Lenalidomide Derivative and PROTAC for Controlling Neosubstrate Degradation

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Abstract

Lenalidomide, an immunomodulatory drug (IMiD), is commonly used as first-line therapy in many haematological cancer diseases, such as multiple myeloma (MM) and 5q myelodysplastic syndromes (5q MDS), and it functions as a molecular glue for the protein degradation of neosubstrates by CRL4<sup>CRBN</sup><sup>1−4</sup>. Proteolysis-targeting chimeras (PROTACs) using IMiDs with a target protein binder also induce the degradation of target proteins<sup>5,6</sup>. The targeted protein degradation (TPD) of neosubstrates is crucial for IMiD therapy<sup>2−4,7</sup>. However, current IMiDs and IMiD-based PROTACs also unexpectedly break down neosubstrates involved in embryonic development and disease progression<sup>2−4,8−10</sup>. Here, we showed that 6-position modifications of lenalidomide are essential for controlling neosubstrate selectivity; 6-fluoro lenalidomide induced the selective degradation of IKZF1, IZKF3, and CK1α, which are involved in anti-haematological cancer activity<sup>2−4</sup>, and showed stronger antiproliferative effects on MM and 5q MDS cell lines than lenalidomide. PROTACs using the new lenalidomide derivatives for BET proteins induced the selective degradation of BET proteins with the same neosubstrate selectivity. PROTACs also exerted antiproliferative effects in all cell lines examined, including MM and neuroblastoma cell lines. Thus, 6-position-modified lenalidomide is a key strategy for selective TPD using thalidomide derivatives and PROTACs.

Main

Lenalidomide, a thalidomide derivative, is an immunomodulatory drug (IMiD) widely used for treating several haematological cancers<sup>11−13</sup>, such as multiple myeloma (MM) and 5q myelodysplastic syndromes (5q MDS). IMiDs are a class of drugs that function as a molecular glue and induce 26S proteasomal degradation of neosubstrates by hijacking E3 ubiquitin ligase CRL4<sup>CRBN</sup> via interactions between IMiDs and cereblon (CRBN). In the last decade, many neosubstrates involved in the molecular action of IMiDs have been reported<sup>2−4,8−10</sup>. For example, degradation of IKZF1 and IZKF3 or IKZF1 and CK1α is involved in anti-MM<sup>2,3</sup> activity or anti-5qMDS activity<sup>4</sup>, respectively. However, SALL4 and PLZF degradation is considered the cause of thalidomide teratogenicity<sup>8−10</sup>, although this has not been proven in mammalian models.

Targeted protein degradation (TPD) is a powerful mechanism of action of drugs and a fundamental approach to developing drugs for undruggable proteins, including transcription factors. Many next-generation thalidomide derivatives, such as CC-90009<sup>14</sup> and CC-122<sup>15</sup>, are actively being developed. However, thalidomide derivatives have not been reported to induce selective degradation of therapeutic target proteins without the degradation of neosubstrates involved in teratogenicity.

Proteolysis-targeting chimera (PROTAC) protein degraders are an alternative approach for TPD<sup>6,7</sup>. PROTACs are synthesized from two functional compounds: E3 ligase binder-like IMiDs and a target binder such as a target protein inhibitor<sup>5−7</sup>. PROTACs can theoretically many target proteins by using diverse target binders<sup>6,7</sup>. Owing to the remarkable clinical success of IMiDs, TPD is a promising approach
for treating several diseases, and many PROTACs\textsuperscript{16} are being developed worldwide. Several E3 binders have been developed, including the CRBN binder, von Hippel–Lindau (VHL) binder\textsuperscript{17}, and cellular inhibitor of apoptosis protein (cIAP) binder\textsuperscript{18}. Because the CRBN binder has the smallest molecular weight, and CRBN is ubiquitously expressed in diverse tissues, IMiD-based PROTACs can be applied to PROTACs for diverse target proteins\textsuperscript{16,19}. However, IMiD-based PROTACs induce the protein degradation of neosubstrates, such as IKZF1, SALL4, and PLZF\textsuperscript{7,21,22}.

Many structural studies have shown that IMiDs bind to a hydrophobic pocket in the C-terminal domain of CRBN\textsuperscript{18,19}. Thalidomide and thalidomide derivatives have two chemical rings: glutarimide and phthalimide rings. The former binds to the C-terminal region of CRBN\textsuperscript{22,23}, and the latter leads to selective interactions with neosubstrates\textsuperscript{23,24}. The phthalimide ring differs between thalidomide and thalidomide derivatives; thus, the chemical functional groups in this ring are crucial to the selectivity of the neosubstrate\textsuperscript{23,24}. In addition, we recently demonstrated that 5-hydroxylation of the phthalimide ring alters neosubstrate selectivity, resulting in 5-hydroxythalidomide, which strongly degrades SALL4 but not IKZF1\textsuperscript{10,25}. This evidence leads us to the simple hypothesis that sophisticated chemical modulation of the phthalimide ring in thalidomide derivatives could tightly control the selectivity of the neosubstrate with the construction of a CRBN binder for highly selective TPD.

### 6-position Modifications On Lenalidomide

To identify thalidomide derivatives that selectively induce protein degradation of therapeutic targets for haematological cancer, we synthesized 10 thalidomide derivatives modified on a phthalimide ring (NE-001 to NE-010; Extended Data Fig. 1a). An AlphaScreen-based interaction assay using a wheat cell-free system (Extended Data Fig. 1b) established in our previous studies\textsuperscript{10,25} showed that thalidomide, lenalidomide, pomalidomide and 5-hydroxythalidomide showed stronger or equal ability to interact with SALL4/PLZF than IKZF1(Extended Data Fig. 1c). On the other hand, 6-fluoro lenalidomide (NE-005) interacted more strongly with IKZF1 than with SALL4 and PLZF (Extended Data Fig. 1c). Furthermore, immunoblot analysis showed that NE-005 induced the protein degradation of exogenous Myc-IKZF1, but not AGIA-SALL4 (Extended Data Fig. 1d).

In contrast, 5-fluoro lenalidomide (NE-008) and 7-fluoro lenalidomide (NE-006) barely induced interactions between CRBN neosubstrates and could not degrade Myc-IKZF1 and AGIA-SALL4 (Extended Data Fig. 1c and d). 6-fluoro pomalidomide (NE-003) strongly induced protein degradation of both Myc-IKZF1 and SALL4 (Extended Data Fig. 1d). Furthermore, NE-005 induced selective and strong degradation of endogenous IKZF1, IKZF3, and CK1\textalpha, but not SALL4 and PLZF, in cultured cells (Extended Data Fig. 1e and f). Notably, NE-005 induced drastic degradation of CK1\textalpha, which is involved in the anti-5q MDS activity of lenalidomide, at 10-fold lower concentrations than lenalidomide (Extended Data Fig. 1e and f).

Therefore, we synthesized 6-position-modified lenalidomide and pomalidomide (NE-011–NE-014) using halogen atoms (Extended Data Fig. 2a). AlphaScreen-based interaction assays showed that 6-chloro
lenalidomide (NE-013) selectively interacted with IKZF1, whereas 6-bromo lenalidomide (NE-014) scarcely interacted with neosubstrates (Extended Data Fig. 2b). Conversely, the 6-position-modification on pomalidomide did not increase selectivity to IKZF1 and reduced binding ability towards IKZF1, SALL4, and PLZF (Extended Data Fig. 2b). These results suggest that 6-position-modification of lenalidomide is the optimal approach for selective thalidomide derivatives. Previous studies have reported that protein degradation ability of SALL4 by lenalidomide is the lowest among thalidomide derivatives currently in use. Lenalidomide is the first-line treatment for MM and 5q MDS and is the most widely used IMiD. Therefore, modifying lenalidomide to increase selectivity for anti-haematological cancers is reasonable.

We then characterized the 6-position-modified lenalidomide using biochemical and cell-based experiments. The biochemical interaction assay revealed that 6-fluoro lenalidomide (NE-005/F-Le) interacted with SALL4 at the same level as lenalidomide (Le) but interacted more strongly with IKZF1 than Le (Fig. 1a and b). However, 6-chloro lenalidomide (NE-013/Cl-Le) did not interact with SALL4, but its affinity for IKZF1 was lower than that for Le (Fig. 1b). These differences in the binding ability for neosubstrates to CRBN were also validated via an in vitro pull-down assay using recombinant proteins (Extended Data Fig. 2c).

In addition, the in vitro ubiquitination assay showed that the polyubiquitination level of SALL4 by F-Le was weaker than that of Le, while the polyubiquitination level of IKZF1 was the same as that of Le (Fig. 1c). Consistent with results of the in vitro ubiquitination assay, the polyubiquitination of SALL4 by F-Le was very weak. However, polyubiquitination of IKZF1 was the strongest in HEK293T cells (Fig. 1d). We then investigated the degradation of neosubstrates by lenalidomide derivatives using cell lines expressing these neosubstrates. Immunoblot analyses confirmed that F-Le strongly induced the protein degradation of IKZF1, IKZF3, and CK1α (Fig. 1e), but the induction of SALL4 and PLZF degradation by F-Le was weaker than that by Le (Fig. 1f and Extended Data Fig. 2d). Cl-Le was more selective, but its degradation ability for IKZF1, IKZF3, and CK1α was lower than that of Le (Fig. 1e and f, Extended Data Fig. 2d). Br-Le barely induced the protein degradation of IKZF1, IKZF3, and CK1α. Minor changes in substituent size at the 6-position of lenalidomide significantly alter neosubstrate selectivity, and 6-fluoro lenalidomide and 6-chloro lenalidomide may be more selective thalidomide derivatives for neosubstrates involved in anti-haematological cancer activity.

6-fluoro Lenalidomide Shows Antiproliferative Effects

The 6-position-modified lenalidomides induced selective protein degradation of neosubstrates involved in anti-MM and anti-5q MDS activities (Fig. 1c–d). Therefore, we investigated whether lenalidomide derivatives exerted antiproliferative effects in both MM and 5q MDS cell lines. Cell-Titer Glo assays showed that F-Le strongly reduced cell growth in the Le-sensitive MM cell lines MM1.S, H929, and U266, but not in the Le-insensitive cell line RPMI8226 (Extended Data Fig. 3a). In the Le-sensitive 5q MDS-L cell line, F-Le showed a stronger antiproliferative effect than Le, and Cl-Le at the same level as Le (Extended Data Fig. 3b). It has been reported that the downregulation of IRF4 and MYC via IKZF1 and
IKZF3 degradation is a key mechanism underlying the antiproliferative effect of Le and pomalidomide (Po) in MM cells\textsuperscript{2,3,26}.

Immunoblot analysis revealed that F-Le and Cl-Le treatments also reduced the protein expression levels of IRF4 and MYC in MM1.S and H929 cell lines (Fig. 2a and Extended Data Fig. 3c). Regarding the mechanism of action of lenalidomide in 5q MDS cells, the protein degradation of both IKZF1 and CK1\(\alpha\) was required for the antiproliferative effect of lenalidomide\textsuperscript{4,27}. First, IKZF1 degradation by lenalidomide induced upregulation of RUNX1, followed by induction of differentiation into megakaryocytes\textsuperscript{27}.

Subsequently, lenalidomide induced the protein degradation of CK1\(\alpha\), which has a low expression level in 5q MDS cells, and induced apoptosis\textsuperscript{27}. Po, Le, F-Le, and Cl-Le induced the protein degradation of IKZF1 and upregulation of RUNX1 (Fig. 2b) and increased the mRNA expression levels of \textit{SELP} and \textit{ITGB3}, which are induced by lenalidomide for differentiation into megakaryocytes\textsuperscript{27} (Fig. 2c). These results suggest that lenalidomide derivatives have the same mechanism of action as lenalidomide and pomalidomide. We then used dose-dependent analyses to compare the antiproliferative effects of lenalidomide, pomalidomide, and lenalidomide derivatives. F-Le and Cl-Le showed dose-dependent antiproliferative effects. F-Le exerted a stronger antiproliferative effect on MM1.S and H929 cells than Le (Fig. 2d and e, Extended Data Fig. 3d).

Furthermore, F-Le showed the same efficiency in MDS-L cells at doses 10–100 times lower than that of lenalidomide (Fig. 2e), consistent with the degradation of CK1\(\alpha\) by F-Le (Fig. 2a and b, Extended Data Fig. 3c). These results suggest that 6-fluoro lenalidomide is a selective and highly effective lenalidomide derivative for treating MM and 5q MDS.

**Protacs Based On 6-modified Lenalidomide**

IMiD-based PROTACs with various linkers suitable for TPD have been developed. First, we confirmed the degradation of target proteins and neosubstrates by IMiD-based PROTACs with different linkers (Extended Data Fig. 4a). Immunoblot analyses showed that PROTACs induced protein degradation in neosubstrates with different neosubstrate selectivities (Extended Data Fig. 4b and c). These results suggest that not only the linker but also the optimization of the CRBN binder is required for selective TPD. IKZF1 and IKZF3 play pivotal roles in the development and function of haematological cells\textsuperscript{28–30}. Therefore, it is suggested that protein degradation of IKZF1 and IKZF3 is undesirable in the case of PROTACs for non-haematological cancers.

Based on the results shown in Fig. 1, it is expected that 6-position-modified lenalidomides with bulky molecules cannot induce protein degradation in any neosubstrate. Therefore, we synthesized 6-trifluoromethyl lenalidomide (NE-015/F\(3\)C-Le) and 6-trifluoromethoxy lenalidomide (NE-016/F\(3\)CO-Le) and investigated the CRBN-neosubstrate interactions and protein degradation of neosubstrates (Extended Data Fig. 5a). We found that CRBN did not interact with IKZF1, SALL4, and PLZF in the presence of F\(3\)C-
Le and F₃CO-Le (Extended Data Fig. 5b) and did not induce protein degradation of neosubstrates in cells (Extended Data Fig. 5c–e).

In previous studies, we reported that the neosubstrate selectivity of IMiDs in cells could be evaluated using ancestral BirA for proximity-dependent biotin identification (AirID)-fused CRBN³¹,³² (Extended Data Fig. 5f). The streptavidin pull-down assay (STA-PDA) validated that 6-position-modified lenalidomides with bulky molecules cannot interact with any neosubstrate in the cells (Extended Data Fig. 5g). To examine whether lenalidomide derivatives interact with CRBN, we generated thalidomide-immobilized magnetic beads using a thalidomide derivative (Extended Data Fig. 6a). Recombinant FLAG-GST-CRBN was pulled down using thalidomide-immobilized beads, followed by competitive elution of FLAG-GST-CRBN by lenalidomide derivatives. Immunoblot analysis revealed that the 6-position-modified lenalidomides interacted with CRBN (Extended Data Fig. 6b).

Furthermore, we quantitatively investigated the binding ability of lenalidomide derivatives using isothermal titration calorimetry (ITC) analysis, which was established in a previous study²⁵. The $K_D$ values showed that the affinities of F-Le, Cl-Le, Br-Le, and F₃CO-Le were 2–3 times lower than that of Le (Extended Data Fig. 6c). We could not evaluate the binding ability of F₃C-Le because the interaction between CRBN and F₃C-Le was not detected under ITC assay conditions (Extended Data Fig. 6c). Given that the affinity of F₃CO-Le ($K_D = 2.83 ± 0.17 \, \mu M$) was the highest among the lenalidomide derivatives and higher than that of CRBN–(S)-thalidomide ($K_D = 4.00 ± 0.36 \, \mu M$²⁵), 6-position modification with a bulky molecule did not significantly affect the binding ability to CRBN. These results indicate that 6-position-modified lenalidomides can interact with CRBN and are available as CRBN binders for PROTACs.

Next, we purchased or synthesized PROTACs for BET proteins using a BET inhibitor (OTX-015) and pomalidomide (ARV-825/Po-P), lenalidomide (Le-P) or 6-position-modified lenalidomides (F-P, Cl-P and F₃C-P) (Fig. 3a) and examined the degradation of both BET proteins and neosubstrates. As expected, PROTACs induced the protein degradation of BRD2, BRD3, and BRD4 in NTERA-2 cells (Extended Data Fig. 7a). Consistent with the neosubstrate selectivity of lenalidomide derivatives, the degradation level of SALL4 by F-P and Cl-P was lower than that of Le-P, and F₃C-P scarcely degraded SALL4 (Fig. 3b and Extended Data Fig. 7a). Further, PROTACs did not induce protein degradation in HEK293T and MDS-L cells (Extended Data Fig. 7b and c). These results were validated via dose-dependent experiments using HuH7 cells, which expressed both SALL4 and PLZF (Fig. 3c).

Additionally, immunoblot analysis showed that the protein degradation levels of IKZF1 and IKZF3 by Cl-P and F₃C-P were lower than those in MM1.S, H929, and U266 cells (Fig. 3d and Extended Data Fig. 7d). Due to STA-PDA using AirID-CRBN, the biotinylation level of SALL4 and PLZF by F-P, Cl-P, and F₃C-P was lower than that by Le-P in HuH7 cells (Fig. 3e), and Cl-P and F₃C-P scarcely biotinylated IKZF1 and IKZF3 in MM1.S cells (Fig. 3e). In both cell lines, the biotinylation level of BET proteins by F₃C-P was slightly lower than that of the other PROTACs (Fig. 3e). To analyse protein degradation globally and quantitatively, we performed 18-plex tandem mass tag (TMT) labelling and mass spectrometry (MS)
analysis of NTERA-2 and MM1.S cells (Extended Data Fig. 7e). Lenalidomide and lenalidomide derivative-based PROTACs more selectively induced the degradation of BET proteins than pomalidomide-based PROTACs (Extended Data Fig. 8 and Supplementary Data 1–2). Furthermore, protein degradation levels of neosubstrates by F-P, Cl-P, and F₃C-P were lower than those by Le-P. However, that of BET proteins was at the same level among all PROTACs (Fig. 3f), supporting that the 6-position-modified lenalidomides could be CRBN binders for selective TPD.

Antiproliferative Effect Of Protacs

Next, we investigated whether PROTACs had an antiproliferative effect on cultured cells. Expectedly, the PROTACs showed an antiproliferative effect on MM1.S cells (Extended Data Fig. 9a). This effect on MM1.S and H929 cells occurred in a dose-dependent manner (Fig. 4a). Because Po, Le, and F-Le are effective in MM cell lines (Extended Data Fig. 9b), it is predicted that Po-P, Le-P, and F-P showed stronger antiproliferative effects than Cl-P and F₃C-P due to the dual protein degradation of IKZF1/IKZF3 and BET proteins (Fig. 2d and Extended Data Fig. 9b). Therefore, to evaluate lenalidomide derivatives as CRBN binders, we investigated protein degradation and antiproliferative effects in non-haematological cancer cell lines.

BET proteins have been attractive targets for treating diverse cancers, including neuroblastoma. In the neuroblastoma cell line IMR32, F-P, Cl-P, and F₃C-P degraded BET proteins at the same dose range as Le-P (Fig. 4b). In the CellTiter-Glo assay, F-P and Cl-P showed antiproliferative effects at the same dose as Le-P (Fig. 4c and Extended Data Fig. 9c). The maximum antiproliferative effect of F₃C-P was similar to that of other PROTACs. However, the antiproliferative effect of F₃C-P at low doses (10 to 100 nM) was lower than that of other PROTACs (Fig. 4c and Extended Data Fig. 9c).

Furthermore, in the pluripotent human embryonal carcinoma cell line NTERA-2 and colon cancer cell line HCT116, F-P, Cl-P, and F₃C-P induced degradation of BET proteins in the same dose range as Le-P (Extended Data Fig. 9d). In NTERA-2 cells, F-P showed an antiproliferative effect at the same level as Le-P, but that of Cl-P and F₃C-P at low concentrations was lower than that of other PROTACs (Fig. 4d and Extended Data Fig. 9e). In the case of HCT116 cells, antiproliferative effects of F-P and Cl-P were observed at the same dose as Le-P. The antiproliferative effect of F₃C-P at 10 µM was the highest among all PROTACs (Fig. 4e and Extended Data Fig. 9f). These results indicate that F-Le is a more selective and effective CRBN binder for the treatment of haematological cancers, including MM, and that Cl-Le and F₃C-Le are selective CRBN binders for the treatment of non-haematological cancers. These findings demonstrate that 6-position-modified lenalidomide controls protein degradation of off-target proteins.

Discussion

Lenalidomide and pomalidomide are small molecule drugs used at a scale of 16 billion US dollars annually worldwide. In particular, lenalidomide is the leading IMiD, associated with a cost of
approximately 13 billion US dollars annually, because it is used for treating MM and 5q MDS. In this study, we revealed that 6-position modification with a small substituent of lenalidomide increases the selectivity for IKZF1, IKZF3, and CK1α, which are involved in anti-MM and anti-5q MDS activity. Importantly, 6-fluoro lenalidomide strongly induced protein degradation as a therapeutic target. However, the degradation of SALL4 and PLZF, which are involved in thalidomide teratogenicity, was weak (Fig. 1e–g). In fact, 6-fluoro lenalidomide was more effective than lenalidomide in MM and 5q MDS cells (Fig. 1d and e). Therefore, our findings strongly suggest that 6-fluoro lenalidomide is a more selective and effective lenalidomide derivative for treating MM and 5q MDS. However, more detailed investigations, such as those for bioactivity and bioavailability, are required.

IMiDs demonstrated that protein degradation can be a potent drug mechanism of action and that undruggable proteins, including transcription factors, can be targeted. Owing to the remarkable clinical success of IMiDs, novel thalidomide derivatives, such as CC-220, are actively being developed. However, no reported derivatives avoid the protein degradation of teratogenic targets. Interestingly, the chemical structures of CC-220 and CC-92480 were modified with each substituent on the amino group at the 4-position on lenalidomide. Since CC-220 and CC-92480 show considerable potential for drastic protein degradation of IKZF1 and IKZF3 at low doses, 6-fluoro CC-220 or CC-92480 may be a more selective and effective lenalidomide derivative for the treatment of MM. Based on this evidence, our results provide a promising direction for the development of selective and effective lenalidomide derivatives for future IMiDs.

PROTACs are an alternative approach to inducing the degradation of target proteins. Since the target binder does not need to be an inhibitor of target proteins, PROTACs enable us to target many types of proteins, including transcription factors, using many target binders. Thalidomide derivatives are the smallest among previously developed E3 binders. The small molecular weight is a major advantage for developing PROTACs because a large molecular weight often causes problems in the cell permeability of the drug. However, it is a significant challenge to overcome the induction of degradation of neosubstrates by thalidomide derivative-based PROTACs. This study showed that more selective PROTACs could be developed using 6-position-modified lenalidomide (Fig. 3b–f). Based on previous reports, many researchers have revealed that linker optimization could increase the selectivity of PROTACs.

On the other hand, we revealed that thalidomide and its derivatives alter neosubstrate selectivity by metabolism, and the 5-hydroxyl metabolite selectively and strongly induces protein degradation of SALL4. Although there is no report on whether the E3 binder generated as a metabolite of PROTACs induces protein degradation of neosubstrates, several studies have reported that the E3 binder is generated by metabolizing PROTACs. Therefore, improving the neosubstrate selectivity of the CRBN binder is crucial for developing more selective thalidomide derivative-based PROTACs in vivo.
In conclusion, more ideal thalidomide derivative-based PROTACs for TPD can be developed by combining the optimized linker and CRBN binders developed in this study. Our results provide crucial information for selective and effective TPD using thalidomide derivatives and thalidomide derivative-based PROTACs.

References


methods

Reagents

Thalidomide (Tokyo Chemical Industry), pomalidomide (Tokyo Chemical Industry), lenalidomide (FUJIFILM Wako Pure Chemical Corporation), thalidomide-O-COOH (MedChemExpress), dBET1 (MedChemExpress), dBET6 (MedChemExpress), ARV-825 (MedChemExpress), MD-224 (MedChemExpress), ARV-110 (MedChemExpress) and MG132 (Peptide Institute) were purchased from each manufacturer. The procedure and physical data of thalidomide derivatives and PROTACs synthesized in this study were described in Supplementary Information. All drugs were dissolved in DMSO (FUJIFILM Wako Pure Chemical Corporation) at 10–200 mM and stored at -30°C as stock solutions and diluted 1,000-fold for in vivo experiments or 100–200-fold for in vitro experiments.

Plasmids

The pDONR221 and pcDNA3.1(+) plasmids were purchased from Invitrogen. The pEU plasmid for wheat cell-free protein synthesis was constructed in our laboratory. The pcDNA3.1(+)−FLAG-GW, pcDNA3.1(+)−Myc-MCS, pcDNA3.1(+)−AGIA-MCS, pEU-blsls-GW, pEU-FLAG-GST-GW and pEU-FLAG-GST-MCS plasmids were constructed by polymerase chain reaction (PCR) using the In-Fusion system (Takara Bio) or PCR and restriction enzymes. The pEU-FLAG-GST-SALL4, -IKZF1 and -PLZF plasmids were purchased from the Kazusa DNA Research Institute. AirID was purchased as an artificial gene from Thermo Fisher Scientific. The open reading frames (ORFs) of SALL4 and IKZF1 were amplified, and restriction enzyme sites were added by PCR and cloned into pcDNA3.1(+)−AGIA-MCS or pcDNA3.1(+)−Myc-MCS. The ORF of CRBN was purchased from the Mammalian Gene Collection (MGC). The BP reaction sequence (attB and attP) was added to CRBN by PCR and cloned into pDONR221 using BP recombination (Thermo Fisher Scientific). Then, pDONR221-CRBN was recombined into pEU-blsls-GW, pEU-FLAG-GST-GW or pcDNA3.1(+)−
FLAG-GW using LR recombination (attL and attR). The pcDNA3-3× HA-ubiquitin plasmid was kindly provided from Dr. Atsuo T. Sasaki (University of Cincinnati College of Medicine). For the generation of lentivirus for stable cell lines, AGIA-AirID-CRBN was cloned into the pCSII-CMV-MCS-IRES2-Bsd vector using restriction enzymes32.

Cell culture and transfection

HEK293T and MCF-7 cells were cultured in low-glucose DMEM (FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% fetal bovine serum (FUJIFILM Wako Pure Chemical Corporation), 100 U/mL penicillin, and 100 µg/mL streptomycin (Thermo Fisher Scientific) at 37°C under 5% CO2. HEK293T cells were transiently transfected using polyethyleneimine (PEI) Max (MW 40,000) (PolyScience, Inc.). HuH7 cells were cultured in DMEM (high glucose) medium (FUJIFILM Wako) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate (Thermo Fisher Scientific), 10 mM HEPES (Thermo Fisher Scientific), and 1× MEM NEAA (Thermo Fisher Scientific) at 37°C under 5% CO2. MM1.S, H929, U266, RPMI8226, SKM-1 and KG-1 cells were cultured in RPMI 1640 GlutaMAX medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FUJIFILM Wako Pure Chemical Corporation), 100 U/mL penicillin, 100 µg/mL streptomycin, and 55 µM 2-mercaptoethanol (Thermo Fisher Scientific) at 37°C under 5% CO2. KG-1a cells were cultured in RPMI 1640 GlutaMAX medium (Thermo Fisher Scientific) supplemented with 20% fetal bovine serum (FUJIFILM Wako Pure Chemical Corporation), 100 U/mL penicillin, 100 µg/mL streptomycin, and 55 µM 2-mercaptoethanol (Thermo Fisher Scientific) at 37°C under 5% CO2. MDS-L cells were cultured in RPMI 1640 GlutaMAX medium (Thermo Fisher Scientific) supplemented with 20% fetal bovine serum (FUJIFILM Wako Pure Chemical Corporation), 100 U/mL penicillin, 100 µg/mL streptomycin, 20 ng/ml IL-3 (Biolegend) and 55 µM 2-mercaptoethanol (Thermo Fisher Scientific) at 37°C under 5% CO2. NTERA-2 cells were cultured in DMEM (high glucose) (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 2mM L-glutamine (Thermo Fisher Scientific) and 1× MEM NEAA at 37°C under 5% CO2. IMR32 cells were cultured in MEM GlutaMAX medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 1× MEM NEAA at 37°C under 5% CO2. For generation of cell line stably expressing AGIA-AirID-CRBN, lentivirus was produced in HEK293T cells by transfection of pCSII-CMV-AirID-CRBN-IRES2-Bsd expression vector together with pCMV-VSV-G-RSV-Rev and pCAG-HIVgp as described in previous study32. HuH7 and MM1.S cells were infected with the lentivirus and selected using blasticidin S (Thermo Fisher Scientific) in previous study32.

Antibodies

The following horseradish peroxidase (HRP)-conjugated antibodies were used: FLAG (Sigma-Aldrich, #A8592, 1:5000), AGIA44 (produced in our laboratory, 1:5000), Myc-tag (Cell Signaling Technology, #2040, 1:1000), HA-tag (Roche, #12013819001, 1:5000), α-tubulin (MBL, #PM054-7, 1:5000), GAPDH (MBL, #M171-7, 1:5000), streptavidin (Abcam, ab7403, 1:5000) and biotin (Cell Signaling Technology, #7075,
The following primary antibodies were used: CRBN (#71810, 1:1000), IKZF1/Ikaros (#14859, 1:1000), IKZF3/Aiolos (#15103, 1:1000), IRF4 (#62834, 1:1000), c-Myc (#18583, 1:1000), RUNX1 (#4336, 1:1000), BRD4 (#13440, 1:1000), GAPDH (#5174, 1:1000), (all from Cell Signaling Technology); BRD4 (#A301-985A, 1:1000), BRD2 (#A302-583A, 1:1000) (all from Bethyl Laboratories); PLZF (R&D System, #AF2944, 1:1000); SALL4 (#sc-101147, 1:500), BRD3 (#2088C3a, 1:500) (all from Santa Cruz Biotechnology); CK1α (Abcam, #ab108296, 1:1000) and α-tubulin (LI-COR Biosciences, #926-42213, 1:1000). Anti-rabbit IgG (HRP-conjugated, Cell Signaling Technology, #7074, 1:5000), anti-mouse IgG (HRP-conjugated, Cell Signaling Technology, #7076, 1:5000), anti-goat IgG (HRP-conjugated, Thermo Fisher Scientific, #81-1620, 1:10000), IRDye 800CW goat anti-rabbit IgG (LI-COR Biosciences, #925-32211, 1:10000), IRDye 680RD goat anti-mouse IgG (LI-COR Biosciences, #925-68070, 1:10000), IRDye 800CW goat anti-mouse IgG (LI-COR Biosciences, #925-32210, 1:10000) and IRDye 680RD goat anti-rabbit IgG (LI-COR Biosciences, #925-68071, 1:10000) were used as secondary antibodies.

**Immunoblot analysis**

Each cell pellet was lysed in RIPA buffer (25 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA) containing protease inhibitor cocktail (Sigma-Aldrich). The cell lysates were centrifuged at 16,100 × g for 15 min, and protein concentration in the supernatant was quantified by BCA assay kit (Thermo Fisher Scientific). Then, the lysates were denatured by boiling in 1× sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol) containing 5% 2-mercaptoethanol. The equal amount of lysate was separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were blocked using 5% skim milk (Megmilk Snow Brand) in TBST (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Tween20) or Intercept (TBS) Blocking Buffer/TBS (LI-COR Biosciences) at room temperature for 1 h, and incubated with primary antibodies overnight at 4°C. Then, the membranes were washed using TBST for 15 min and incubated with secondary antibodies at room temperature for 1h. ImmunoStar LD (FUJIFILM Wako), or EzWestLumi plus (Atto) were used as a substrate for HRP, and the luminescence signal was detected using an ImageQuant LAS 4000 mini (GE Healthcare). In some blots, the membrane was stripped with stripping solution (FUJIFILM Wako Pure Chemical Corporation) and re-probed with other antibodies. For fluorescent immunoblot analysis, the fluorescent signal was detected using an Odyssey Fc (LI-COR Biosciences).

**Production of recombinant proteins using a cell-free system**

Recombinant protein synthesis was conducted using a wheat cell-free system. In vitro transcription and wheat cell-free protein synthesis were performed using the WEPRO1240 expression kit (Cell-Free Sciences). Transcription was performed using SP6 RNA polymerase with the plasmids or DNA fragments as templates. The translation reaction was performed in bilayer mode using the WEPRO1240 expression kit (Cell-Free Sciences), according to the manufacturer's protocol. For biotin labelling of bls-CRBN, cell-free synthesised crude biotin ligase (BirA) produced using the wheat cell-free expression system was added to the bottom layer, and 0.5 µM (final concentration) of d-biotin (Nacalai Tesque) was added to both the upper and lower layers, as described previously45. For production and purification of recombinant SALL4
and IKZF1, FLAG-GST-SALL4 or -IKZF1 were synthesized on 6-ml scale using the WEPRO1240G expression kit (Cell-Free Sciences). Then, the crude protein solutions containing NaCl (final conc. 100 mM) and DTT (final conc. 10 mM) were rotated with 200 μl MagneGST Glutathione Particles (Promega) at 4°C for 3 h. The beads were washed three times with 800 μl PBS and incubated two times with 150 μl elution buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 10 mM reduced glutathione) on ice for 15 min. The purified proteins were confirmed by CBB-staining and protein concentration was calculated by the band intensity of each purified protein using Image J software.

**AlphaScreen-based biochemical assays using recombinant proteins**

In vitro biochemical interaction assay was performed as described previously. 10 μL CRBN mixtures containing 0.5 μl biotinylated bls-CRBN in AlphaScreen buffer (100 mM Tris [pH 8.0], 0.01% Tween20, 100 mM NaCl, and 1 mg/mL BSA) were prepared. 5 μL compound mixtures containing thalidomide derivatives were prepared in AlphaScreen buffer. 5 μL substrate mixtures containing 0.8 μL FLAG-GST-SALL4, -IKZF1 or -PLZF in AlphaScreen buffer were prepared. Then, the three mixtures were dispensed and incubated at 26°C for 1 h in a 384-well AlphaPlate (PerkinElmer). Subsequently, 5 μL detection mixture containing 0.2 μg/mL anti-DYKDDDDK mouse mAb (FUJIFILM Wako Pure Chemical Corporation), 0.08 μL streptavidin-coated donor beads, and 0.08 μL Protein A-coated acceptor beads (μL) in AlphaScreen buffer were added to each well and incubated. After incubation at 26°C for 1 h, luminescence signals were detected using an EnVision plate reader (PerkinElmer).

**In vitro pull-down assay of CRBN and neosubstrate**

10 μl biotinylated bls-CRBN and 10 μl Dynabeads M-280 Streptavidin were mixed and rotated at room temperature for 1 h. The beads were washed three times with 500 μl PBS containing 0.05% Tween20 and then 300 μl reaction solutions containing 20 μl FLAG-GST-SALL4 or -IKZF1 and DMSO or 100 μM thalidomide derivatives (0.5% DMSO) in AlphaScreen buffer were added. After incubation at room temperature for 90 min, the beads were washed three times with 500 μl IP Lysis buffer (Pierce) (25 mM Tris-HCl pH [7.5], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol). The proteins were eluted by boiling with 1× sample buffer containing 5% 2-mercaptoethanol and analysed by immunoblot.

**In vitro ubiquitination assays**

FLAG-GST-SALL4 and -IKZF1 were obtained by protein synthesis and purification using wheat cell-free system described above. The recombinant CRL4<sup>CRBN</sup> complex was purchased from R&D system (E3-650). 100 nM FLAG-GST-SALL4 or -IKZF1 in 30 μl 1× ubiquitination reaction buffer (20 mM HEPES pH [7.5], 150 mM NaCl and 10 mM MgCl<sub>2</sub>) containing 200 nM UBE1 E1 (R&D systems, U-110), 1 μM UbcH5a E2 (Enzo, BML-UW9050-100), 10 μM HA- ubiquitin (Boston Biochem, U-110) and DMSO or 20 μM thalidomide derivatives (1% DMSO) was incubated at 30°C for 30 min. Then, 100 mM ATP (final conc. 5 mM) was mixed and in vitro ubiquitination reaction was performed at 30°C for 3 h. The proteins were denatured in 1% SDS by boiling at 95°C for 15 min. The proteins were diluted 10-fold with IP Lysis buffer (Pierce) and immunoprecipitated anti-FLAG M2 magnetic beads (Sigma-Aldrich) at 4°C for 4 h. The beads were
washed three times with 800 µl of IP Lysis buffer (Pierce), and the proteins were eluted by boiling with 20 µl 1× sample buffer containing 5% 2-mercaptoethanol and analysed by immunoblot.

**In cells ubiquitination assays**

HEK293T cells were cultured in a 6-well plate and transfected with 500 ng pcDNA3.1(+)−FLAG-CRBN, 500 ng pcDNA3.1(+)−AGIA-SALL4 or -IKZF1 and 400 ng pcDNA3 3× HA-ubiquitin. After 16 h of incubation from transfection, the cells were treated with DMSO, 1 µM pomalidomide, 10 µM lenalidomide or 10 µM lenalidomide derivatives in the presence of 10 µM MG132 for 8 h. The cells were lysed in 150 µl of SDS lysis buffer (50 mM Tris–HCl pH [7.5], 1% SDS) containing a protease inhibitor cocktail (Sigma-Aldrich) and denatured at 90°C for 15 min. The lysates were treated with Benzonase Nuclease (Sigma-Aldrich) at 37°C for 30 min, and 120 µl the lysates were centrifuged at 16,100 × g for 15 min and then diluted 10-fold with IP Lysis buffer (Pierce). The proteins were immunoprecipitated overnight with Dynabeads Protein G (Thermo Fisher Scientific) interacting anti-AGIA antibody at 4°C, which were then washed three times with 800 µl of IP Lysis buffer (Pierce). Proteins were eluted by boiling in 25 µl 1× sample buffer containing 5% 2-mercaptoethanol. The proteins were then analysed by immunoblot.

**In vitro competition assay by thalidomide derivatives**

4 mM thalidomide-immobilized magnetic beads were generated by using thalidomide-O-COOH (MedChemExpress) and FG-beads (TAMAGAWA SEILI CO., LTD.) according to manufacturer’s instruction. 10 µl FLAG-GST-CRBN and 10 µl 4 mM thalidomide-immobilized magnetic beads in 500 µl IP Lysis buffer (Pierce) were rotated at room temperature for 2 h. The beads were washed four times with 800 µl IP Lysis buffer (Pierce) and eluted with 20 µl IP Lysis buffer (Pierce) containing DMSO or 200 µM thalidomide derivatives (1% DMSO) by vortex at 26°C for 30 min. The eluted proteins were denatured by boiling with 1× sample buffer containing 5% 2-mercaptoethanol and analysed by immunoblot.

**CRBN TBD expression and purification**

For ITC measurements, DNA sequences encoded human CRBN TBD (318–426 and C366S mutation) was cloned into pGEX6P-3 (GE Healthcare), and the recombinant CRBN TBD was expressed in *E. coli* Rossetta(DE3) cells (Novagen) using lysogeny-broth (LB) media supplemented with 100 µg ml⁻¹ ampicillin and 17 µg ml⁻¹ chloramphenicol. Protein expression was induced by adding 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 18°C when the optical density at 600 nm (OD600) reached ~ 0.6. The cells were collected by centrifugation and were then resuspended in buffer containing 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 0.1 mM tris(2-carboxyethyl)phosphine (TCEP). After sonication and centrifugation, the supernatant of the cell lysate was passed over glutathione Sepharose 4B resin (GE Healthcare), and resin-bound protein was cleaved overnight by human rhinovirus 3C (HRV3C) protease. Proteins eluted from the resin were purified by size-exclusion chromatography with Superdex 75 10/300 GL (GE Healthcare) in 50 mM sodium phosphate, pH 7.4, 200 mM NaCl and 0.1 mM TCEP. The fractions containing the CRBN TBD were pooled and concentrated by ultrafiltration with Vivaspin 20 (MWCO 3000,
Sartorius). The concentration of the CRBN TBD was determined by measuring the ultraviolet absorbance at 280 nm, and the molecular extinction coefficient was 27,960 M⁻¹ cm⁻¹.

**ITC measurements**

The binding affinity of (R/S)-lenalidomide, (R/S)-F-lenalidomide, (R/S)-Cl-lenalidomide, (R/S)-Br-lenalidomide, (R/S)-F₃C-lenalidomide and (R/S)-F₃CO-lenalidomide to the CRBN TBD was measured by using an isothermal titration calorimeter (MicroCal iTC₂₀₀, Malvern). The CRBN TBD was dialysed in a binding buffer containing 50 mM sodium phosphate, pH 7.4, 200 mM NaCl, and 0.1 mM TCEP, and then, DMSO was added to the protein solution at a final concentration of 0.2%. (R/S)-lenalidomide, (R/S)-F-lenalidomide, (R/S)-Cl-lenalidomide, (R/S)-Br-lenalidomide, (R/S)-F₃C-lenalidomide or (R/S)-F₃CO-lenalidomide was dissolved in DMSO, and the solution was mixed with binding buffer with the DMSO concentration adjusted to 0.2%. For titrations, the (R/S)-lenalidomide solution (400 µM, in the syringe), (R/S)-F-lenalidomide solution (400 µM, in the syringe), (R/S)-Cl-lenalidomide solution (400 µM, in the syringe), (R/S)-Br-lenalidomide solution (400 µM, in the syringe), (R/S)-F₃C-lenalidomide solution (400 µM, in the syringe) or (R/S)-F₃CO-lenalidomide solution (300 µM, in the syringe) was injected into the sample cell filled with the CRBN TBD solution (10 µM or 20 µM, in the cell) in 37 consecutive 1.0 µl aliquots at 120 s intervals. The first injection volume was 0.4 µl, and the observed thermal peak was excluded from the data analyses. All experiments were performed at 25°C with a reference power of 5 µcal sec⁻¹ and stirring speed of 750 rpm. Data fitting was performed using Origin 7.0 software (OriginLab) in the “one set of sites” mode. The values of the dissociation constant (K_D) and molar binding ratio (n) for each lenalidomide-derivatives were calculated from the data obtained in triplicate experiments (means ± SD).

**Streptavidin pull-down assay using BioID**

Streptavidin pull-down assays using AirID-CRBN were performed as described previously. MM1.S or HuH7 cells stably expressing AGIA-AirID-CRBN were cultured in a 6-well plate and treated with DMSO or 10 µM thalidomide derivatives in the presence of 10 µM biotin and 5 µM MG132 for 6 h. The cells were harvested using cell scraper and lysed in 300 µL SDS lysis buffer (50 mM Tris-HCl pH 7.5, 1% SDS) containing a protease inhibitor cocktail (Sigma-Aldrich), and then the lysates were denatured by boiling at 95°C for 15 min. The lysates were treated with Benzonase Nuclease (Sigma-Aldrich) at 37°C for 30 min and centrifuged at 16,100 × g for 15 min. Subsequently, 250 µL lysate was added to 1 mL IP Lysis buffer (Pierce) containing 20 µL streptavidin sepharose beads (GE Healthcare) and rotated at 4°C overnight. The beads were washed three times with 600 µL IP Lysis buffer (Pierce), and the proteins were eluted by boiling with 40 µL 2 × sample buffer containing 5% 2-mercaptoethanol.

For the streptavidin pull-down assay using PROTAC, MM1.S or HuH7 cells stably expressing AGIA-AirID-CRBN were cultured in a 10-cm dish and treated with DMSO or 100 nM PROTAC in the presence of 10 µM biotin and 5 µM MG132 for 6 h. The cells were harvested using cell scraper and lysed in 600 µL SDS lysis buffer (50 mM Tris-HCl pH 7.5, 1% SDS) containing a protease inhibitor cocktail (Sigma-Aldrich), and then the lysates were denatured by boiling at 95°C for 15 min. The lysates were treated with Benzonase
Nuclease (Sigma-Aldrich) at 37°C for 30 min and centrifuged at 16,100 × g for 15 min. Subsequently, 560 µl the lysate was added to 1 mL IP Lysis buffer (Pierce) containing 25 µL streptavidin sepharose beads (GE Healthcare) and rotated at 4°C overnight. The beads were washed three times with 800 µL IP Lysis buffer (Pierce), and the proteins were eluted by boiling with 40 µL 2 × sample buffer containing 5% 2-mercaptoethanol.

Quantitative RT-PCR

MDS-L cells were cultured in 48-well plates and treated with DMSO, 10 µM pomalidomide, 10 µM lenalidomide or 10 µM lenalidomide derivatives for 3 days. Then, half of the cells were collected into a tube, and the remaining cells were diluted 2-fold with culture medium. The diluted cells were cultured for up to 6 days and collected in a tube. All collected cells were lysis using SuperPrep II Cell Lysis Kit for qPCR (Toyobo) and total RNA was isolated using SuperPrep II Cell RT Kit for qPCR (Toyobo), according to the manufacturer’s instructions. RT-PCR was performed using KOD SYBR qPCR Mix (Toyobo) and the data were normalised against GAPDH mRNA levels. PCR primers were as follows: SELP sense 5´-TCCGCTGCATTGACTCTGGACA −3´, SELP anti-sense 5´-CTGAAACGCTCTCAAGGATGGAG −3´, ITGB3 sense 5´-CATGGATTCCAGCAATGTCCTCC −3´, ITGB3 antisense 5´-TTGAGGCAGGTGGCATTGAAGG −3´, GAPDH sense 5´-AGCAACAGGGTGTTGGAC −3´, and GAPDH antisense 5´-GTGTGGTGGGGGACTGAG −3´.

Cell viability assay

To evaluate the anti-proliferative effect of thalidomide derivatives on multiple myeloma and 5q myelodysplastic syndrome cell line, MM1.S, U266, H929, RPMI8226, KG-1, KG-1a, SKM-1 or MDS-L cells were cultured in 24-well plates in the presence of DMSO, thalidomide, pomalidomide, lenalidomide or lenalidomide derivatives at indicated concentration. The cells were cultured for 5 days or diluted 4-fold every 3 days and cultured for 9 days. The cells were lysed using Cell-Titer-Glo assay kit (Promega) and dispensed in 384-well OptiPlate (PerkinElmer). Luminescent signal was detected using SpectraMax iD3 (Molecular Devise) according to the manufacturer’s instruction.

To evaluate anti-proliferative effect of lenalidomide derivatives or PROTACs on diverse cultured-cells, MM1.S, H929, IMR32, NTERA-2 or HCT116 cells were cultured in 96-well plates in the presence of DMSO, thalidomide, pomalidomide, lenalidomide, lenalidomide derivatives or PROTACs at indicated concentration for 5 days. The cells were lysed using Cell-Titer-Glo assay kit (Promega) and dispensed in 384-well OptiPlate (PerkinElmer). Luminescent signal was detected using SpectraMax iD3 (Molecular Devise) according to the manufacturer's instruction.

TMT-Based Quantitative Proteomics

For global investigation of protein degradation by PROTACs, MM1.S or NTERA-2 cells were cultured in 6-well plates and treated with DMSO or 300 nM PROTACs for 16 h. MM1.S or NTERA-2 cells were harvested by suspending or scraping and centrifuged at 400 × g for 3 min, after which the cell pellets were washed...
with PBS. Then, the cell pellets were lysed in 100 µL guanidine buffer (6 M guanidine-HCl, 100 mM HEPES-NaOH, pH 7.5, 10 mM TCEP, 40 mM chloroacetamide). After heating and sonication, proteins (100 µg each) were purified by methanol–chloroform precipitation and resuspended in 20 µL 0.1% RapiGest SF (Waters) in 50 mM triethylammonium bicarbonate. After sonication and heating at 95°C for 10 min, the proteins were digested with 2 µg trypsin/Lys-C mix (Promega) at 37°C overnight. The digested peptides (40 µg each) were labeled with 0.5 mg TMTpro-18plex reagents (Thermo Fisher Scientific) for 1 h at 25°C. After the reaction was quenched with hydroxylamine, all the TMT-labeled samples were pooled, acidified with TFA, and fractionated by offline high-pH reversed-phase chromatography on a Vanquish DUO UHPLC (Thermo Fisher Scientific), as previously reported with slight modifications. Briefly, the peptides were loaded onto a 4.6 × 250 mm Xbridge BEH130 C18 column with 3.5 mm particles (Waters) and separated using a 30 min multistep gradient of solvents A (10 mM ammonium formate at pH 9.0 in 2% ACN) and B (10 mM ammonium formate pH 9.0 in 80% ACN), at a flow rate of 1 mL/min. Peptides were separated into 48 fractions, which were consolidated into 16 fractions. Each fraction was evaporated in a SpeedVac concentrator and dissolved in 0.1% TFA and 3% ACN. LC-MS/MS analysis of the resultant peptides (500 ng each) was performed on an EASY-nLC 1200 UHPLC connected to a Q Exactive Plus mass spectrometer through a nanoelectrospray ion source (Thermo Fisher Scientific). The peptides were separated on the analytical column (75 µm × 15 cm, 3 µm; Nikkyo Technos) with a linear gradient of 4–20% for 0–115 min and 20–32% for 115–160 min, followed by an increase to 80% ACN for 10 min and finally held at 80% ACN for 10 min. The mass spectrometer was operated in data-dependent acquisition mode with a top 10 MS/MS method. MS1 spectra were measured with a resolution of 70,000, an AGC target of 3e6, and a mass range from 375 to 1,400 m/z. MS/MS spectra were triggered at a resolution of 35,000, an AGC target of 1e5, an isolation window of 0.7 m/z, a maximum injection time of 150 ms, and a normalized collision energy of 33. Dynamic exclusion was set to 20 s. Raw data were directly analyzed against the SwissProt database restricted to Homo sapiens using Proteome Discoverer version 2.4 with the Sequest HT search engine for identification and TMT quantification. The search parameters were as follows: (a) trypsin as an enzyme with up to two missed cleavages; (b) precursor mass tolerance of 10 ppm; (c) fragment mass tolerance of 0.02 Da; (d) TMT of lysine and peptide N-terminus and carbamidomethylation of cysteine as fixed modifications; (e) oxidation of methionine as a variable modification. Peptides were filtered at an FDR of 1% using the Percolator node. TMT quantification was performed using the Reporter Ions Quantifier node. Normalization was performed such that the total sum of the abundance values for each TMT channel over all peptides was the same.

Statistical analysis

The data are presented as the mean ± standard deviation (SD). Significant changes were analysed by Student’s t-tests or one-way analysis of variance (ANOVA), followed by Tukey’s tests using GraphPad Prism (version 8) software (GraphPad, Inc.).

Data availability
The MS proteomics data have been provided in Supplementary Data 1–2 and deposited to the ProteomeXchange Consortium via the jPOST partner repository with the dataset identifiers PXD037178 (MM1.S cells) and PXD037179 (NTERA-2 cells). All data supporting the findings of this study are provided in the main text and Extended Data. Source data are provided with this paper.

**Code availability**

No new algorithms were developed for this manuscript. All code generated for analysis is available from the authors upon request.

**Methods references**


**Declarations**

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**Author contributions**
S.Yamanaka, K.N. and Y.S, performed the biochemical, molecular, and cellular biology experiments. H.F., M.T. and T.M. performed ITC experiments. T.N., M.U. and N.S. synthesised and analysed the thalidomide derivatives and PROTACs. S.Yamanaka, Y.Y., S.Yoshida and Y.I. analysed anti-proliferative effect of PROTACs. K.N. and H.K. performed TMT-based proteomics analyses. S.Yamanaka and T.S. analysed the data, designed the study, wrote the paper, and all authors contributed to the manuscript.

**Competing interest declaration**

The authors declare no competing financial interests.

**Additional information**

**Supplementary information** The online version contains supplementary material available at https://XXXX.

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**Figures**
Figure 1

6-position modification on lenalidomide alter neosubstrate selectivity.

a, Chemical structures of lenalidomide (Le), 6-fluoro lenalidomide (F-Le), 6-chloro lenalidomide (Cl-Le) and 6-bromo lenalidomide (Br-Le). b, Dose-dependent interaction assay using recombinant proteins. The CRBN–IKZF1, CRBN–SALL4, and CRBN–PLZF complex formation was analysed using AlphaScreen (AS) technology. The relative AS signals are expressed as the luminescence signal relative to the luminescence signal of DMSO, which is considered 1. Error bars denote standard deviations (independent experiments, n = 3). c, In vitro ubiquitination assay of SALL4 and IKZF1 by CRL4<sup>CRBN</sup>. Purified FLAG-GST-IKZF1 or SALL4 were mixed with recombinant CRL4<sup>CRBN</sup>, E1, E2, and HA-Ub, and ubiquitination reactions were performed in the presence of DMSO, 20 µM pomalidomide (Po), 20 µM Le, 20 µM F-Le, 20 µM Cl-Le, or 20 µM Br-Le.
μM Br-Le. Ubiquitinated SALL4 and IKZF1 were immunoprecipitated using an anti-FLAG antibody and analysed by immunoblotting. The experiment was repeated twice independently, with similar results. d, In the cell ubiquitination assay of SALL4 and IKZF1 by CRL4\textsuperscript{CRBN}. HEK293T cells were transfected with pcDNA3-HA-ubiquitin and pcDNA3.1-FLAG-CRBN and pcDNA3.1-AGIA-SALL4 or -IKZF1 and treated with DMSO, 1 μM Po, 10 μM Le, 10 μM F-Le, 10 μM Cl-Le, or 10 μM Br-Le in the presence of 10 μM MG132 for 8 h. Ubiquitinated SALL4 and IKZF1 were immuno precipitated using an anti-AGIA antibody and analysed through immunoblotting. The experiment was repeated twice independently, with similar results. e–g, Immunoblot analysis of dose-dependent neosubstrate degradation in (e) MM1.S, (f) HuH7, or (g) NTERA-2 cells. Each cell line was treated with DMSO, Po, Le, F-Le, Cl-Le, or Br-Le for 24 h, and the protein expression levels of neosubstrates were analysed via immunoblotting. The experiment was independently repeated thrice, with similar results.

Figure 2

Antiproliferative effect by 6-position-modified lenalidomide on multiple myeloma and 5q myelodysplastic syndromes.
a, Immunoblot analysis of IRF4 and c-Myc. MM1.S cells were treated with DMSO, Po, Le, F-Le, Cl-Le, or Br-Le for 72 h; cell lysates were analysed through immunoblotting. The experiment was independently repeated thrice, with similar results. b, Immunoblot analysis of RUNX1. MDS-L cells were treated with DMSO, Po, Le, F-Le, Cl-Le, or Br-Le for 24 h, and cell lysates were analysed through immunoblotting. The experiment was repeated twice independently, with similar results. c, Expression of SELP and ITGB3, which are upregulated in 5q MDS cells treated with lenalidomide. MDS-L cells were treated with DMSO, 10 µM Po, 10 µM Le, 10 µM F-Le, 10 µM Cl-Le, or 10 µM Br-Le for 3 or 6 days, and mRNA expression was measured using quantitative RT-PCR. Relative mRNA expression was determined using the expression level following DMSO treatment. Error bars denote standard deviation (biological replicates; n = 3). d, Dose-dependent antiproliferative effect of lenalidomide derivatives on MM cell lines. MM1.S and H929 cells were treated with DMSO, Po, Le, F-Le, Cl-Le, or Br-Le for 9 days, and cell viability was analysed using the CellTiter-Glo assay kit. Cell viability was expressed as the luminescence signal relative to the luminescence signal of DMSO, which was considered 100. Error bars denote standard deviation (biological replicates; n = 4). e, Dose-dependent antiproliferative effect of lenalidomide derivatives on 5q MDS cell lines. MDS-L cells were treated with DMSO, Po, Le, F-Le, Cl-Le, or Br-Le for 20 days, and cell viability was analysed using the CellTiter-Glo assay kit. Cell viability was expressed as the luminescence signal relative to the luminescence signal of DMSO, which was considered 100. Error bars denote standard deviation (biological replicates; n = 4).
Figure 3

Neosubstrate selectivity of PROTACs using 6-position-modified lenalidomides.

a, Chemical structures of ARV-825 (Po-P), NE-017 (Le-P), NE-018 (F-P), NE-019 (Cl-P), and NE-020 (F₃C-P).
b, Neosubstrate selectivity of PROTACs using lenalidomide derivatives. NTERA-2 cells were treated with DMSO, 0.1 µM Po, 0.1 µM Le, 0.1 µM F-Le, 0.1 µM Cl-Le, 0.1 µM F₃C-Le, 0.1 µM Po-P, 0.1 µM Le-P, 0.1 µM F-P, 0.1 µM Cl-P, or 0.1 µM F₃C-P for 24 h; SALL4 protein expression was analysed via immunoblot. The experiment was independently repeated thrice, with similar results.
c, Immunoblot analysis of BET proteins SALL4 and PLZF. HuH7 cells were treated with DMSO, Po-P, Le-P, F-P, Cl-P, or F₃C-P for 24 h; expression of proteins and neosubstrates was analysed through immunoblotting. The experiment was...
independently repeated thrice, with similar results. **d**, Immunoblot analysis of BET proteins IKZF1 and IKZF3. MM1.S and H929 cells were treated with DMSO, Po-P, Le-P, F-P, Cl-P, or F₃C-P for 24 h; expression of proteins and neosubstrates was analysed via immunoblotting. The experiment was independently repeated thrice, with similar results. **e**, In-cell proximity-dependent biotinylation of BET proteins and neosubstrates through AirID-CRBN. HuH7 and MM1.S cells expressing AGIA-AirID-CRBN were treated with DMSO, 0.1 µM Po-P, 0.1 µM Le-P, 0.1 µM F-P, 0.1 µM Cl-P, or 0.1 µM F₃C-P in the presence of 10 µM biotin and 5 µM MG132 for 6 h; biotinylated proteins were pulled down by streptavidin Sepharose and analysed using immunoblot. The experiment was repeated twice independently, with similar results. **f**, Heatmap showing the ratio of BET proteins to neosubstrates in whole-proteome quantification. NTERA-2 and MM1.S cells were treated with DMSO, 0.3 µM Po-P, 0.3 µM Le-P, 0.3 µM F-P, 0.3 µM Cl-P, or 0.3 µM F₃C-P for 16 h; quantitative proteomics analysis was performed using a tandem mass tag (biological replicates, n = 3).

**Figure 4**

Antiproliferative effect by PROTACs using 6-position-modified lenalidomides on diverse cancer cell lines.
a. Antiproliferative effects of PROTACs using lenalidomide derivatives on MM cell lines. MM1.S and H929 cells were treated with DMSO, Po-P, Le-P, F-P, Cl-P, or F₃C-P for 5 days, and cell viability was analysed using the CellTiter-Glo assay kit. Cell viability was expressed as the luminescence signal relative to the luminescence signal of DMSO, which was considered 100. Error bars denote standard deviation (biological replicates; n = 3).

b. Degradation of BET proteins by PROTACs using lenalidomide derivatives in a neuroblastoma cell line. IMR32 cells were treated with DMSO, Po-P, Le-P, F-P, Cl-P, or F₃C-P for 24 h, and the expression levels of BET proteins were analysed through immunoblotting. The experiment was independently repeated thrice, with similar results.

c–e, Antiproliferative effects of PROTACs using lenalidomide derivatives on (c) neuroblastoma, (d) pluripotent human embryonal carcinoma, and (e) colon cancer cell lines. (c) IMR32, (d) NTERA-2, or (e) HCT116 cells were treated with DMSO, Po-P, Le-P, F-P, Cl-P, or F₃C-P for 2 days (HCT116 cells), 3 days (NTERA-2 cells), or 5 days (IMR32 cells), and cell viability was analysed using the Cell Titer-Glo assay kit. Cell viability was expressed as the luminescence signal relative to the luminescence signal of DMSO, which was considered 100. Error bars denote standard deviation (biological replicates; n = 3).

**Supplementary Files**

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