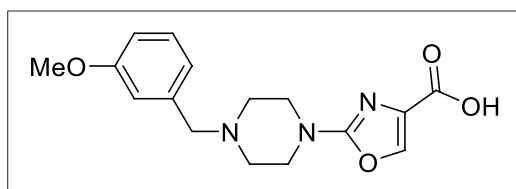


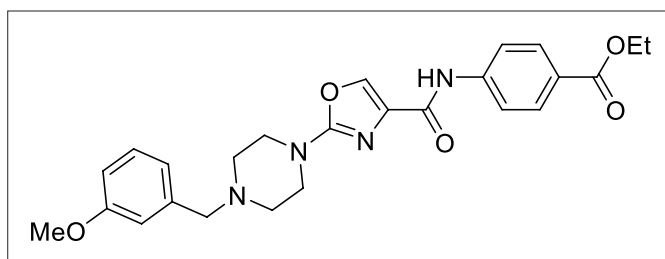
Figure 6—figure supplement 1. Caspase 7 and cleaved caspase 7 in Jurkat and KOPT-K1 cells after the treatment of doxorubicin. Western blot data showed caspase 7 and cleaved caspase 7 after the treatment with 1 μ M doxorubicin from 0 to 48 hr. At 48 hr treatment, doxorubicin at 1 μ M induced 100% cell death in KOPT-K1 cells (A). Cell viability after the treatment at 4, 6, 12, 20, 24, and 30 hr in Jurkat and KOPT-K1 cells was determined using CellTiter-Glo assay (B). Data represent mean + SEM (n=3).



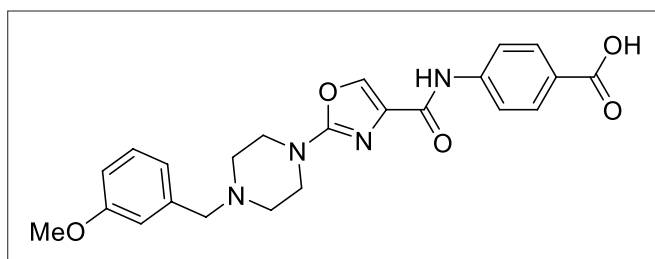
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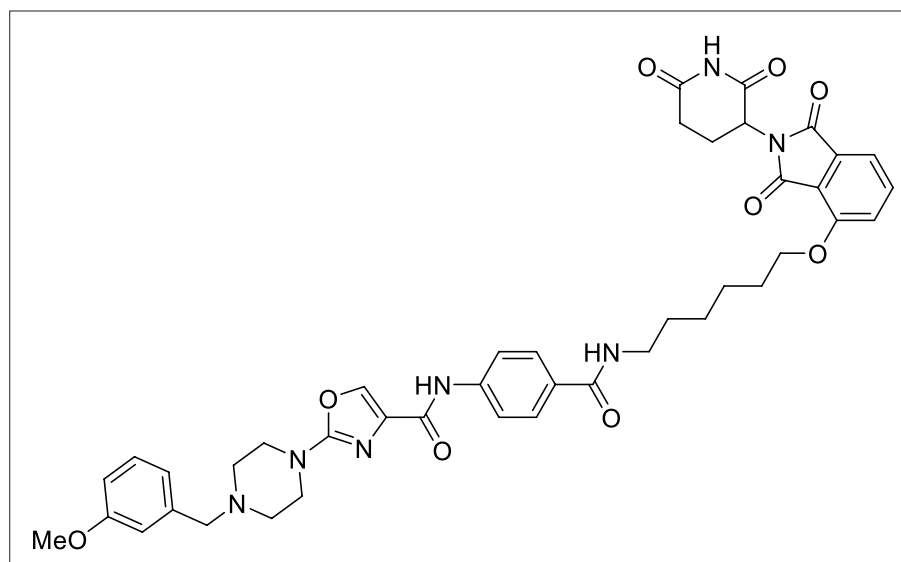
Chemical structure 2. ethyl 2-(4-(3-methoxybenzyl)piperazin-1-yl)oxazole-4-carboxylate.



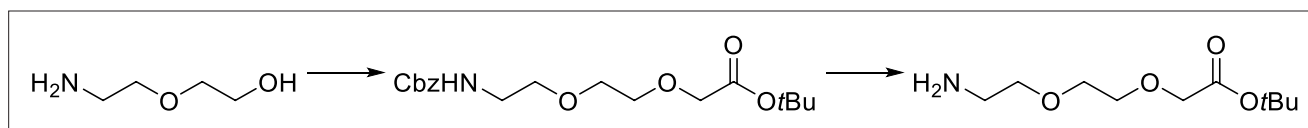
Chemical structure 3. ethyl 4-(2-(4-(3-methoxybenzyl)piperazin-1-yl)oxazole-4-carboxamido)benzoate.



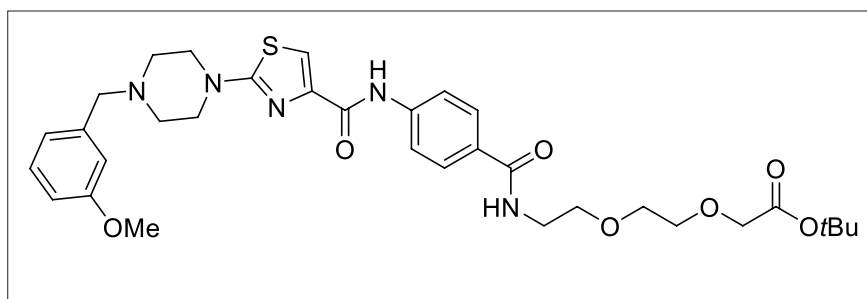
Chemical structure 4. 4-(2-(4-(3-methoxybenzyl)piperazin-1-yl)oxazole-4-carboxamido)benzoic acid.



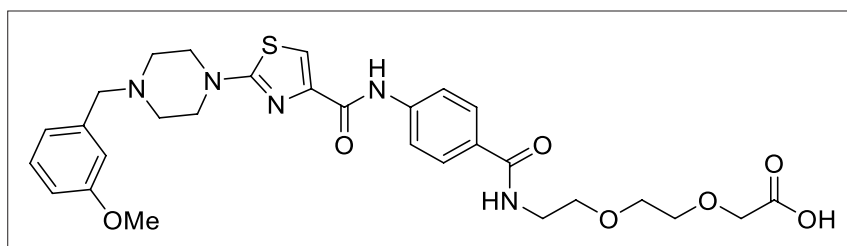
Chemical structure 5. N-(4-((6-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)oxy)hexyl)carbonyl)phenyl)-2-(4-(3-methoxybenzyl)piperazin-1-yl)oxazole-4-carboxamide.



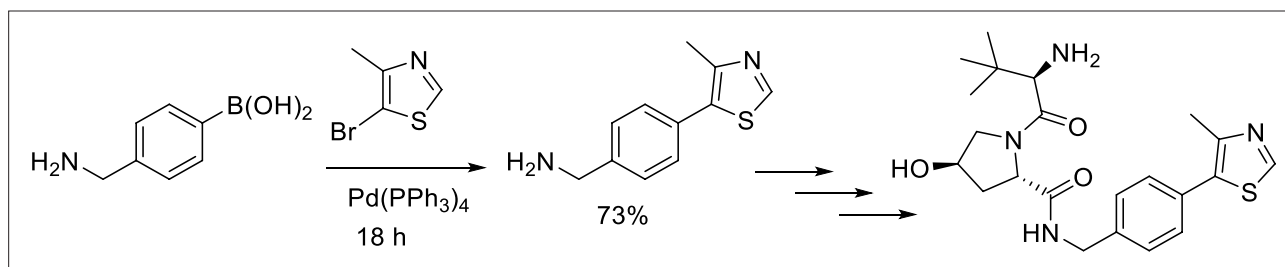
Chemical structure 6. Synthesis of Abd-VHL.



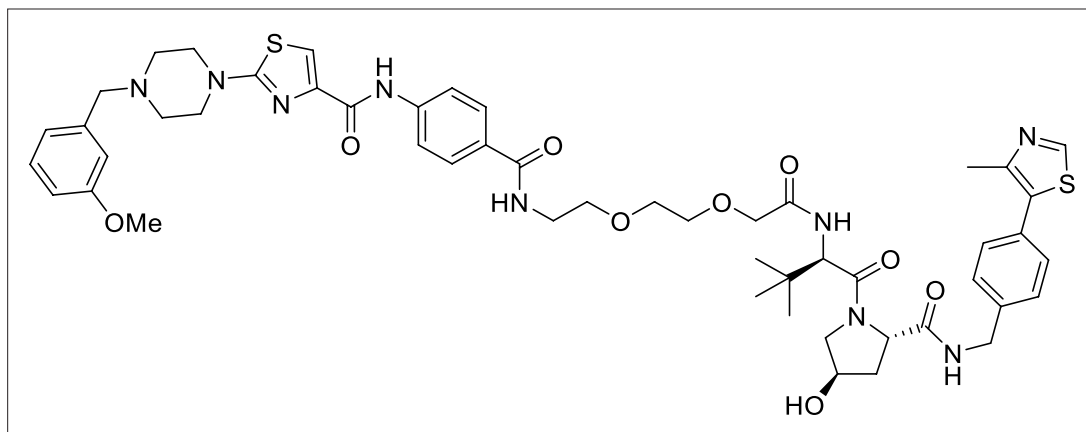
Chemical structure 7. tert-butyl 2-(2-(2-(4-(2-(4-(3-methoxybenzyl)piperazin-1-yl)thiazole-4-carboxamido)benzamido)ethoxy)ethoxy)acetate.



Chemical structure 8. 2-(2-(2-(4-(2-(4-(3-methoxybenzyl)piperazin-1-yl)thiazole-4-carboxamido)benzamido)ethoxy)ethoxy)acetic acid.



Chemical structure 9. VHL ligand.



Chemical structure 10. N-((4-((2-(2-(((R)-1-((2S,4R)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidine-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-2-oxoethoxy)ethoxy)ethyl)carbamoyl)phenyl)-2-(4-(3-methoxybenzyl)piperazin-1-yl)thiazole-4-carboxamide.