Modulation of protein abundance using tag-Targeted Protein Degrader (tTPD) systems targeting FKBP12\textsuperscript{F36V} (dTAGs) or HaloTag\textsuperscript{7} (HaloPROTACs) are powerful approaches for preclinical target validation. Interchanging tags and tag-targeting degraders is important to achieve efficient substrate degradation, yet limited degrader/tag pairs are available and side-by-side comparisons have not been performed. To expand the tTPD repertoire we developed catalytic NanoLuc-targeting PROTACs (NanoTACs) to hijack the CRL4\textsuperscript{CRBN} complex and degrade NanoLuc tagged substrates, enabling rapid luminescence-based degradation screening. To benchmark NanoTACs against existing tTPD systems we use an interchangeable reporter system to comparatively test optimal degrader/tag pairs. Overall, we find the dTAG system exhibits superior degradation. To align tag-induced degradation with physiology we demonstrate that NanoTACs limit MLKL-driven necroptosis. In this work we extend the tTPD platform to include NanoTACs adding flexibility to tTPD studies, and benchmark each tTPD system to highlight the importance of comparing each system against each substrate.
Proteolysis targeting chimeras (PROTACs)/degraders are heterobifunctional molecules that trigger ubiquitination of cellular targets by hijacking E3 ubiquitin ligases resulting in rapid proteasomal degradation. Powerful genetic tools exist to experimentally modulate the abundance of gene products, such as CRISPR-Cas9 genome editing and RNA interference. However, genetic technologies are limited in their ability to assess acute changes in protein levels, and present additional challenges as protein depletion may take days depending on protein half-life and protein loss is typically irreversible, which may in turn impart a selection pressure for compensatory mechanisms. Small-molecule perturbations on the other hand are popular as they can be used to rapidly, and for the most part, reversibly assess biological responses. Yet, small-molecule inhibitors can be limited by off-target effects, the disruption of discrete domain function, occupancy-driven modes of action and the cost and time associated with their development.

Degrader-mediated degradation of proteins presents exciting opportunities to explore the not-yet-drugged proteome as the majority of human proteins, such as transcription factors, non-enzymatic proteins and scaffolding proteins lack active sites rendering them difficult to inhibit with small-molecule inhibitors. Degraders consist of a target-binding ligand and an E3-ligase-binding ligand separated by a chemical linker. The design and optimisation of each of these elements can be critical to achieve efficient substrate degradation as the ligand and the linker can form favourable inter- and intramolecular interactions within the E3-degrader-substrate ternary complex. For example, varying the linker length can enhance the degradation of substrates by reducing steric clashes that can occur between the E3 and substrate.

Tag-Targeted Protein Degrader (tTPD) systems direct degrader compounds to protein tags using tag-targeting heterobifunctional molecules (Fig. 1a). tTPD systems are remarkable technologies that permit rapid and reversible degradation of tagged substrate proteins through cell-permeable and in vivo compatible degraders. tTPD systems include the monovalent molecular glue-based auxin-inducible degron (AID) ligand-inducible affinity-directed protein missile (L-AdPROM), CH6-tag directed specific and nongenetic inhibitors of apoptosis protein (IAP)-dependent protein erasers (SNIPERs). BromoTag, FK506-binding protein 12 (FKBP12) and HaloTag targeting Cereblon (CRBN)- or Von Hippel-Lindau (VHL)-based systems. Degrader molecules that target FKBP12WT or FKBP12F36V (hereafter referred to as FKBP12F36V) and recruit the cullin-RING ligase (CRL) complexes, CRL4CRBN and CRL2VHL, to an FKBP12-tagged substrate have the prefix dTAG (dTAG13, dTAG48, dTAG71)⁹,¹⁰,¹⁶,¹⁷. HaloTag targeting heterobifunctional degraders have been referred to as HaloPROTACs¹⁸,¹⁹, which utilise the CRL2VHL system, or HaloTag-binding degradation inducers, which utilise the IAP proteins.

For simplicity, we refer to all tag-targeting heterobifunctional degrader systems listed above as tTPD systems and have abbreviated each degrader name and the targeted tag/E3s used in this study (Table 1). We have also abbreviated all degrader names to mark each tTPD system in identical cellular settings. We show that all of the tTPD systems offer up the prospect of selectively and reversibly depleting proteins of interest at will. However, each of the three tags offers advantages and disadvantages (Fig. 1b), and which tag to choose really hinges on the researcher’s intentions for their targeted protein of interest.

Results
Development of NanoTACs; a NanoLuc-targeting degrader system. We developed heterobifunctional degraders that bind NanoLuc (NanoTACs) to trigger protein degradation of NanoLuc-tagged substrates building upon the existing tTPD systems that direct degraders to FKBP12F36V or Halo-tagged substrates (Fig. 1a). While each tTPD system has specific advantages, NanoLuc is the only tag that offers luminescence properties. Additionally, NanoTACs are catalytic and can act as degradation catalysts, unlike HaloPROTACs (Fig. 1b). Our most potent NanoTAC recruits the CRL4CRBN E3-ligase complex to trigger the degradation of NanoLuc-tagged substrate proteins (Fig. 1c). To generate this NanoTAC (NC4) (Fig. 1d), we synthesised two series of NanoTAC analogues. NC1 and NC2 were generated by linking NanoLuc inhibitor 1, a small-molecule inhibitor of NanoLuc with a low nanomolar IC₅₀ (31 nM) against the enzymatic activity of NanoLuc²⁷ (Supplementary Fig. 1a), with the CRBN ligand thalidomide using varied alkyl chain linker lengths (Supplementary Fig. 1b, c). NC3, NC4, NC5 and the structurally related control analogue unable to recruit CRBN, NC₆, were
Fig. 1 Development of NanoTACs: a NanoLuc-targeting degrader system. a Schematic depicting the tTPD systems. FKBP12F36V, NanoLuc or Halo epitope tags are fused to proteins of interest (POI) and tag-targeting heterobifunctional degrader compounds are employed to hijack cullin-RING ligase (CRL) complexes to trigger proteasomal degradation of the tagged POI. b Comparison of tools and properties of each tag for tag-targeted protein degradation. NT not tested. Asterisk (*) see discussion for further information. 

c Schematic depicting the NanoLuc-CRBN tTPD system. 
d Chemical structure of the heterobifunctional NanoLuc-targeting PROTAC; NanoTAC4 (NC4).

Table 1 tTPD system nomenclature.

| Abb. | Tag/E3 Abb. | Tag-targeting degrader | Target (Tag) | E3-ligase complex |
|------|-------------|-------------------------|-------------|-------------------|
| FC1  | F36V-CRBN   | dTAG13                  | FKB12F36V   | CUL4CRBN          |
| FC2  | F36V-CRBN   | dTAG48                  | FKB12F36V   | CUL4CRBN          |
| FV1  | F36V-VHL    | dTAGV1                  | FKB12F36V   | CUL2VHL           |
| NC1  | NanoLuc-CRBN| NanoTAC1                | NanoLuc     | CUL4CRBN          |
| NC2  | NanoLuc-CRBN| NanoTAC2                | NanoLuc     | CUL4CRBN          |
| NC3  | NanoLuc-CRBN| NanoTAC3                | NanoLuc     | CUL4CRBN          |
| NC4  | NanoLuc-CRBN| NanoTAC4                | NanoLuc     | CUL4CRBN          |
| NC5  | NanoLuc-CRBN| NanoTAC5                | NanoLuc     | CUL4CRBN          |
| NC*  | NanoLuc-CRBN| NanoTAC*                | NanoLuc     | CUL4CRBN-ME Inactive |
| NV1  | NanoLuc-VHL | NanoTACV1               | NanoLuc     | CUL2VHL           |
| NV2  | NanoLuc-VHL | NanoTACV2               | NanoLuc     | CUL2VHL           |
| HC1  | Halo-CRBN   | HaloPROTAC-1            | HaloTag7    | CUL4CRBN          |
| HC2  | Halo-CRBN   | HaloPROTAC-2            | HaloTag7    | CUL4CRBN          |
| HC3  | Halo-CRBN   | HaloPROTAC-3            | HaloTag7    | CUL4CRBN          |
| HV1  | Halo-VHL    | HaloPROTAC-A            | HaloTag7    | CUL2VHL           |
| HV2  | Halo-VHL    | HaloPROTAC-E            | HaloTag7    | CUL2VHL           |

Abb. abbreviation.
prepared by coupling a derivative of the more potent NanoLuc inhibitor 2, with a single-digit nanomolar IC_{50} (4.2 nM) against the enzymatic activity of NanoLuc, and NanoLuc in addition to the fluorescent protein EGFP to create the artificial recombinant fusion protein Halo-EGFP-NanoLuc-FKBPF36V (H-E-N-F) (Supplementary Fig. 2a). All graphs in this study have been colour-coded corresponding to the targeted tag to ease interpretation. The F36V-CRBN degrader FC1 almost completely degraded the fusion protein at a concentration of 100 nM as judged by Western blot while the NanoLuc-CRBN targeting NanoTACs NC1 and NC2 led to partial degradation of the fusion protein at 500 nM and 1 μM (Supplementary Fig. 2b).

We next tested the second series of NanoTAC analogues. To explore whether NanoTAC degraders could be improved by recruiting a different E3 ligase, we employed NV1 and NV2 that hijack the CRL2VHL complex (Supplementary Fig. 2c). NV1 and NV2 were unable to trigger degradation of the H-E-N-F fusion protein over the wide range of tested concentrations up to 16 μM (Supplementary Fig. 2d, e). The lack of degradation was not due to an absence of VHL activity, as the F36V-VHL targeting degrader (FV1) triggered complete degradation of the fusion protein by Western blot (Supplementary Fig. 2d, e).

Luminescence is a higher throughput readout than Western blotting, however, NanoTAC degraders compete with NanoLuc substrates as they interfere with the conversion of furimazine to furimamide (Supplementary Fig. 2f). To address this, we generated a fusion protein that incorporates Firefly: Halo-Firefly-NanoLuc-FKBPF36V (H-FF-N-F) (Fig. 2a). This construct allows for the direct side-by-side comparison of all tTPD systems including the NanoLuc tTPD system using a Firefly luciferase, which emits a luminescence signal that can be assayed independently from NanoLuc through the use of distinct substrates. Using the H-FF-N-F reporter protein we tested the second series of NanoLuc-CRBN targeting NanoTAC analogues NC3, NC4, NC5 and NC* (inactive control). We observed a greater reduction in Firefly luminescence, and protein levels by Western blot, upon NanoTAC treatment of cells expressing the H-FF-N-F fusion protein (Fig. 2b, c). Specifically, NC3 and NC4 showed a marked improvement in the extent of substrate degradation compared to the first series of analogues NC1 and NC2, particularly at lower concentrations (Fig. 2b, c). At higher concentrations, NC4 demonstrated the hook effect (Fig. 2c), which is a characteristic feature of heterobifunctional degraders whereby high concentrations block degradation due to ligand saturation and inability to form a ternary complex. Interestingly, NC5 which harbour a linker that is just one carbon length shorter than NC4 was incapable of triggering degradation of the fusion protein, possibly due to unfavourable steric interactions as a result of the decreased linker length (Fig. 2b, c). As expected, the control compound NC* did not trigger degradation of the fusion protein (Fig. 2b, c). NanoTAC degraders were equally capable of triggering degradation of the H-E-N-F substrate protein with NC3 and NC4 consistently outperforming the first analogue NC1 (Fig. 2d). Overall, NC4 displayed the most effective degradation profile and was therefore characterised further.

NC4 exhibited approximately a 30-fold reduced inhibition of NanoLuc enzymatic activity compared to the parent inhibitor (NanoLuc inhibitor 2) indicating that conjugation of the CRBN ligand altered the binding kinetics of NC4 (Supplementary Fig. 2g). Strikingly, even though NC4 exhibited reduced inhibition of NanoLuc enzymatic activity NC4 was still capable of rapidly reducing protein levels within 2 h and degradation was sustained over a period of 10 h (Fig. 2e). Further to this, degradation by NC4 was sustained over 24 h when stimulation was maintained in the culture medium, and reversible by 24 h when stimulation was removed after 5 h of initial treatment (Fig. 2f). NC4 was also capable of triggering degradation of the fusion protein at low concentrations, ~30 nM, with a slight hook effect observed at higher concentrations, similar to that observed in Firefly luminescence-based readouts (Fig. 2e). Importantly, the global proteomics analysis demonstrated that NC4 triggers specific degradation of the target substrate with no significant off-target degradations observed when assayed against 591 proteins (Fig. 2b). Notably, our proteomics analysis did not detect the Thalidomide-induced CRBN neosubstrates, IKZF1 or IKZF3, and therefore we cannot comment on whether these neosubstrates are altered upon NC4 treatment.

To explore whether NanoLuc luminescence could be used in conjunction with NanoTAC treatment we titrated NC4 against NC* from 2–10 h. We reasoned that the catalytic nature of NC4 would allow for substrate degradation at concentrations below that required to inhibit NanoLuc’s enzymatic luminescence activity. To this extent we compared the ability of NC4 to reduce luminescence with the control compound, NC*, which lacks the degradative capability. As predicted, we observed clear differences in NanoLuc luminescence at 31.2 nM and 15.6 nM between NC4 and NC* (Supplementary Fig. 2h). Importantly, the reduction in NanoLuc luminescence and the observed depletion of the reporter by Western blot was indicative of cullin-based proteasome-mediated degradation, as pre-treatment with the proteasome inhibitor MG132 or the NEDD8-activating enzyme inhibitor MLN4924 could rescue both the reduction in luminescence and protein levels (Fig. 2i, j). Notably, a slightly enhanced rescue was observed when the substrate furimazine concentration was increased for NanoLuc detection, consistent with the competitive nature of NanoLuc inhibition by NC4 (Fig. 2i).
the H-FF-N-F or the H-E-N-F fusion proteins (Fig. 2a and Supplementary Fig. 2a). Using Firefly luminescence, we were able to directly compare the degradative capacity of the F36V-CRBN, F36V-VHL, Halo-VHL and NanoLuc-CRBN systems against the same substrate. Overall, F36V-CRBN degraders (FC1 and FC2) outperformed the Halo-VHL degraders (HV1 and HV2), while the F36V-VHL degrader (FV1) triggered the most efficient substrate depletion over a wide concentration range after 5 h (Fig. 3b). A similar degradation profile was observed from the corresponding NanoLuc luminescence detected from the H-FF-N-F and H-E-N-F substrates in the same assay (Supplementary Fig. 3c, d). Halo-VHL degraders, although less efficient at triggering substrate depletion compared to the F36V-CRBN and F36V-VHL degraders, particularly at lower concentrations,
displayed slightly improved substrate degradation over 8 and 24 h at relatively low concentrations (Fig. 3c, Supplementary Fig. 3e). The NanoLuc-CRBN targeting NanoTAC, NC4, was capable of triggering degradation of the fusion protein over a wide concentration range and to levels comparable to that of the Halo and FKBP36V tTPD systems displaying a calculated Dmax of 77% and a DC50 of 21.6 nM against our fusion (Fig. 3b, c). NC4, displayed a similar degradation profile to that of the HaloPROTACs (Fig. 3b), but reproducibly appeared to lose efficacy after 24 h (Fig. 3c). Consistent with our luminescence results, we observed reduced levels of the fusion proteins with all tested tTPD degrader systems by Western blot (Fig. 3d, Supplementary Fig. 3f), and importantly the observed reduction in luminescence could be rescued by co-treatment with the proteasome inhibitor MG132 (Fig. 3e, Supplementary Fig. 3g). Next we compared the durability of degradation across all tTPD systems by performing wash-out recovery experiments. We transitioned to a constitutive system where the H-E-N-F protein was expressed under the control of a ubiquitin promoter to avoid foreseeable complications with wash-out of the doxycycline in our inducible systems. Interestingly FC1 and FV1 maintained near complete degradation up to 48 h (Fig. 4a), and were capable of maintaining clear degradation for up to 48 h when the degrader was removed from the culture medium at 5 h post-treatment (Fig. 4b). FC2 and NC4 displayed very similar durability profiles with clear degradation seen up to 24 h (Fig. 4a), however, this was not maintained when the degrader was removed from the culture medium after 24 h (Fig. 4b). HaloPROTACs HV1 and HV2 were less durable in their response with depletion not maintained upon removal of the degrader from the culture medium and overall slower degradation kinetics (Fig. 4a, b).

To functionally assess the impact of the different tTPDs on a model substrate, we selected the well-characterised pro-necrotic pseudokinase MLKL. MLKL is the terminal effector protein for necrotic cell death30 which can be induced by the combined treatment of TNF, Smac mimetic (SM); compound A31 and z-VAD-fmk (TSZ)32. C-terminally tagged MLKL has previously been shown to be fully functional33 and MLKL knockout cells cannot undergo necroptosis unless reconstituted with an active MLKL construct34. We therefore generated C-terminal fusion proteins of MLKL containing either FKBP36V, Halo or NanoLuc tags and stably expressed doxycycline-inducible versions of these proteins in MLKL−/− HT29 cells that were generated by CRISPR/Cas9 targeting. The HT29 cell line is widely used and can undergo rapid necroptosis when stimulated with TSZ35. MLKL-NanoLuc expression was independently confirmed by NanoLuc luminescence (Supplementary Fig. 4a). The tagged versions of MLKL displayed reduced expression compared to untagged MLKL in MLKL−/− HT29 cells, with the NanoLuc tag leading to a strong reduction in expression compared to adding a Halo or FKBP36V tag (Supplementary Fig. 4b). From this data it is unclear to what extent this will limit the application of the NanoLuc tag to other targets, and what causes the reduced expression. Nevertheless, the expression level of MLKL-NanoLuc was sufficient as MLKL-Halo, MLKL-FKBP36V and MLKL-NanoLuc could confer comparable sensitivity to necroptosis (Supplementary Fig. 4c).

The tagged MLKL substrates afforded us the opportunity to directly compare the efficiency of the CRBN and VHL degrader systems against a biologically-relevant substrate with the tags positioned at the same terminus (Fig. 4c). Interestingly the FV1 degrader that recruits F36V-VHL was faster at triggering degradation of MLKL compared to the F36V-CRBN degrader FC1, acting within 1 h (Supplementary Fig. 4d). FV1 was also more effective at triggering degradation of MLKL-FKBP36V compared to the F36V-CRBN degrader FC1 resulting in no detectable protein at 5 h at low nM concentrations (Fig. 4d). To directly compare whether the FKBP36V or Halo epitope tags could influence the degradation of tagged MLKL with the same CRL system we tested the F36V-VHL and Halo-VHL systems side-by-side (Fig. 4e). Consistent with our previous results we observed that the F36V-VHL recruiting degrader FV1 was the most effective compound (Fig. 4f). Interestingly, we observed comparable degradation of murine MLKL-FKBP36V reconstituted into MLKL−/− mouse dermal fibroblasts (MDFs) with F36V-CRBN degraders (FC1, FC2) and the F36V-VHL degrader (FV1) (Supplementary Fig. 4e), suggesting that even slight changes in the substrate or cell type might influence the effectiveness of degrader compounds. To explore this further, we inserted each tag into the genomic locus of the FBL gene that encodes for the protein Fibrillarin, which is exclusively expressed in the nucleolus. We performed concentration titrations and time courses on Fibrillarin-NanoLuc, Fibrillarin-Halo and Fibrillarin-FKBP36V expressing cell lines and calculated Dmax values based on densitometry measurements (Supplementary Fig. 4f–m). Consistent with our results for MLKL and our fusion protein, we observed that FV1 was superior in degradation capacity compared to the other tag degraders displaying a Dmax of ~95.9% after 10 h. FC1, HV1, HV2 and NC4 all demonstrated clear degradation of FBL over 10 h with calculated Dmax values of ~70%, ~93.2%, ~94.8% and ~52.9%, respectively. Fibrillarin-NanoLuc predominantly localises to the nucleus (Supplementary Fig. 5a) suggesting that NC4 was capable of degrading substrates that primarily reside in the nucleus. To determine whether substrates in other cellular compartments could also be targeted by NC4 we generated a fusion construct containing NanoLuc and...
the plasma membrane transporter protein SLC38A2. We confirmed the location of NanoLuc-SLC38A2 in the membrane compartment (Supplementary Fig. 5b), and consistent with our previous results we observed a reduction in NanoLuc-SLC38A2 upon treatment with NC4 (Supplementary Fig. 5c).

tTPD systems trigger physiologically relevant degradation. To compare the biological activity of tTPD-mediated degradation across all tTPD platforms, we assessed whether our most potent NanoTAC could degrade substrates to a level that prevents necroptosis to achieve a response similar to protein knockout. Consistent with our previous results, NanoTAC degraders triggered efficient degradation of MLKL-NanoLuc, with NC4 displaying the most effective degradation profile (Supplementary Fig. 5d). Similar to the degradation of the Halo-EGFP-NanoLuc-FKBPF36V fusion protein, NC4 triggered degradation of MLKL-NanoLuc within 2 h.

![Fig. 3 FKBPF36V tTPD systems outperform other tTPD systems.](image)
with sustained degradation over 10 h (Supplementary Fig. 5e). Impressively, NC4-induced degradation of MLKL-NanoLuc was sufficient to reduce MLKL-NanoLuc-driven necroptosis in HT29 cells in a dose-dependent manner (Fig. 5a), and importantly, NanoTAC degraders displayed no anti-necroptotic activity in MLKL−/− cells expressing untagged MLKL (Fig. 5a) or wild-type HT29 cells (Supplementary Fig. 5f). F36V-CRBN targeting degraders (FC1, FC2) were capable of reducing necroptosis driven by MLKL-FKBPF36V in MLKL−/− HT29 cells in a dose-dependent manner, sustaining cell survival over 24 h at 62.5 and 15.6 nM, respectively (Fig. 5b, c). The F36V-VHL targeting degrader FV1 was extremely efficient at preventing necroptosis driven by MLKL-FKBPF36V, demonstrating a sustained and prolonged reduction in cell death at 3.9 nM over 24 h (Fig. 5d). HaloPROTACs (HV1 and HV2), although capable of reducing necroptosis driven by Halo-MLKL in MLKL−/− HT29 cells, required much higher concentrations to maintain cell survival; 250 and 125 nM, respectively (Fig. 5e, f). Interestingly, HaloPROTACs also exhibited steep...
reductions in cell survival at lower concentrations, which may be attributed to the non-catalytic nature of these degrader compounds (Fig. 5e, f). Like NanoTAC degraders, FKBP\text{F36V} degraders and HaloPROTACs displayed no anti-necroptotic activity in MLKL\textsuperscript{−/−} cells expressing untagged MLKL (Figs 5b–f) or wild-type HT29 cells (Supplementary Fig. 5g, h). A representative flow cytometry gating strategy that was used to analyse the induction of necroptosis is provided (Supplementary Fig. 5i). To test whether NanoTACs could be combined with other iTPD systems to modulate the levels of multiple proteins, we deleted endogenous caspase-8 via CRISPR/Cas9 targeting in HT29 cells expressing MLKL-NLuc, and re-expressed caspase-8-FKBP\text{F36V}. Both caspase-8-FKBP\text{F36V} and MLKL-NLuc could be depleted by FV1 and NC4 treatment in these cells, respectively (Fig. 5g). TNF and Smac mimetic (TNF + SM)
Fig. 5 NanoTAC degraders trigger MLKL degradation and block necroptotic cell death. a MLKL−/− HT29 cells stably expressing the doxycycline-inducible untagged MLKL or the C-terminal fusion protein MLKL-NanoLuc were induced with 40 ng/mL doxycycline overnight, then stimulated with NC4 (NanoLuc-CRB) or 5 h before the addition of TNF (100 ng/mL) + Smac mimetic (compound A; 500 nM) + caspase inhibitor; z-VAD-fmk (10 μM), for 24 h. Cell death was assessed by flow cytometric analysis of PI exclusion. N = 3 independent experiments (symbols), EB represent mean ± SD. b–d MLKL−/− HT29 cells expressing doxycycline-inducible untagged MLKL or the C-terminal fusion protein MLKL-FKBP36V were induced with 40 ng/mL doxycycline overnight, then stimulated with F1, F2 or F5 for 5 h before the addition of TNF (100 ng/mL) + Smac mimetic (compound A; 500 nM) + caspase inhibitor; z-VAD-fmk (10 μM), for 24 h. Cell death was assessed by flow cytometric analysis of PI exclusion. N = 3 independent experiments (symbols), EB represent mean ± SD. e MLKL−/− HT29 cells expressing doxycycline-inducible untagged MLKL or the C-terminal fusion protein MLKL-Halo were treated with 40 ng/mL doxycycline overnight to induce the constructs, then stimulated with the indicated concentrations HVI or HV2 for 5 h before the addition of TNF (100 ng/mL) + Smac mimetic (compound A; 500 nM) + caspase inhibitor; z-VAD-fmk (10 μM), for 24 h. Cell death was assessed by flow cytometric analysis of PI exclusion. N = 3 independent experiments (symbols), EB represent mean ± SD. f Western from MLKL−/−/CASPB−/− HT29 cells stably expressing the doxycycline-inducible C-terminal fusion protein-fusion, MLKL-NanoLuc (MLKL-Nluc) and constitutively expressing the C-terminal fusion protein caspase-8-FKBP36V. Cells were induced with 40 ng/mL doxycycline overnight, then stimulated with FVI and NC4 for 5 h. g Cells from f were treated with 40 ng/mL doxycycline and stimulated with 125 nM of FVI, NC4, or NC* for 5 h before the addition of TNF (100 ng/mL) + Smac mimetic (compound A; 500 nM) for 19.5 h. Cell death was assessed using an IncuCyte and Cytotox-Red uptake. N = 3 independent experiments, one representative experiment shown, EB represent mean ± SD. Source data are provided as a Source Data file.

Discussion

To add to the current toolbox of available tTPD systems and to compare the utility of different degrader/tag combinations we developed a new NanoLuc-targeted degrader system and a new reporter system. Our reporter system enables rapid comparative testing of optimal degrader/tag combinations that will advance the field by providing an interchangeable tag system that is amenable to a limitless number of degrader/tag pairs. We use this reporter system to test and develop NanoTACs, demonstrating that the reporter can facilitate the development of new tTPD systems. We believe this reporter system will increase the ease with which the powerful tTPD approach can be adopted and extended by other laboratories.

Using our reporter system and test substrates (MLKL & Fibrinllarin) we provide side-by-side comparisons of the Halo-VHL, Halo-CRBN, F36V-CRBN and F36V-VHL tTPD systems, and directly compare these to the newly developed NanoLuc-CRBN and NanoLuc-VHL systems. We highlight the importance of comparing all tTPD systems against each substrate as each system does not degrade equally, and efficient tTPD-mediated degradation is extremely substrate-specific.

Interestingly, when we compared the degradation of human MLKL-FKBP36V and mouse MLKL-FKBP36V we observed that the CRBN-recruiting degraders were less effective at targeting human MLKL-FKBP36V compared to VHL degraders, yet mouse MLKL-FKBP36V was degraded by both systems equally. We also observed that endogenously tagged-Fibrinllarin, which is localised in the nucleolus, was preferentially degraded by VHL-recruiting degraders compared to CRBN-recruiting degraders. This effect is potentially due to differences in the formation or stability of the ternary complex between the CRBN and VHL containing E3-ligase complexes and different substrate proteins, or subcellular location of VHL and CRBN, which may dictate substrate specificity for these E3-ligase complexes.

If a ternary complex is unstable or exhibits poor cooperative binding then certain degraders could be less efficient at triggering degradation, which would be consistent with previous reports that detail the importance of cooperativity for degrader-mediated protein degradation. Our data highlights that each substrate needs to be assessed against all tTPD systems as it is extremely challenging to predict which substrate-CRL complex and which degrader/tag pair will lead to optimal degradation of a particular substrate. Another important consideration that may influence ubiquitination and substrate degradation is which protein terminus the tag is attached to as this may alter lysine accessibility. The optimal terminus for a particular tag is ultimately substrate-specific and some substrates cannot tolerate a tag attached to a particular terminus. However, if ubiquitin is primarily conjugated to the tag lysine residues rather than substrate lysines then the position of the tag would be unlikely to influence degradation but may still impact protein function.

Importantly, we find that the newly developed NanoLuc-CRBN degrader system functions comparably to the other tTPD systems, triggering degradation at low nM concentrations of degrader and to physiologically relevant levels. NanoTACs, however, also have some drawbacks that are important to highlight. We currently do not have efficient NanoLuc-VHL degraders in hand that would potentially increase the substrate scope of protein targets for NanoTAC degradation. NanoTACs are based on a NanoLuc inhibitor warhead, therefore, at intra-cellular concentrations approximating the IC50, NanoTACs interfere with nanoluciferase activity by inhibiting the conversion of furimazine to furimamide, preventing the ability to perform high-throughput degradation assays via a luminescence readout. Notably, since NanoTACs achieve efficient degradation of NLuc-tagged proteins resulting in a low cellular DC50, nanoluciferase luminescence can still be used to reliably read out degradation when used well below the NLuc IC50. However, for this purpose, we recommend that titrations are performed against the inactive analogue (NC*) and that critical controls i.e. MG132 or MLN4924 are included to separate degradation from NLuc inhibitory activity. Further investigation
is also required to determine whether NanoTACs can be compatible with the in vivo nanoluminescence substrates. NanoTACs are functionally similar to FKBP36V, targeting degraders as NanoTACs are catalytic by nature and are not consumed during the reaction. The catalytic property of degraders is an important feature as degraders that exhibit poor cellular penetration or weak target-binding affinity can still be highly efficient degraders. It is, therefore, intriguing that NanoTACs are less effective compared to FKBP36V, targeting degraders and might suggest that there are differences in the cellular accumulation of these molecules. The catalytic property of the NanoLuc/NanoTAC tTPD system is a major advantage over non-catalytic tTPD systems and further optimisation of NanoTACs to achieve improved degradation efficiency of NLuc-tagged substrates presents an exciting opportunity.

The importance of catalytic degraders for substrate degradation was highlighted by elegant studies into Bruton’s tyrosine kinase (BTK) binding ibrutinib-derived degraders, demonstrating that reversible binding to BTK was essential to trigger BTK degradation. It was postulated that irreversible covalent drug–protein adducts such as HaloPROTACs are consumed once they engage and react with their target protein and hence cannot reach degradation efficiency comparable to catalytic degraders. Conversely, a number of studies argue against this theory.

Furthermore, our data and those of others suggest that covalent HaloPROTACs can trigger efficient degradation of Halo-tagged substrates and we show the comparable activity of non-catalytic HaloPROTACs to catalytic NanoTAC degraders. Moreover, HaloPROTACs have high affinity and selectivity for the Halo tag and we observed that HaloPROTACs maintained substrate depletion over 24 h.

Overall, we find that the FKBP36V tTPD systems are generally superior at triggering degradation of FKBP36V tagged substrates with regard to the minimal effective concentration and the time taken to achieve protein loss respective to controls, compared to the Halo- and NanoLuc-targeting systems, in our hands. Our observation that NanoTACs and HaloPROTACs degrade with slower kinetics and require higher minimal concentrations to achieve effective degradation compared to FKBP36V degraders might be explained by a variety of factors that alter the intracellular accumulation of these compounds including cell permeability, drug efflux pumps, cellular metabolism or interactions with intracellular proteins and other biomolecules. Another possible limitation in the effectiveness of HaloPROTACs is their potential reactivity with strong nucleophiles present in the cell (e.g. Glutathione) due to the chloroalkyl moiety. This irreversible side-reaction would consume the HaloPROTAC and decrease the effective concentration of the target Halo-tagged protein. HaloPROTACs and NanoTACs likely do not reach sufficient intracellular concentration in our necrotic assays at lower assay concentrations, as our data show that these degraders are unable to provide complete protection against MLKL-Halo induced necroptosis.

The FKBP36V system is excellent for triggering degradation of FKBP36V-tagged substrates, and the FKBP36V tag is small compared to the other tags at just 12 kDa, having minimal impact on fusion partner proteins. If targeted protein degradation is the primary goal, then this tTPD system is an ideal choice as the experimenter can easily and rapidly switch between the CRBN and VHL systems through the use of different degrader compounds. However, if the experimenter is interested in also understanding tagged protein biochemistry/biology, (cellular localisation, protein interactomes, tissue expression profiles etc.) then the NanoLuc (catalytic degrader available) and Halo (only non-catalytic degraders available) tags are superior as they offer the best of both worlds due to the numerous commercial tools available for these two protein tags.

Consistent with previous reports, our data detail that all current tag-targeting degraders trigger proteasomal degradation of tagged substrates. However, we did observe that the degradation of the reporter construct by FV1 was only partially reduced in the presence of the proteasome inhibitor MG132. Given that we pre-incubated the cells with MG132 for 1 hour prior to the addition of FV1, which is sufficient to block degradation triggered by the other tag-targeting degraders, it is possible that FV1 might also trigger degradation through alternative pathways, for example, the lysosomal degradation pathway. Alternatively, FV1 could simply be very efficient at triggering substrate ubiquitination and degradation, and this degradation might precede complete proteasomal inhibition by MG132, even after pre-incubation.

A comprehensive analysis in animals is yet to be conducted to compare the tTPD systems side-by-side, and in vivo pharmacokinetic and pharmacodynamic studies will need to be performed on the NanoTAC compounds. Regardless, studies that have tested individual tag-targeting degraders, or similar degraders such as RC32 comprised of the FKBP12 ligand Rapamycin conjugated to the CRBN ligand pomalidomide, in animals, detail that they are well tolerated and can trigger efficient depletion of substrates. Notably, in vivo pharmacokinetic analysis and activity studies for the dTAG degraders FC1 and FV1 have been performed, and one group has reported in vivo activity for one HaloPROTAC, HaloPROTAC-38, which was not used in our study. Tag-targeting degrader compounds are not designed to be used therapeutically; however, tTPD systems do allow for the assessment of chemical-induced protein depletion in animal disease models, that will closely mimic targeted protein degradation of endogenous targets.

With the advancement of the tTPD systems to include NanoLuc in the armamentarium, tTPD technology is leading the way for comprehensive validation studies to be conducted on prospective therapeutic targets to rationally determine which targets warrant drug discovery investment to identify ligands targeting equivalent endogenous proteins.

### Methods

#### Cell culture and maintenance.

HEK293T (ATCC; CRL3216), HT29 (ATCC; HTB-38) and immortalized (SV40 large-T antigen) mouse dermal fibroblast (MDF) cells (generated in-house from C57BL/6 mouse tails) were cultured in Dubecco’s modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS, Sigma), 50 U/ml penicillin (Gibco) and 50 μg/ml streptomycin (Gibco). IMDFs were isolated from C57BL/6 mouse tails and transformed with SV40 Large-T antigen. All cells were maintained at 37°C with 10% CO2, in a humidified incubator.

#### Plasmid constructs and stable cell lines.

The cDNA for human or mouse Mlk1 was cloned from synthetic gBlock fragments (Integrated DNA Technologies) to generate fusion proteins harbouring a Halo, FKBP36V, or NanoLuc (NLuc) tag at the C-terminus of MLK1. The individual tags in the Halo-EGFP-NanoLuc- FKBP36V (H-E-N-F) reporter construct were generated as gBlock fragments (Integrated DNA Technologies) and made into a single reporter construct using restriction digestion and ligation cloning. Halo refers to HaloTag7, FKBP36V refers to FKBP1236, each protein in the reporter construct is separated by a 9-10 amino acid glycine-serine linker. To generate the H-F-F-N reporter construct, the EGFP was excised from the H-E-N-F reporter construct and replaced with Firefly luciferase (kind gift from Joao Heath) using In-Fusion cloning (Takara Bio). MLK1+/− HT29 cells were generated by CRISPR-Cas9 targeting using lentiviral transduction, reported previously, and Mlk1−/− mouse dermal fibroblasts (MDFs) were generated from Mlk1−/− mice described previously. Stable cell lines were generated using the Lentiviral packaging constructs (VSVG, RSV-Rev, PMDL) and the pFIRE3G doxycycline-inducible vector and selected with Puromycin (2 μg/ml). pX330A-FBL/PITC was a gift from Takashi Yamamoto (Addgene plasmid # 63671; http://n2t.net/addgene:63671; RRID:Addgene_63671) and pCRIS-PITCChv2-FBL was a gift from Takashi Yamamoto (Addgene plasmid # 63672; http://n2t.net/addgene:63672; RRID:Addgene_63672) and pCRIS-PITCChv2-FBL was modified to contain either the NanoLuc, Halo or FKBP36V tag sequences to enable genomic insertion of a tag-

### T2A-PuroR sequence at the FBL locus to produce C-terminal NanoLuc-, Halo- or FKBP36V-tagged Fibbrillarin. For knock-in cell lines, 63671 or modified 63672
vectors were co-transfected into 293T cells with lipofectamine, stably integrated cells were selected with puromycin (1 μg/mL).

**NanoLuc and Firefly luciferase assays.** For high-throughput assessment of tPDD-mediated degradation, cells expressing NanoLuc or Firefly fusion proteins were seeded into 384-well flat bottom, clear bottom white-walled plates (Corning) at 1–1.5 × 10^4 cells/well in 50 μL of DMEM/FCS. A final concentration of 20 ng/mL and 40 ng/mL of doxycycline was used for HEK293T and HT29 cells, respectively, followed by overnight incubation (16–24 h) to induce construct expression. Cells were then treated with either vehicle control (DMSO) or degrader compound as indicated. In compound titration experiments, all vehicle control amounts were indicated. In compound titration experiments, all vehicle control amounts were (Sigma) or MLN4924 (1 μM; Tocris) prior to the addition of the degrader compound. Time-course experiments were conducted as reverse time course so that luminescence could be detected at the same cell density. At the stated time-points, luminescence was induced using the Nano-GLO Luciferase assay system (Promega) for NanoLuc luminescence, or Nano-Glo Dual-Luciferase Reporter assay system (Promega) for NanoLuc/Firefly dual luminescence. Clear plate bottoms were tap washed with coloured tape to prevent bleeding between wells. For IC₅₀ measurements recombinant NanoLuc enzyme was prepared to a concentration of 0.2 nM, and NanoLuc LCS dilution buffer was diluted 1:30 in TBS + 0.1% BSA. NC4 or NanoLuc inhibitor 2 were diluted in the LCS solution and combined with the diluted NanoLuc NanoLuc enzyme. Samples were incubated for 6 min at room temperature before assaying for luminescence. Luminescence was then measured (0.1 s/well, filter 470–480) on a microplate reader (CLARIOstar Plus, BMG Labtech).

**Immunoblotting.** Cells were seeded in 24-well plates and doxycycline (20 ng/mL for HEK293T, 40 ng/mL for HT29) and incubated overnight (16–24 h) prior to degrader treatments, as stated in the Fig. legends. All timepoints were performed as reverse timepoints in order to harvest all cells at the same time. At the indicated time points cells were lysed in NuPAGE LDS lysis buffer (Invitrogen) diluted to 1× in DSSC lysate buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA pH 8.0, 1% Triton X-100, 10% glycerol, H₂O) supplemented with β-Mercaptoethanol (2%), protease and phosphatase inhibitors. Lysates were run through polyacrylamide columns (Pierce) to shred DNA. Proteins were separated by SDS-PAGE on 4–12% gradient gels (Invitrogen) and transferred onto Immobilon-E membranes (Merck). Membranes were blocked in 5% skim milk (Devondale) in TBS-T (TBS, 0.1% Tween-20) for 1 h prior to immunoblotting with primary antibodies overnight at 4 °C. Unless stated otherwise, all primary antibodies were diluted in TBS-T (TBS, 0.1% Tween-20) containing 5% BSA (Sigma A8022) and 0.04% sodium azide: HaloTag (1:1000, Promega; G9211), β-actin (1:20,000, Sigma; A-1798), MLK3 (1:1000, produced in-house; H31 clone)39, FKBP1 (1:1000, R&D systems; 422513), caspase-8 (1:1000, Proteintech; 13423-2-AP), Histone H3 (1:1000, Abcam; ab10799), Cadherin (1:1000, Cell Signalling Technologies; 4068T). Membranes were washed 3 × 5–10 min in TBS-T (TBS, 0.1% Tween-20) prior to the addition of the appropriate secondary antibodies conjugated to horseradish peroxidase (Jaxon laboratories). All secondary antibodies were diluted at 1:10,000 in TBS-T (TBS, 0.1% Tween-20) containing 5% skim milk and incubated at room temperature for 1 h. Final washes of 4 × 5–10 min with TBS-T (TBS, 0.1% Tween-20) were conducted before ECL development (Millipore, Bio-Rad) and protein detection using the ChemiDoc Touch Imaging System (Bio-Rad). All images were processed using Image Lab software.

**Necroptosis assays.** To simulate necroptosis, cells were seeded into 96-well plates ±40 ng/mL doxycycline, to induce construct expression, and treated with 100 ng/mL FLAG-TNF (recombinant human, in-house), 500 nM compound A Smac mimetic (kindly gifted by TetraLogics Pharmaceuticals) and 5 μM compound IDN-6556 (Cayman) or 10 μM Z-VAD-fmk (Selleckchem) overnight (16–24 h). Degrader treatment of either vehicle control (DMSO) or degrader compound was added for 5 h, as stated in the Fig. legends. Cells were detached using Trypsin-EDTA (Merck) and resuspended in cell recruitment dTAG molecules.

**Quantitative proteomics.** Cell pellets were extracted with 5% SDS (including 100 mM TEAB) and processed using micro S-traps as described by the manufacturer (ProtiFi). For liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, approximately 200 ng of sample was injected onto an Acquity M-class UPLC (Waters) connected to a timsTOF pro II (Bruker). Peptides were separated using a 112 min gradient (solvent A: 0.1% formic acid; solvent B: 99.9% acetonitrile/0.1% formic acid) on a C18 analytical column (IonOpticks, Aurora Series Emitter Column, AUR2-25075C18A 25 cm × 75 μm). Data-dependent PASEF acquisition was performed (100–1700 m/z scan range and 0.6–1.6 mobility range) and the data searched against the reviewed Homo sapiens uniprot database (UP000005640) with MSfragger (v.3.13) within the Fragpipe workflow (v17.0) using strict trypsin cleavage and up to two missed cleavages. Precursor and fragment ion tolerances were both set to 20 ppm and the minimum peptide length set at seven. Peptide and protein level FDR was set at 1% and protein quantification determined using the MaxLFQ algorithm40. Data were processed and visualised with the DEP R package, where statistical significance was determined using the moderated t-statistic from the limma package 41. Unadjusted P-values were plotted and statistical significance was determined using the density-based q-value in fdrtool42.

**Animal handling.** C57BL/6J mice were maintained in-house under specific pathogen-free conditions at the Walter and Eliza Hall Institute of Medical Research (WEHI), Australia. Animal rooms were maintained at approximately 21 °C ± 3 °C at 40–70% humidity with a timed 14:10 h light-dark cycle. Wild-type C57BL/6J mice were bred at WEHI and/or obtained from WEHI animal supplies (Kew, Australia). None of the mice used in our experiments had been previously used for other procedures. The animals presented with a healthy weight and were selected independently of their gender for generating MDFs. Female and male mice were at least 6 weeks old at the time of experimentation. All procedures for this study were approved by the Walter and Eliza Hall Institute (WEHI) Animal Ethics Committee, Australia. All research complied with all relevant ethical regulations for animal testing and research.

**Generation of NanoTAC heterobifunctional compounds.** See supplementary methods.

**Data availability**

The data that support this study are available from the Reagents are available upon request. All NanoTACs are available upon request. Unprocessed Western blots are provided in the source data file. The mass spectrometry proteomics data generated in this study have been deposited in the ProteomeXchange Consortium via the PRIDE42 partner repository with the dataset identifier PXD031371. Source data are provided with this paper.

Received: 22 April 2021; Accepted: 25 March 2022; Published online: 19 April 2022

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