**BREAKTHROUGH REPORT**

# Rapid depletion of target proteins in plants by an inducible protein degradation system

**Linzhou Huang[1,2] and Marcela Rojas-Pierce[1]**

[1]Department of Plant and Microbial Biology, North Carolina State University, Raleigh, NC 27695
[2]Current address: College of Advanced Agricultural Sciences, Zhejiang A&F University, Hangzhou, Zhejiang 311300, China
Address correspondence to mrojasp@ncsu.edu and lzhuang@zafu.edu.cn

Short title: E3-DART for target protein degradation
One-sentence summary: The E3-DART system can be used to target proteins for degradation in plant cells.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plcell/pages/General-Instructions) is: Marcela Rojas-Pierce (mrojasp@ncsu.edu).

**Abstract**

Inducible protein knockdowns are excellent tools to test the function of essential proteins in short time scales and to capture the role of proteins in dynamic events. Current approaches destroy or sequester proteins by exploiting plant biological mechanisms such as the activity of photoreceptors for optogenetics or auxin-mediated ubiquitination in auxin degrons. It follows that these are not applicable for plants as light and auxin are strong signals for plant cells. We describe here an inducible protein degradation system in plants named E3-DART for **E3**-targeted **D**egradation of Pl**a**nt P**r**ot**e**ins. The E3-DART system is based on the specific and well-characterized interaction between the Salmonella secreted protein H1 (SspH1) and its human target protein kinase N1 (PKN1). This system harnesses the E3 catalytic activity of SspH1 and the SspH1-binding activity of the Homology Region 1b (HR1b) domain from PKN1. Using *Nicotiana benthamiana* and Arabidopsis (*Arabidopsis thaliana*), we show that a chimeric protein containing the Leucine-Rich Repeat (LRR) and novel E3 ligase (NEL) domains of SspH1 efficiently targets protein fusions of varying sizes containing HR1b for degradation. Target protein degradation was induced by transcriptional control of the chimeric E3 ligase using a glucocorticoid transactivation system and target protein depletion was detected as early as 3 h after induction. This system could be used to study the loss of any plant protein with high temporal resolution and may become an important tool in plant cell biology.

## Introduction

Functional studies of proteins of interest (POI) can be achieved by conditional manipulation at the DNA, RNA and protein levels (Sauer and Henderson, 1988; Gatz et al., 1992; Gossen and Bujard, 1992; Zuo and Chua, 2000; Elbashir et al., 2001; Guo et al., 2003; Gilbert et al., 2013; Housden et al., 2017; Natsume and Kanemaki, 2017; Dai et al., 2018; Galizi and Jaramillo, 2019). One major disadvantage of pre-translational strategies for POI depletion is the length of time to achieve full depletion because inactivation is determined by the half-life of the POI (Nishimura et al., 2009; Natsume and Kanemaki, 2017). By contrast, protein depletion at the post-translational level allows the rapid knock-down of POI directly, thus greatly facilitating the interpretation of altered phenotypes with minimal secondary effects. Eukaryotic cells have evolved dynamic protein quality control systems for the precise elimination of proteins and organelles in response to environmental changes, and the ubiquitin-proteasome system is a major player in the specific degradation of selected proteins (Pohl and Dikic, 2019).

Protein ubiquitination in eukaryotes is carried out by an ATP-dependent enzymatic cascade that sequentially involves the activity of an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase, which ultimately conjugates the ubiquitin moiety to the lysine residue of substrate proteins (Scheffner et al., 1995; Pickart, 2001). Based on the ubiquitin chain length and linkage type, ubiquitinated proteins are subject to different fates, including the degradation mediated by the 26S-proteasome system (Sullivan et al., 2003; Vierstra, 2009; Bedford et al., 2010; Livneh et al., 2016; Collins and Goldberg, 2017; Bard et al., 2018). Substrate recognition in ubiquitination is mediated by E3 ubiquitin ligases, a large and diverse family of proteins in eukaryotes that determine both the efficiency and substrate specificity of ubiquitination reactions (Zheng and Shabek, 2017). E3 ligases in eukaryotes can be divided into RING (really interesting new gene)-type, HECT (homologous to the E6AP carboxyl terminus)-type and RING-IBR-RING (RBR)-type E3 ligases (Zheng and Shabek, 2017). HECT-type and

RBR E3 ligases contain an invariant cysteine residue that accepts ubiquitin (Ub) from charged E2 conjugating enzymes before transfer to the target, while RING-domain ligases recruit targets via specific target-binding surfaces but the Ub is transferred directly from the E2 to the substrate (Bernassola et al., 2008; Deshaies and Joazeiro, 2009; Rotin and Kumar, 2009; Budhidarmo et al., 2012; Lechtenberg et al., 2016; Zheng and Shabek, 2017). E3 ligases in plants can be grouped into four classes, the HECT, RING, U-box and Cullin-RING ligases (CRLs), based on their subunit composition and their mechanism of action. The last two classes have gained much attention as they mediate important hormone perception and environmental stress responses (Vierstra, 2009; Shabek and Zheng, 2014; Trujillo, 2018; Blazquez et al., 2020).

Some bacterial pathogen effectors functionally resemble eukaryotic E3 ligases and suppress the host immune system. Bacterial E3 ligases have been identified in plant and animal pathogens as part of the RING, HECT and Novel E3 Ligase (NEL) classes (Huibregtse and Rohde, 2014; Maculins et al., 2016; Ashida and Sasakawa, 2017). The first identified NELs were the type III effectors of invasive plasmid antigen H (IpaH) family, including IpaH9.8 from *Shigella* and Salmonella secreted protein H1 (SspH1) from *Salmonella,* which contain a N-terminal leucine-reach repeat (LRR) domain for substrate recognition and a C-terminal domain (NEL) with E3 catalytic activity (Rohde et al., 2007). NEL E3 ligases function similarly to HECT-type E3 Ligases in which ubiquitin is transferred to their substrate from a cysteine residue in the E3 (Rohde et al., 2007; Singer et al., 2008; Zhu et al., 2008; Keszei et al., 2014; Takagi et al., 2016). Salmonella SspH1 targets human protein kinase N1 (PKN1) for ubiquitination, and the E3 ligase-substrate interaction is mediated by the N-terminal LRR domain of SspH1 and the Homology Region 1b (HR1b) domain of PKN1(Haraga and Miller, 2006; Keszei et al., 2014). The structural basis of SspH1-PKN1 interaction is well-characterized, and a short HR1b domain can be ubiquitinated *in vitro* by the fragment of SspH1 containing only the LRR and NEL domains (LRR-NEL) (Keszei et al., 2014). Ludwicki *et. al.* (2019) fused the NEL domains of bacterial IpaH family members including *Shigella* IpaHs and SspH1 to the GFP-specific

fibronectin type III (FN3) monobody (GS2). The IpaH-GS2 chimeras were efficient in targeting a wide range of GFP-tagged mammalian substrates with different subcellular localizations (Ludwicki et al., 2019). This suggests remarkable modularity and plasticity for creating synthetic degron systems based on bacterial NELs.

Targeting POIs for degradation by hijacking the endogenous ubiquitin-proteasome system has been exploited by cell biologists, with very few examples in plants (Nishimura et al., 2009; Natsume and Kanemaki, 2017; Yesbolatova et al., 2020; Phanindhar and Mishra, 2023). Most systems utilize a ligand and ligand-binding domains to recruit the target protein to an E3 ligase, while others take advantage of instability domains, or degrons, to trigger protein degradation. Several ligand-based systems take advantage of the plant hormones auxin and jasmonate and cognate receptors TRANSPORT INHIBITOR RESPONSE1 (TIR1) and CORONATINE INSENSITIVE1 (COI). For example, the auxin-induced degron (AID) system uses the AUXIN/INDOLE-3-ACETIC ACID (IAA17) degron sequence to recruit a target protein for ubiquitination by an heterologous SCF-TIR1 E3 ligase (Nishimura et al., 2009). IAA or NAA treatment results in depletion of POIs within 30-60 minutes (Nishimura et al., 2009). Subsequent improvements and applications of the AID system for rapid and tight inactivation of POIs have been documented in multiple organisms including *Plasmodium falciparum*, *Toxoplasma gondii*, yeast, human and mouse (Kreidenweiss et al., 2013; Morawska and Ulrich, 2013; Tanaka et al., 2015; Natsume et al., 2016; Brown et al., 2017; Papagiannakis et al., 2017; Brown et al., 2018; Miura et al., 2018; Brocas et al., 2019; Li et al., 2019; Mendoza-Ochoa et al., 2019; Ng et al., 2019; Sathyan et al., 2019; Yesbolatova et al., 2019). Similarly, the pJAZ degron system is based on the AtCOI1 receptor and a 23-AA AtJAZ1 (JASMONATE ZIM-DOMAIN1) degron sequence to induced target degradation by coronatine, the bacterial analog of jasmonic acid isoleucine (Brosh et al., 2016). Other systems take advantage of anti-GFP nanobodies to recruit GFP protein fusions to E3 ligases. The deGradFP system uses the anti-GFP nanobody VhhGFP4 fused to the F-box domain of Drosophila F-box protein Slimb (NSlmb) to direct GFP fusion

proteins to the endogenous mammalian SCF E3 ligase complex (Saerens et al., 2005; Rothbauer et al., 2006; Caussinus et al., 2011). Replacement of the F-box domain of the deGradFP allows the efficient depletion of target proteins in nematodes and zebrafish, and temporal and spatial removal of POIs can be achieved with tissue-specific and inducible promoters (Shin et al., 2015; Wang et al., 2017; Yamaguchi et al., 2019). Combinations of an AID system with the nanobody-mediated recruitment of targets makes very robust inducible degrons in human cells and zebrafish (Daniel et al., 2018). The proteolysis-targeting chimera (PROTAC) is based on a small bifunctional chemical probe that binds specifically to the POI and to an E3 ligase. Binding of the PROTAC to the POI results in recruitment of the Skp1-Cullin-F-box (SCF) E3 ligase complex, POI ubiquitination and subsequent degradation (Sakamoto et al., 2001). Although this technology has now matured and showed its great values in drug discovery and clinic therapies of diseases (Lai and Crews, 2017; Bushweller, 2019; Burslem and Crews, 2020; Liu et al., 2020), it is not applicable for POIs for which a specific ligand has not been identified. To overcome this shortcoming, Portnoff et al. (2014) developed bifunctional protein chimeras that combine E3 ligase activity with a designer binding protein. Here, the E3 ligase domain of Hsc70 interacting protein (CHIP) is fused to either an antibody single-chain variable fragment (scFv) or a fibronectin type III domain monobody, both of which target their respective antigens with high specificity and affinity (Paul and Ghosh, 2014; Portnoff et al., 2014). Depletion of different POIs can be achieved by domain swapping within the ubiquitin body sequence with the customized binding protein or E3 ligase (Shin et al., 2015; Fulcher et al., 2016; Fulcher et al., 2017; Kanner et al., 2017). Temperature-sensitive degrons take advantage of the N-end rule (also known as N-degron) pathway (Bachmair et al., 1986; Varshavsky, 1991; Gibbs et al., 2014; Varshavsky, 2017, 2019) to conditionally inactivate yeast target proteins at 37°C via the ubiquitin-proteasome (Dohmen et al., 1994; Kanemaki et al., 2003; Rajagopalan et al., 2004; Sanchez-Diaz et al., 2004; Dohmen and Varshavsky, 2005; Sanchez-Diaz et al., 2008; Kearsey and Gregan, 2009). These systems, however, have limited use in animals and plants that are sensitive to the high

temperature treatment required for POI degradation.

Many cellular processes in plant cells are extremely dynamic (Szymanski et al., 2018). Capturing the dynamics of these processes by functional studies of POIs require perturbation systems with high-temporal resolution. In addition, disruption of essential proteins in plants is hampered by the lethality of loss-of-function mutants. Reliable systems for inducible protein depletion to test the function of essential proteins in short time scales and to capture dynamic events, however, are not available in plants. The deGradFP fusion was expressed in tobacco (*Nicotiana tabacum*) and no GFP was detected when co-expressed with the NSlmb-VHHGFP4 fusion (Baudisch et al., 2018) but this fusion was not effective at degrading a CENTROMERIC HISTONE H3 (CENH3) fused to EYFP (Sorge et al., 2021). This fusion, however, was shown to induce haploids in plants that degrade CENH-EYFP (Sorge et al., 2021). None of these examples are inducible. An ethanol-inducible system was used in Arabidopsis (*Arabidopsis thaliana*) meristems to degrade WUS-GFP with deGradFP (Ma et al., 2019). So far, the only inducible protein depletion system in stably-transformed plants, an lt-degron, required incubations at 13°C and 27-29°C to either maintain the resting state or activate protein degradation, respectively (Faden et al., 2016). These temperatures are not optimal for plant growth, and are likely to induce stress responses in Arabidopsis. To fill this gap, we developed a chemical-inducible degron system for plant protein depletion based on the E3 ligase activity of the LRR-NEL domain of *Salmonella* SspH1 and its interaction with the HR1b domain of human PKN1 (Keszei et al., 2014) . In this system, named E3-DART, the expression of the E3 ligase module was controlled by a well-established dexamethasone-inducible system (Doumane et al., 2021) and the HR1b domain was used as a degron to instill instability of POIs in the presence of the E3 Ligase. This system allows rapid degradation of target proteins in response to dexamethasone (DEX).

## Results

**Rationale on plant degron system design based on SspH1**

E3-DART combines the E3 catalytic activity of Salmonella SspH1 and the cognate SspH1-interacting domain of its human target PKN1 (Fig 1A). SspH1 is a Novel E3 Ligase (NEL) that contains a Leucine-Rich Repeat (LRR) domain for PKN1 recruitment and an NEL catalytic domain for ubiquitination (Keszei et al., 2014). Specifically, the LRR domain from SspH1 binds to the Homology Region 1b (HR1b) domain of PKN1. The HR1b-binding surface in the LRR domain is comprised of a concave face from 10 parallel β-sheets, and the specificity of this interaction results from 13 (out of 15) unique contact residues in the LRR which are not present in closely-related NEL domain proteins (Keszei et al., 2014). This and the fact that SspH1 evolved to target a human protein, suggest high levels of specificity, and imply that LRR is unlikely to recognize an endogenous protein in plants. Remarkably, the HR1b domain can be ubiquitinated *in vitro* by a fragment of SspH1 containing only the LRR and NEL domains (LRR-NEL) (Keszei et al., 2014). Therefore, in this context, the HR1b domain of PKN1 and the LRR-NEL fragment of SspH1 are necessary and sufficient for PKN1 ubiquitination (Keszei et al., 2014), and excellent candidates to develop a synthetic protein degron in plants.

The E3-DART design harnesses the E3 catalytic activity of the SspH1$^{\text{LRR-NEL}}$ fragment to target ubiquitination of a POI fused to the HR1b domain (Fig. 1A). To test the functionality of each module in plants, we generated tagged protein fusions for transient expression. The LRR-NEL fragment was fused to a hemagglutinin (HA) tag and the mCherry fluorescent protein at the N and C-termini, respectively, to generate a HA-LRR-NEL-mCherry E3 ligase chimera (*here in* referred to as E3-DART, Fig. 1B). The HR1b domain was fused to GFP for visualization of target degradation. Given that a longer fragment containing HR1a and HR1b resulted in larger ubiquitinated conjugates *in vitro* (Keszei et al., 2014), we also tested HR1b in combination with HR1a in GFP fusions. Mutations in C492, which is part of the NEL domain catalytic site, renders SspH1 inactive (Keszei et al., 2014) , and alanine (A) substitutions in R181 and R185 of PKN1 abolish its interaction with SspH1 (Keszei et al., 2014). The corresponding mutations were

generated to be used as negative controls including HA-LRR-NEL$^{C492A}$-mCherry (*here in* referred to as E3-DART$^{C492A}$) and non-interacting HR1b$^{R181/185A}$-GFP (Fig 1B). These constructs were expressed independently in transient assays in *Nicotiana benthamiana* under the control of an *UBIQUITIN10* promoter to test whether they could be readily detected in plant cells. E3-DART and both C- and N-terminal GFP fusions of HR1b accumulated in the cytosol and nucleus in a similar pattern as free GFP (Fig. 1C). Degron fusions that included both HR1a and HR1b resulted in punctate patterns in the cytosol indicating possible protein aggregation (Fig. 1C) and these constructs were not used further.

**E3-DART targets HR1b-GFP for degradation in plants**

We first tested whether the E3-DART interacted with HR1b-GFP in plants using co-immunoprecipitation (co-IP) analysis with crude extracts from infiltrated leaves. Since the longevity of the HR1b fusions were likely dependent on the presence of E3-DART, leaves were transformed with each construct separately and protein extracts were mixed to test for protein-protein interactions. As shown in Fig. 2A, HR1b-GFP interacts with the catalytically active and inactive forms of E3-DART (HA-LRR-NEL-mCherry fusion and HA-LRR-NEL$^{C492A}$-mCherry). In contrast, the HR1b$^{R181/185A}$ mutations abolished the LRR-HR1b binding, which supports the specificity of the interaction between HR1b-GFP and E3-DART in plant cells. Confocal microscopy was used to test whether expression of both HR1b-GFP and E3-DART would result in protein degradation. Co-expression of E3-DART and HR1b-GFP resulted in very low accumulation of HR1b-GFP in *Nicotiana benthamiana* epidermal cells (Fig. 2B). In contrast, HR1b$^{R181/185A}$-GFP fluorescence was detected when co-expressed with the active E3-DART indicating that degradation of HR1b-GFP is dependent on its interaction with the LRR domain *in planta*. Strong HR1b-GFP fluorescence was also detected when co-expressed with the catalytically inactive E3-DART$^{C492A}$, indicating that the accumulation of HR1b-GFP is sensitive

to NEL E3 enzyme activity (Fig. 2B). Immunoblot analysis was used to confirm that the levels of fluorescence from HR1b-GFP reflected protein abundance instead of improper GFP folding. Similar to the confocal analysis, when E3-DART was co-expressed with either HR1b-GFP or HR1b$^{R181/185A}$-GFP, only the mutant HR1b$^{R181/185A}$-GFP was detected by immunoblotting. HR1b-GFP was again detected when co-expressed with the catalytically inactive E3-DART$^{C492A}$. RT-PCR experiments showed that HR1b-GFP and HR1b$^{R181/185A}$-GFP constructs were transcribed efficiently (Fig. 2C), which supported the conclusion that E3-DART targets HR1b-GFP for degradation in co-expression experiments. Co-expression with a Flag-RFP-Myc construct was used as an internal expression control (Fig. 2C). To gain further evidence that E3-DART targets HR1b-GFP *in planta*, a dose-dependent expression analysis (Yu et al., 2020) was performed by co-infiltration of equal amounts of *Agrobacterium tumefaciens* carrying the *HR1b-GFP* construct with increasing amounts of *A. tumefaciens* carrying the *HA-LRR-NEL-mCherry* construct. Samples were collected 3 days after infiltration and subjected to immunoblot analysis. As shown in Fig. 2D, increased expression of E3-DART resulted in reduced accumulation of HR1b-GFP. Collectively, these results indicate that HR1b-GFP can be efficiently targeted for degradation by E3-DART in *N. benthamiana* transient assays.

We next tested whether E3-DART targets proteins for degradation via the ubiquitin-proteasome pathway. The 26S-proteasome inhibitor MG132 was applied to *N. benthamiana* leaves previously co-infiltrated with *Agrobacterium* carrying *E3-DART* and *HR1b-GFP* constructs. MG132 substantially attenuated the depletion of HR1b-GFP (Fig. 2E) suggesting that the E3-DART targets HR1b-GFP for degradation via the 26S proteasome. Moreover, high-molecular-weight ubiquitin conjugates could be detected in HR1b-GFP extracts after immunoprecipitation with anti-GFP antibody, and MG132 treatment enhanced the accumulation of these conjugates (Fig. 2F). Taken together, these results suggested that E3-DART can hijack the plant endogenous ubiquitin-26S proteasome pathway for targeted degradation of HR1b-GFP.

To test whether the degron system works efficiently in Arabidopsis, we generated stable-

expression constructs of HR1b-GFP, HR1b$^{R181/185A}$-GFP, E3-DART and the catalytically inactive E3-DART$^{C492A}$ under the control of the Arabidopsis *UBIQUITIN 10* (*UBQ10*) promoter. Constructs were transformed sequentially into wild-type Col-0 plants such that homozygous lines with similar levels of HR1b-GFP or HR1b$^{R181/185A}$-GFP fluorescence were later transformed with E3-DART or E3-DART$^{C492A}$ cassettes. Roots from $T_2$ seedlings from at least 8 different $T_1$ lines per combination were tested by fluorescence microscopy. Like the transient assays, very weak fluorescence was detected from HR1b-GFP in roots that expressed functional E3-DART (Fig. 2G). Strong fluorescence was detected from lines carrying the HR1b$^{R181/185A}$-GFP mutant degron together with E3-DART or in roots that expressed HR1b-GFP and the catalytically inactive E3-DART$^{C492A}$ (Fig. 2G). Immunoblot experiments with these transgenic lines further demonstrated the efficient depletion of HR1b-GFP when the catalytically active enzyme is present but not with the E3-DART$^{C492A}$ mutant (Fig. 2H). Taken together, these results suggested that the *Salmonella* SspH1$^{LRR-NEL}$ fragment can target HR1b-GFP for degradation in Arabidopsis and the degradation is dependent on the catalytic activity of the E3-DART ligase and the HR1b interaction.

**E3-DART targets endogenous proteins in the nucleus and cytosol**

So far, we have shown that E3-DART enables the degradation of the HR1b-GFP, which diffuses freely in the nucleus and cytoplasm in *N. benthamiana*. To test whether E3-DART can target HR1b fusions to endogenous proteins that localize to the nucleus or the cytosol, we fused HR1b or the mutant degron HR1b$^{R181/185A}$ to the Arabidopsis cytoplasmic protein VACUOLAR PROTEIN SORTING 34 (VPS34) (Lee et al., 2008) and the transcription factor ROOT HAIR DEFECTIVE6 (RHD6) (Menand et al., 2007). *VPS34* encodes Phosphatidylinositol 3-Kinase, a protein of 814 amino acids that functions in the cytosol (Lee et al., 2008), while RHD6 is a small transcription factor that is localized to the nucleus. These fusions were transiently co-expressed with E3-DART in *N. benthamiana*. Confocal imaging showed that both the VPS34-GFP-HR1b and HR1b-GFP-RHD6 fusions were efficiently degraded via the E3-DART system. Using the

catalytically-inactive enzyme (E3-DART$^{C492A}$) or the mutant HR1b$^{R181/185A}$ degron resulted in long-lived target proteins as expected (Fig. 3A-C), and these results were validated by immunoblot analysis (Fig. 3B, D). The effective degradation of VPS34-GFP-HR1b indicates that HR1b can target large proteins for degradation. We then fused a triple nuclear-localization sequence (NLS) to the N-terminus of E3-DART and co-expressed it with HR1b-GFP-RHD6 in *N. benthamiana* to test its activity in the nucleus. As expected, the HR1b-GFP-RHD6 was again degraded by the nuclear-localized E3-DART (3×NLS-HA-LRR-NEL-mCherry) in a similar fashion (Fig. 3E-F). These results indicate that the E3-DART system can be used to efficiently target endogenous proteins of different sizes in plants, either in the nucleus or cytosol.

**Anti-GFP Nanobodies are not compatible with E3-DART for target recruitment**

A targeted degron system that recruits protein targets by direct interaction with GFP would be immediately applicable to any plant GFP-fusion line with a single cross. We attempted such a strategy by fusing anti-GFP nanobodies (nb) (Kubala et al., 2010; Caussinus et al., 2011; Daniel et al., 2018) to E3-DART. The LRR domain was still included in this design given the strong autocatalytic activity of the NEL domain which is inhibited by the LRR domain (Chou et al., 2012). Two nanobodies were tested, vhhGFP4 (Kubala et al., 2010; Caussinus et al., 2011; Daniel et al., 2018) which has been used effectively in combination with E3 ligases in mammalian and drosophila cells (Caussinus et al., 2011) and GS2 (Koide et al., 2012) which was also effective in mammalian cells in fusions with two NEL E3 ligases including SspH1 (GS2: Ludwicki et al., 2019). Unfortunately, neither of the two nanobodies were sufficient to target GFP as transient co-expression of a vhhGFP4-LRR-NEL-mCherry fusion with free GFP resulted in strong GFP fluorescence (Fig. S1A). This result could not be explained by reduced E3 ligase activity in the new fusion because this E3 ligase still targets the HR1b-GFP fusion for degradation, probably due to the LRR-HR1b interaction. Moreover, co-expression of the vhhGFP4-LRR-NEL-mCherry fusion does not affect the accumulation of the mutant degron HR1b$^{R181/185A}$ -GFP fusion. Similar results were observed when the GS2 nanobody was fused to

the LRR-NEL fragment (Fig. S1B). These results suggest that the nanobody-GFP interaction may be precluded by steric hindrance of the LRR-NEL fusion at least in the current designs.

**Establishment of an inducible plant protein degron system**

The plant degron system is effective in degrading HR1b-tagged proteins with different subcellular localizations, but making it inducible is important for its functionality. We first attempted to establish the inducibility of target protein degradation by harnessing the mode of action of steroid hormone receptors which have been well-exploited in plants (Aoyama and Chua, 1997; Zuo et al., 2000; Borghi, 2010; Doumane et al., 2021; Samalova and Moore, 2021). In the absence of the hormone, ligand binding domains (LBDs) of steroid hormone receptors are bound by a cytosolic inhibitory complex composed of heat shock protein 90 (HSP90), HSP70, P23 and other components (Pratt and Toft, 1997; Schopf et al., 2017). This large complex dissociates upon hormone binding to the LBDs, which makes these domains chemically inducible, and therefore, LBDs have been used as regulators *in cis* of other protein domains including nuclear targeting signals and enzymatic catalytic sites (Picard, 1993; Pratt and Toft, 1997; Kawai-Yamada et al., 2001; Boe et al., 2008; Nguyen et al., 2022). We initially tested whether steroid hormone binding domains could be used to control the interaction between E3-DART and HR1b-GFP. We fused the ligand binding domain of the glucocorticoid receptor (GRBD) to the LRR-NEL-mCherry fusion and tested whether DEX treatment could be used to control degradation of HR1b-GFP. As shown in Fig. S2, the HR1b-GFP was degraded by the GRBD-LRR-NEL-mCherry fusion in the absence of DEX, suggesting that HR1b binding to the LRR domain is not precluded by the HSP inhibitory complex that binds to GRBD. An additional GRBD domain at the C terminus of the E3 ligase was also insufficient (Fig. S2), and similar fusions between the human estrogen receptor ligand binding domain hERBD (Zuo et al., 2000) and the NEL E3 ligase were also insufficient to block the targeting of HR1b-GFP (Fig. S3). We conclude that ligand binding domains of steroid hormone receptors are not suitable for controlling the inducibility of the plant degron at the post-translational level.

LBDs from the human estrogen receptor (ER) which binds estradiol (Zuo et al., 2000) and the rat glucocorticoid receptor (GR) which binds dexamethasone (Aoyama and Chua, 1997), have been used successfully in plants for controlled gene expression (Zuo and Chua, 2000). To realize the inducible degradation of HR1b-fusions, we developed a transcriptionally-induced degron system by taking advantage of the well-known GAL4-VP16-GR (GVG) transcription induction system (Aoyama and Chua, 1997; Doumane et al., 2021) to control the expression of E3-DART with dexamethasone (Fig. 4A). In this system, expression of E3-DART is driven by a promoter containing six copies of the GAL4 upstream activating sequences (6×UAS) which are bound by the GAL4 DNA binding domain of the GVG chimeric transcription factor. DEX treatment triggers GVG nuclear targeting and transcription of the downstream gene encoding the NEL E3 ligase (Fig. 4A). The GVG-based *HA-LRR-NEL-mCherry* inducible expression cassette and the *HR1b-GFP* expression cassette were cloned in tandem into the same vector and then transiently expressed in *N. benthamiana*. Time-course DEX treatments with the agroinfiltrated *N. benthamiana* leaves were used to assess the induction of E3-DART and the longevity of HR1b targets by immunoblotting (Fig. 4B). RT-PCR from infiltrated tissues was used to confirm similar levels of expression of the GVG transcription factor and the HR1b fusions in all samples. When the inducible E3-DART was co-expressed with HR1b-GFP (lanes 1-3), the E3 ligase fusion was detected 3 and 6 h after DEX treatment and this increase was concomitant with a decrease in HR1b-GFP levels. This decrease suggested induced HR1b-GFP degradation as the result of DEX treatment. This conclusion was supported by the insensitivity of the mutant $HR1b^{R181/185A}$-GFP fusion at the same time points (lanes 4-6). When the inducible version of the inactive E3-$DART^{C492A}$ was co-expressed with HR1b-GFP, there was no substantial change in GFP abundance even after 6 h of DEX treatment (lanes 7-9). These results demonstrate that our DEX-induced plant degron allows the chemically-controlled depletion of HR1b-fusions by E3-DART and this is dependent on the LRR-HR1b interaction.

**Rapid degradation of plant proteins by a DEX-inducible E3-DART**

We then wanted to test the efficiency of the inducible degron when HR1b-GFP is fused to native proteins in different locations. To this end, the expression cassettes for *VPS34-GFP-HR1b* and *HR1b-GFP-RHD6* fusions were cloned downstream of the inducible *HA-LRR-NEL-mCherry* expression cassettes in the same plasmid. DEX treatment of *N. benthamiana* leaves agroinfiltrated with constructs containing inducible E3-DARTs and the *VPS34-GFP-HR1b* construct showed accumulation of E3-DART at 3 and 6 h with concomitant and almost complete depletion of the VPS34 fusion (Fig. 5A, lanes 1-3). Again, this is most likely due to protein degradation because the VPS34 fused to the mutant HR1b$^{R181/185A}$ fragment was unaffected by DEX treatment even in the presence of E3-DART (lanes 4-6). Moreover, the VPS34-GFP-HR1b was detected when the catalytically inactive E3-DART$^{C492A}$ was induced by DEX (lanes 7-9). Faster inducible degradation was detected with the nuclear fusion protein HR1b-GFP-RHD6 (Fig. 5B). When this protein was expressed together with the inducible E3-DART, 3 h of DEX treatment were sufficient to deplete the HR1b-GFP-RHD6 protein (lanes 1-2), demonstrating the rapid and robust degradation of HR1b-GFP-tagged proteins by the inducible degron system. By contrast, neither the mutant degron fragment (HR1b$^{R181/185A}$) nor the catalytically inactive E3-DART$^{C492A}$ resulted in degradation of RHD6 fusion targets (lanes 3-7). These results combined demonstrate the feasibility of the DEX-inducible E3-DART system to target plant protein degradation with strong specificity and with proteins of varying sizes that function either in the nucleus or cytosol.

## Discussion

Here we showed that a synthetic bacterial effector E3 ligase, SspH1$^{LRR-NEL}$, can be used to target chimeric proteins bearing the cognate HR1b degron sequence in plants. This interaction is specific and dependent on the E3 ligase catalytic activity as well as the HR1b-LRR interaction. Therefore, this system provides a robust mode of protein abundance control in plant cells. The

HR1b domain was functional at either N or C termini of target proteins and therefore the system provides flexibility for targets that have strict requirements for synthetic fusion constructs. Keszei et al (2014) showed that the HR1b domain alone can be ubiquitinated by the LRR-NEL domain of SspH1, so most likely HR1b contains sufficient ubiquitination sites for most protein fusions. And since the HR1b-LRR interaction is a requirement for E3-DART functionality, protein-fusion topologies that prevent HR1b binding to the LRR may not operate as expected. As SspH1 functions as a HECT E3 ligase, a Cys residue that receives a charged ubiquitin before transfer to the target is required for ubiquitination. Moreover, two amino acid residues, R181 and R185 within the HR1b domain in SspH1, were previously shown to be required for HR1b-LRR interactions (Keszei et al., 2014) . Therefore, mutant versions of the NEL-LRR fragment and HR1b can be used as precise negative controls in cell biology experiments to demonstrate specificity of observed phenotypes. We have not observed morphological or developmental defects in over 50 Arabidopsis plants expressing E3-DART, which suggest minimal off-target effects on endogenous proteins. Given its robust catalytic activity, it is likely that the enzyme folds correctly at the temperatures in which plants are grown (22°C) which is more than 15 degrees lower than its normal temperature. Overall, the NEL-LRR fragment and the HR1b degron represent a functional protein degron system for plants.

One advantage of the E3-DART system compared to other degron designs such as the degradFP (Caussinus et al., 2011) is that a single protein (LRR-NEL) provides target recruitment and E3 ligase functions. This means that the E3 ligase does not compete with native proteins for establishment of the E3 ligase complex. Given that SspH1 is a bacterial effector from a human pathogen capable of recruiting charged E2 enzymes from mammalian cells, we hypothesized that it could recruit plant E2s efficiently. This was demonstrated by the effective degradation of HR1b fusions in *N. benthamiana* and Arabidopsis.

The current version of this degron can be induced at the transcriptional level by dexamethasone using the GVG system in *N. benthamiana* (Aoyama and Chua, 1997; Doumane

et al., 2021). Transcriptional induction in this system is well documented in plants and the earliest detection of proteins after induction range from 3 h to several days across plant species (Kawai-Yamada et al., 2001; Tang et al., 2004; Park et al., 2012; Tsuda et al., 2012; Li et al., 2016; Nguyen et al., 2022). While this timeline is not ideal for fast post-translational control of target protein function, it allowed us to generate robust proof-of-concept datasets that showed the effectiveness and specificity of the degron. There is only one other example of inducible protein degradation in plants which used the deGradFP in Arabidopsis but protein degradation was only observed 24 h after induction (Ma et al., 2019) . Adoption of E3-DART requires a robust DEX-inducible cassette that is effective in the plant of study and in some cases as a stable transgenic line. The current version described here, where GVG is driven by the Arabidopsis *pUBQ10* promoter, was highly efficient for E3-DART induction in *N. benthamiana*, but experiments in Arabidopsis transgenic plants showed low accumulation of the GVG transcription factor and inconsistent transcriptional induction of E3-DART. Future experiments beyond the scope of this work will entail optimization of GVG expression with different promoters or transactivation systems. Consistent cellular accumulation of DEX is also needed, and we found DEX treatment via the petiole to be highly reproducible. While this method may not be suitable for some applications, syringe or vacuum infiltration of DEX into leaves appear to induce cellular damage as we observed rapid decreases in GFP fluorescence even in the absence of any E3-DART ligase. Potential applications of E3-DART also include expression of the E3 ligase under control of stress-inducible or cell-type specific promoters for temporal and spatial depletion of protein targets.

Binding domains of steroid hormone receptors have been used successfully to control the nuclear accumulation of transcription factors (Kodaira et al., 2011; Marques-Bueno et al., 2016; Kim et al., 2022), genome editing tools such as Cas9 (Nguyen et al., 2016) and the activity of kinases and other signaling proteins (Boe et al., 2008; Pai et al., 2012) . This control is driven by steric interference of functional protein domains by inhibitory HSP90 complexes that bind to the

binding domain in the absence of steroid hormone. Except for transcription factor fusions (Aoyama and Chua, 1997; Kodaira et al., 2011; Kim et al., 2022 ), no other protein functions have been controlled by HBDs in plants. An early version of our design attempted to control the E3 ligase activity by steric inhibition of LRR interactions with GRDB or ERDB domains, but neither of these were successful, regardless of the location in the protein or even the number of steroid hormone binding domains. A possible interpretation of this result is that the affinity of the LRR domain for HR1b-containing targets is higher than that of GRDB or ERDB for the heat shock protein inhibitory complex. In this instance, binding and degradation of the target occurs faster than the association with HSP90. A similar result was found for a Wee1 protein fusion to the estradiol BD in which the protein retained activity in the absence of estradiol in yeast cells (Boe et al., 2008) .

Our design is still bi-partite in which every target needs to be fused to the HR1b domain to initiate ubiquitination. We attempted to make a more universal tool by using an anti-GFP nanobody for target recruitment, but these efforts were unsuccessful. There are several documented examples where GFP nanobodies are sufficient to recruit interacting partners including those where a nanobody is fused to an E3 ligase (Caussinus et al., 2011; Daniel et al., 2018; Prole and Taylor, 2019; Yamaguchi et al., 2019; Sorge et al., 2021). Noticeably, nanobody-based degrons such as mAID-VhhGFP and deGradFP show different rates of degradation of specific target proteins indicating that there may be context-dependent sequences that can affect their efficiency (Daniel et al., 2018). There are possible reasons why a nanobody was not sufficient to promote protein-protein interaction and target ubiquitination. For example, the nanobody fusions could be unavailable due to steric hindrance between the LRR domain and the nanobody. We tried to overcome this issue by placing a short GS linker between the two protein domains but such a linker may not have been sufficient.

## Materials and Methods

### Plant materials and growth conditions

*Nicotiana benthamiana* plants were grown in soil in the lab under a 16-h light/8-h dark photoperiod for 40 days and the abaxial epidermis of *N. benthamiana* leaves were infiltrated with bacteria suspensions as previously reported (Chen et al., 2008). Arabidopsis (*Arabidopsis thaliana*) plants were grown as previously described (Zheng et al., 2014), and the ecotype Columbia (Col-0) was used as wild-type control. All the Arabidopsis transformations were performed by floral dip (Clough and Bent, 1998) using *Agrobacterium tumefaciens* stain C58C1. Plants were transformed sequentially. First, Col-0 wild type plants were transformed with HR1b-GFP or HR1b$^{R181/185A}$-GFP cassettes and homozygous $T_2$ lines with similar levels of GFP fluorescence were identified by kanamycin selection and fluorescence microscopy. Homozygous $T_2$ lines from the same $T_1$ were then transformed with the HA-LRR-NEL-mCherry or HA-LRR-NEL$^{C492A}$-mCherry constructs and 10 independent $T_1$ lines were selected with Basta.

### Chemical treatments

Dexamethasone was purchased from SIGMA-Aldrich ( Cat. No. D1756) and dissolved in DMSO to make 100 mM stock. Leaves were excised below the petiole 72 h after agroinfiltration and immediately placed in a solution of 0.5 × Murashige and Skoog media (MS) containing 100 μM dexamethasone (DEX) for the time indicated.

### Plasmid construction

All plasmids used (Supplemental Dataset S1) were generated by the GoldenBraid system as previously described (Sarrion-Perdigones et al., 2013; Sarrion-Perdigones et al., 2014; Vazquez-Vilar et al., 2015) or obtained from other sources (pUPD2 entry clones listed in Supplemental Dataset S3). Several plasmids were deposited in Addgene (https://www.addgene.org/, Supplemental Datasets S2-S3). Each DNA fragment was domesticated using the GoldenBraid

domestication tool (https://gbcloning.upv.es/tools/domestication/) for either PCR amplification or gene synthesis before cloning into the entry vector pUPD2 (Supplemental Dataset S3). The coding sequences of the LRR-NEL fragment (E162-N700) of *Salmonella enterica* serovar Typhimurium effector protein SspH1, HR1a-1b domains (residues W13-P199) and HR1b domain (residues A122-P199) of human protein kinase N1 (PKN1) (Keszei et al., 2014) and the anti-GFP nanobodies vhhGFP4 Kless (lysine-less) (Caussinus et al., 2011; Daniel et al., 2018) and GS2 (Koide et al., 2012) were codon-optimized for expression in plants and synthesized (Integrated DNA Technologies) before cloning. The Flag-tagged ligand binding domain of rat glucocorticoid receptor (GRBD, residues E508-K795) was amplified from the pSW610-GR-LhG4_BD plasmid (Schurholz et al., 2018) by introducing the coding sequence of triple Flag tags into the forward primer while the Flag-tagged ligand binding domain of human estrogen receptor (hERBD, residues S292-V595) (Zuo et al., 2000) was synthesized.

Starting fragments were cloned into pUPD2 using *Bsm*BI and T4 DNA ligase to generate entry clones (Supplemental Dataset S3) for most constructs. Site-directed mutagenesis or nested PCR were used to generate pUPD2 entry plasmids containing mutant *LRR-NEL*$^{C492A}$ or *HR1b*$^{R181/185A}$ fragments. When used, a Gly/Ser linker (Luker et al., 2004) was introduced as part of either the forward primer or the reverse primer of a GBpart before cloning into pUPD2. To generate the DEX-inducible expression cassettes, the coding sequences of the GAL4-VP16-GR (GVG) (Aoyama and Chua, 1997; Doumane et al., 2021) was codon-optimized and synthesized with a N-terminal Myc tag and a C-terminal Hemagglutinin tag. The *tE9-6xUAS-35Smini* fragment was synthesized directly and all parts were cloned into pUPD2.

Details of expression constructs used in each figure are listed in Supplemental Dataset S1. All transcriptional units (or transgenes) were cloned first in the pGDB3_alpha1 vector and their design is listed in Supplemental Dataset S2. pGDB3_alpha1 plasmids were used for transient expression of a single transgene. GoldenBraid-based ligation reactions from alpha-level plasmids or above were used to combine two or three transcriptional units into pDGB3_omega1 or

pDGB3_alpha1, respectively. The basta resistance expression cassette was amplified from pTNos:BASTA:tNos plasmid (GB0023) and cloned into pDGB3_alpha2 with *Bsm*BI, creating $TNOS_{pro}$:BASTA plasmid. Each transcriptional unit in the alpha1-level degron plasmids above was then inserted into pDGB3_omega1 with basta resistance cassette from $Nos_{pro}$:BASTA plasmid, while the transcription unit of each target plasmid was assembled into pDGB3_omega1 with kanamycin resistance cassette from pEGB Pnos:NptII:Tnos (GB0184) plasmid. *Escherichia coli* strain DH5α was used for the construction and propagation of all plasmids, and all the generated alpha1-level and omega1-level plasmids were transformed into *Agrobacterium tumefaciens* stain C58C1.

**Co-immunoprecipitation Assay**

Immunoprecipitation assays were conducted with crude extracts from agroinfiltrated *N. benthamiana* leaves with overexpressed HA-LRR-NEL-mCherry, HA-LRR-$NEL^{C492A}$-mCherry, HR1b-GFP, $HR1b^{R181/185A}$-GFP and GFP. Total proteins were extracted 72 hours post-agroinfiltration with native buffer (50 mM Tris–MES pH8.0, 0.5 M sucrose, 1 mM $MgCl_2$, 10 mM EDTA, 5 mM DTT, and protease inhibitor cocktail cOmplete Mini tablets (11836170001; Sigma-Aldrich). The supernatants were collected for Co-IP assay after centrifugation at 18,000 × *g* at 4°C three times for 5 min each time. Next, 20 μL of each crude extract were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblotted with an anti-HA or anti-GFP antibody to compare the levels of HA-LRR-NEL-mCherry, HA-LRR-$NEL^{C492A}$-mCherry, HR1b-GFP, and $HR1b^{R181/185A}$ proteins. Equal amounts of each protein were then added to the Co-IP reaction by adjusting the volume of their crude extracts. HA-LRR-NEL-mCherry and HA-LRR-$NEL^{C492A}$-mCherry proteins were mixed with either HR1b-GFP or $HR1b^{R181/185A}$-GFP and incubated with 25 μL of the agarose-conjugated anti-GFP monoclonal antibody (D153-8; MBL) at 4°C for 4 h. After washing three times with phosphate buffered saline (PBS) buffer (pH 7.4), the bead-bound proteins were eluted in 40 μL 2× SDS loading

buffer and then 25 μL of the IP fractions were subjected to SDS-PAGE for immunoblotting analysis. HA-tagged proteins were detected with an anti-HA monoclonal antibody (H9658; Sigma-Aldrich) at a dilution of 1:10000 while the GFP-tagged proteins were detected with an anti-GFP polyclonal antibody (SAB4301138; Sigma-Aldrich) at a dilution of 1:3000. Membranes were probed with anti-mouse (NA931; Cytiva) or anti-rabbit (NA934; Cytiva) peroxidase-linked secondary antibodies at a dilution of 1:10000 for detecting of HA- and GFP-tagged proteins and signal was detected using Clarity™ Western ECL Substrate Kit (1705060; Bio-Rad) with a G:BOX Chemi XRQ gel documentation system.

**Protein degradation analysis**

For *in vivo* protein degradation assays in *N. benthamiana*, the omega1-level plasmids harboring different combinations of HA-tagged E3-DARTs, GFP-tagged targets and Flag-RFP-Myc overexpression cassettes were co-infiltrated into *N. benthamiana* leaves. Transgene cassettes were either encoded in separate plasmids or a single plasmid according to Supplemental Dataset S1. Samples were harvested 3 days after infiltration and total proteins were extracted and immunoblotted with monoclonal anti-GFP antibody (11814460001; Sigma-Aldrich) at 1:3000 dilution and anti-HA antibody (H9658; Sigma-Aldrich) at 1:10000 dilution. The Flag-RFP-Myc was used as the internal expression control and detected with monoclonal anti-Myc (M4439; Sigma-Aldrich) at 1:5000 dilution. Transcript accumulation of the GFP-tagged HR1b and HR1b$^{R181/185A}$ fusions were analyzed by RT-PCR. For analysis of inducible degradation of target proteins, *N. benthamiana* leaves were transiently transformed with plasmids carrying each combination of the Myc-GVG-HA expression cassette, the inducible E3-DARTs and GFP-tagged proteins. Infiltrated leaves were then treated with 100 μM DEX and collected at different time points for degradation analysis. The Myc-GVG-HA was used as internal control.

For analysis of protein accumulation in Arabidopsis, 10-day-old seedlings of mCherry- and GFP-tagged double marker lines grown on 0.5 x MS medium were collected for protein

extraction with native extraction buffer. Tagged protein were detected with either anti-GFP or anti-HA monoclonal antibodies (as described above), and endogenous Actin was detected with rabbit anti-Actin polyclonal antibody (ab197345; Abcam) at 1:5000 dilution.

Secondary antibodies for western blots were anti-mouse (NA931; Cytiva) or anti-rabbit (NA934; Cytiva) peroxidase-linked secondary antibodies at a dilution of 1:10000 for detecting of HA- and GFP-tagged proteins. Chemiluminescence was detected using Clarity™ Western ECL Substrate Kit (1705060; Bio-Rad) with a G:BOX Chemi XRQ gel documentation system.

**In Vivo Ubiquitination Assay**

For *in vivo* ubiquitination analysis of HR1b-GFP, an *Agrobacterium* strain carrying the HA-LRR-NEL-mCherry E3 ligase cassette was co-infiltrated into *N. benthamiana* leaves with an *Agrobacterium* strain carrying HR1b-GFP at an O.D. 600 ratio of 1:8. Equal weights of leaf samples were collected 72 h after agroinfiltration and 100 μM MG132 dissolved in 10 mM $MgCl_2$ was infiltrated into the previously infiltrated leaves 12 h before sample collection. Total proteins were extracted using native buffer supplemented with 100 μM MG132. Next, 25 μL of GFP-Trap® Magnetic Agarose beads (gtma-20; Chromo Tek) was added into 1.5 mL of total protein extracts and incubated at 4°C for 4 h with gentle rotation. The beads were washed four times with PBS buffer containing 100 μM MG132 and eluted with 40 μL 2× SDS loading buffer, boiled for 5 min at 95°C and then subjected to immunoblotting. The polyubiquitination level was detected using mouse anti-ubiquitin monoclonal antibody (eBioP4D1 (P4D1); 14-6078-80; Thermofisher) at a 1:1000 dilution and anti-mouse IgG HRP-linked secondary antibody (NA931; Cytiva) at a 1:10000 dilution. Signal was detected with Clarity™ Western ECL Substrate Kit (1705060; Bio-Rad) with the ChemiDoc MP Imaging System.

**Microscopy**

Epifluorescence microscopy was carried out in a Leica DM5000 compound microscope equipped with a GFP filter cube (EX 470/40 EM 525/50) and TX2 filter cube (Ex 560/40 Em

BP645/75) using a HCX PL APO 20X/0.70 objective. Images were captured using a Leica DFC365 FX monochrome digital camera. Confocal microscopy was done in either a Zeiss LSM710 or Zeiss LSM980 confocal microscope using a Plan-Apochromat 20x/0.8 M27 objective at the Cellular and Molecular Imaging Facility at North Carolina State University. The excitation/emission spectra for detection of GFP and mCherry were 488/482-570 nm and 561/570-685 nm, respectively. Laser power was 1.5-2.0% for the 288 nm laser and 0.6-2.4% for the 561 nm laser.

**Accession Numbers**

Sequence data for all genes examined in this study can be found in the GenBank/EMBL libraries (https://www.ncbi.nlm.nih.gov/) or the Arabidopsis Information Resource (https://www.arabidopsis.org) under the following accession numbers: SspH1 (ACY87967), PKN1 (NP_002732), VPS34 (AT1G60490) and RHD6 (AT1G66470).

**Supplemental Data**

**Supplemental Figure S1.** Anti-GFP nanobodies are insufficient to drive protein degradation via LRR-NEL.

**Supplemental Figure S2.** The GRBD domain is not sufficient to inhibit the LRR-NEL fragment in the absence of dexamethasone.

**Supplemental Figure S3.** The hERBD is insufficient to inhibit the E3 ligase activity of the LRR-NEL fragment in the absence of β-estradiol.

**Supplemental Data Set S1**. Plasmids used in each figure.

**Supplemental Data Set S2**. Alpha level plasmids for all designs used.

**Supplemental Data Set S3**. List of original entry clones in pUPD2 that were used as starting point of domestication and GoldenBraid cloning.

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**Author Contributions**

M.R.P. conceived the original design, acquired funding, analyzed data and-co-wrote the paper. L.H. made degron design modifications, performed research, analyzed data and co-wrote the paper.

*Conflict of interest statement*. M.R.P. and L.H. have filed a patent application based on results reported in this paper.

**Figure Legends**

**Figure 1. Design of the E3-targeted degradation of plant proteins (E3-DART) system.**

(A) The E3-DART system is based on the interaction between the *Salmonella* secreted protein H1 (SspH1) and the human protein kinase N1 (PKN1). The interaction between the HR1b domain of PKN1 and the Leucine-Rich repeat (LRR) domain of SspH1 results in protein binding and ubiquitination by the Novel E3 Ligase (NEL) domain. The HR1b domain is fused to a target protein to recruit the truncated LRR-NEL fragment and induce ubiquitination and target protein degradation.

(B) Protein chimeras used in this study. The LRR-NEL fragment was fused to mCherry and the HR1b domain was fused to GFP. A C492A substitution in the NEL domain corresponds to a catalytically inactive control and the R181A and R185A (R181/185A) mutations in HR1b correspond to non-interacting controls. HA, Hemagglutinin tag; LRR, Leucin-rich repeat; NEL, Novel E3 Ligase, GFP, green fluorescent protein.

(C) Transient expression of degron components in *Nicotiana benthamiana*. Infiltrated leaves were imaged by epifluorescence (LRR-NEL-mCh) or confocal microscopy (GFP fusions) to detect mCherry or GFP signal. The merged image with brightfield acquisition is included. All GFP confocal images were acquired under identical microscope settings. Scale: 40 μm. mCh, mCherry fluorescent protein.

**Figure 2. HA-LRR-NEL-mCherry targets HR1b-GFP for efficient degradation in plants.**

(A) Co-immunoprecipitation (Co-IP) assay using crude extracts from agroinfiltrated *N. benthamiana* leaves confirmed the interaction between HR1b-GFP and both HA-LRR-NEL-mCherry (E3-DART) and HA-LRR-NEL$^{C492A}$-mCherry. Protein extracts from independent transformations were mixed as indicated and binding proteins were immunoprecipitated with anti-GFP and detected by immunoblot.

(B) Confocal microcopy of leaves transiently co-transformed with HR1b-GFP or HR1b$^{R181/185A}$-GFP and HA-LRR-NEL-mCherry or HA-LRR-NEL$^{C492A}$-mCherry. Scale bars: 100 μm. All images were taken with identical microscope settings.

(C) E3-DART targets HR1b-GFP for degradation in plants. Equal amounts of Agrobacterium strains carrying each indicated construct were co-infiltrated in *N. benthamiana* leaves and recombinant proteins were detected by immunoblot.

(D) HR1b stability is dependent on E3-DART. Equal amounts of Agrobacterium strains carrying the HR1b-GFP construct were co-infiltrated with increasing amounts Agrobacteria carrying the

644 HA-LRR-NEL-mCherry (E3-DART) construct. Expressed proteins were detected by
645 immunoblot. Co-transformation with Flag-RFP-Myc was used as a control in (C) and (D). Total
646 RNA was extracted from the agroinfiltrated *N. benthamiana* leaves and RT-PCR was performed
647 to detect HR1b-GFP and HR1b$^{R181/185A}$-GFP transcripts.

648 (E) MG132 treatment attenuates the degradation of HR1b-GFP by E3-DART. *N. benthamiana*
649 leaves were co-infiltrated with HA-LRR-NEL-mCherry and HR1b-GFP cassettes. MG132 (100
650 μM in 10 mM MgCl2) was infiltrated into the same leaves 12 h before sample collection.
651 Proteins were detected by immunoblot.

652 (F) In vivo HR1b-GFP ubiquitination assay. Total proteins were extracted from leaves treated as
653 in (E), immunoprecipitated with an anti-GFP antibody and polyubiquitination was detected with
654 an anti-ubiquitin monoclonal antibody. Ponceau S staining shows equal amounts of inputs were
655 used for immunoprecipitation.

656 (G) E3-DART targets HR1b-GFP for degradation in Arabidopsis. Arabidopsis plants were
657 sequentially transformed with the indicated constructs and roots were imaged by confocal
658 microscopy. All transgenes were driven by the *UBIQUITIN10* promoter and all images were
659 captured with the same microscope settings. Scale bar: 100 μm.

660 (H) HR1b-GFP is unstable in plants expressing the NEL E3 ligase chimera. Proteins were
661 extracted from transgenic seedlings (E) and detected by immunoblot. Actin was used as an
662 endogenous control. HA-LRR-NEL-mCherry and HA-LRRC$^{492A}$-NEL-mCherry proteins were
663 detected with anti-HA monoclonal antibody; HR1b-GFP, HR1b$^{R181/185A}$-GFP and GFP were
664 detected with anti-GFP monoclonal antibody; Flag-GFP-Myc was detected with anti-Myc
665 monoclonal antibody and Arabidopsis actin was detected with anti-Actin polyclonal antibody.

666

667 **Figure 3. Robust degradation of HR1b-tagged plant proteins with E3-DART.**

668 Degradation of a cytoplasmic VPS34-GFP-HR1b (A, B) or nuclear HR1b-GFP-RHD6 (C-F)
669 fusions by E3-DART (HA-LRR-NEL-mCherry, A-D) or a nuclear localized ligase (3xNLS-HA-
670 LRR-NEL-mCherry, E, F). *N. benthamiana* leaves were infiltrated with Agrobacterium carrying
671 each pair of expression cassettes in the same plasmid. The mutant HR1b peptide ($HR1b^{R181/185A}$)
672 and the catalytically inactive E3-DART (HA-LRR-$NEL^{C492A}$-mCherry) were used as controls
673 and all plasmids were infiltrated using equal amounts of Agrobacteria. GFP and mCherry signals
674 were detected by confocal microscopy and the merged image including brightfield acquisition is
675 shown. Scale: 100 μm (A, C, E). Protein abundance from similar transformations was detected
676 by immunoblot and co-transformation with Flag-RFP-Myc was used as a transformation control
677 (B, D, F). RT-PCR was used to confirm expression of the HR1b and $HR1b^{R181/185A}$ fusions.
678 Proteins fused to HA and GFP tags were detected by anti-HA and anti-GFP monoclonal
679 antibodies respectively. The Flag-RFP-Myc was detected by anti-Myc monoclonal antibody.
680 mCh, mCherry.

681

682 **Figure 4. Establishment of a dexamethasone-inducible E3-DART system.**

683 (A) A dexamethasone (DEX)-inducible system was developed by controlling the expression of
684 the NEL E3 ligase chimera with the GVG transactivation system. The GAL4-VP16-GR (GVG)
685 transcription factor is retained in the cytosol in the absence of DEX due to a large inhibitory
686 complex containing HSP70, HSP90 and other proteins. DEX treatment results in its binding to
687 the ligand binding domain of the glucocorticoid receptor (GR), dissociation of GVG from the
688 HSP complex and movement into the nucleus. The GVG transcription factor then binds to the
689 GAL4 upstream activating sequence (*6XUAS*) and triggers the transcription of the NEL E3 ligase
690 coding sequence. Once the ligase is translated, it can bind and ubiquitinate HR1b-bearing targets.
691 (B) DEX-induced degradation of HR1b-GFP fusions by the NEL E3 ligase chimera. *N.*
692 *benthamiana* leaves were infiltrated with Agrobacterium carrying DEX-inducible ligases (HA-

693 LRR-NEL-mCherry or HA-LRR-NEL$^{C492A}$-mCherry) together with the HR1b-GFP or
694 HR1b$^{R181/185A}$-GFP in the same plasmid. 100 μM DEX was applied 48 h after transformation by
695 immersion of leaf petioles and proteins were extracted up to 6 h later. All plasmids were
696 infiltrated using equal amounts of Agrobacteria. Myc-GVG-HA was used as a control. HR1b-
697 GFP and HR1b$^{R181/185A}$-GFP transcripts were detected by RT-PCR.

698

699 **Figure 5. Degradation of degron-tagged plant proteins with the DEX-induced E3-DART**
700 **system.**

701 DEX-induced degradation of HR1b fusions to VPS34 (A) and RHD6 (B) by E3-DART. (A) *N.*
702 *benthamiana* leaves were infiltrated with Agrobacterium carrying DEX-inducible ligases (HA-
703 LRR-NEL-mCherry or HA-LRR-NEL$^{C492A}$-mCherry) together with the target fusions (VPS34-
704 GFP-HR1b or VPS34-GFP-HR1b$^{R181/185A}$-GFP) in the same plasmid. (B) Leaves were infiltrated
705 with Agrobacterium carrying DEX-inducible ligases together with the HR1b-GFP-RHD6 or
706 HR1b$^{R181/185A}$-GFP-RHD6 in the same plasmid. 100 μM DEX was applied 48 h after
707 transformation by immersion of leaf petioles and proteins were extracted up to 6 h later.
708 Detection of Myc-GVG-HA was used as a control. HR1b- and HR1b$^{R181/185A}$-containing
709 transcripts were detected by RT-PCR.

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## Figure Legends

**Figure 1. Design of the E3-targeted degradation of plant proteins (E3-DART) system.**

(A) The E3-DART system is based on the interaction between the *Salmonella* secreted protein H1 (SspH1) and the human protein kinase N1 (PKN1). The interaction between the HR1b domain of PKN1 and the Leucine-Rich repeat (LRR) domain of SspH1 results in protein binding and ubiquitination by the Novel E3 Ligase (NEL) domain. The HR1b domain is fused to a target protein to recruit the truncated LRR-NEL fragment and induce ubiquitination and target protein degradation.

(B) Protein chimeras used in this study. The LRR-NEL fragment was fused to mCherry and the HR1b domain was fused to GFP. A C492A substitution in the NEL domain corresponds to a catalytically inactive control and the R181A and R185A (R181/185A) mutations in HR1b correspond to non-interacting controls. HA, Hemagglutinin tag; LRR, Leucin-rich repeat; NEL, Novel E3 Ligase, GFP, green fluorescent protein.

(C) Transient expression of degron components in *Nicotiana benthamiana*. Infiltrated leaves were imaged by epifluorescence (LRR-NEL-mCh) or confocal microscopy (GFP fusions) to detect mCherry or GFP signal. The merged image with brightfield acquisition is included. All GFP confocal images were acquired under identical microscope settings. Scale: 40 μm. mCh, mCherry fluorescent protein.

**Figure 2. HA-LRR-NEL-mCherry targets HR1b-GFP for efficient degradation in plants.**

(A) Co-immunoprecipitation (Co-IP) assay using crude extracts from agroinfiltrated *N. benthamiana* leaves confirmed the interaction between HR1b-GFP and both HA-LRR-NEL-mCherry (E3-DART) and HA-LRR-NEL$^{C492A}$-mCherry. Protein extracts from independent transformations were mixed as indicated and binding proteins were immunoprecipitated with anti-GFP and detected by immunoblot.

(B) Confocal microcopy of leaves transiently co-transformed with HR1b-GFP or HR1b$^{R181/185A}$-GFP and HA-LRR-NEL-mCherry or HA-LRR-NEL$^{C492A}$-mCherry. Scale bars: 100 μm. All images were taken with identical microscope settings.

(C) E3-DART targets HR1b-GFP for degradation in plants. Equal amounts of Agrobacterium strains carrying each indicated construct were co-infiltrated in *N. benthamiana* leaves and recombinant proteins were detected by immunoblot.

(D) HR1b stability is dependent on E3-DART. Equal amounts of Agrobacterium strains carrying the HR1b-GFP construct were co-infiltrated with increasing amounts Agrobacteria carrying the HA-LRR-NEL-mCherry (E3-DART) construct. Expressed proteins were detected by immunoblot. Co-transformation with Flag-RFP-Myc was used as a control in (C) and (D). Total RNA was extracted from the agroinfiltrated *N. benthamiana* leaves and RT-PCR was performed to detect HR1b-GFP and HR1b$^{R181/185A}$-GFP transcripts.

(E) MG132 treatment attenuates the degradation of HR1b-GFP by E3-DART. *N. benthamiana* leaves were co-infiltrated with HA-LRR-NEL-mCherry and HR1b-GFP cassettes. MG132 (100 μM in 10 mM MgCl2) was infiltrated into the same leaves 12 h before sample collection. Proteins were detected by immunoblot.

(F) In vivo HR1b-GFP ubiquitination assay. Total proteins were extracted from leaves treated as in (E), immunoprecipitated with an anti-GFP antibody and polyubiquitination was detected with an anti-ubiquitin monoclonal antibody. Ponceau S staining shows equal amounts of inputs were used for immunoprecipitation.

(G) E3-DART targets HR1b-GFP for degradation in Arabidopsis. Arabidopsis plants were sequentially transformed with the indicated constructs and roots were imaged by confocal microscopy. All transgenes were driven by the *UBIQUITIN10* promoter and all images were captured with the same microscope settings. Scale bar: 100 μm.

(H) HR1b-GFP is unstable in plants expressing the NEL E3 ligase chimera. Proteins were extracted from transgenic seedlings (E) and detected by immunoblot. Actin was used as an endogenous control. HA-LRR-NEL-mCherry and HA-LRRC$^{492A}$-NEL-mCherry proteins were detected with anti-HA monoclonal antibody; HR1b-GFP, HR1b$^{R181/185A}$-GFP and GFP were detected with anti-GFP monoclonal antibody; Flag-GFP-Myc was detected with anti-Myc monoclonal antibody and Arabidopsis actin was detected with anti-Actin polyclonal antibody.

**Figure 3. Robust degradation of HR1b-tagged plant proteins with E3-DART.**

Degradation of a cytoplasmic VPS34-GFP-HR1b (A, B) or nuclear HR1b-GFP-RHD6 (C-F) fusions by E3-DART (HA-LRR-NEL-mCherry, A-D) or a nuclear localized ligase (3xNLS-HA-LRR-NEL-mCherry, E, F). *N. benthamiana* leaves were infiltrated with Agrobacterium carrying each pair of expression cassettes in the same plasmid. The mutant HR1b peptide (HR1b$^{R181/185A}$) and the catalytically inactive E3-DART (HA-LRR-NEL$^{C492A}$-mCherry) were used as controls and all plasmids were infiltrated using equal amounts of Agrobacteria. GFP and mCherry signals were detected by confocal microscopy and the merged image including brightfield acquisition is shown. Scale: 100 μm (A, C, E). Protein abundance from similar transformations was detected by immunoblot and co-transformation with Flag-RFP-Myc was used as a transformation control (B, D, F). RT-PCR was used to confirm expression of the HR1b and HR1b$^{R181/185A}$ fusions. Proteins fused to HA and GFP tags were detected by anti-HA and anti-GFP monoclonal antibodies respectively. The Flag-RFP-Myc was detected by anti-Myc monoclonal antibody. mCh, mCherry.

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the NEL E3 ligase chimera with the GVG transactivation system. The GAL4-VP16-GR (GVG) transcription factor is retained in the cytosol in the absence of DEX due to a large inhibitory complex containing HSP70, HSP90 and other proteins. DEX treatment results in its binding to the ligand binding domain of the glucocorticoid receptor (GR), dissociation of GVG from the HSP complex and movement into the nucleus. The GVG transcription factor then binds to the GAL4 upstream activating sequence (*6XUAS*) and triggers the transcription of the NEL E3 ligase coding sequence. Once the ligase is translated, it can bind and ubiquitinate HR1b-bearing targets. (B) DEX-induced degradation of HR1b-GFP fusions by the NEL E3 ligase chimera. *N. benthamiana* leaves were infiltrated with Agrobacterium carrying DEX-inducible ligases (HA-LRR-NEL-mCherry or HA-LRR-NEL$^{C492A}$-mCherry) together with the HR1b-GFP or HR1b$^{R181/185A}$-GFP in the same plasmid. 100 μM DEX was applied 48 h after transformation by immersion of leaf petioles and proteins were extracted up to 6 h later. All plasmids were infiltrated using equal amounts of Agrobacteria. Myc-GVG-HA was used as a control. HR1b-GFP and HR1b$^{R181/185A}$-GFP transcripts were detected by RT-PCR.

**Figure 5. Degradation of degron-tagged plant proteins with the DEX-induced E3-DART system.**

DEX-induced degradation of HR1b fusions to VPS34 (A) and RHD6 (B) by E3-DART. (A) *N. benthamiana* leaves were infiltrated with Agrobacterium carrying DEX-inducible ligases (HA-LRR-NEL-mCherry or HA-LRR-NEL$^{C492A}$-mCherry) together with the target fusions (VPS34-GFP-HR1b or VPS34-GFP-HR1b$^{R181/185A}$-GFP) in the same plasmid. (B) Leaves were infiltrated with Agrobacterium carrying DEX-inducible ligases together with the HR1b-GFP-RHD6 or HR1b$^{R181/185A}$-GFP-RHD6 in the same plasmid. 100 μM DEX was applied 48 h after transformation by immersion of leaf petioles and proteins were extracted up to 6 h later. Detection of Myc-GVG-HA was used as a control. HR1b- and HR1b$^{R181/185A}$-containing

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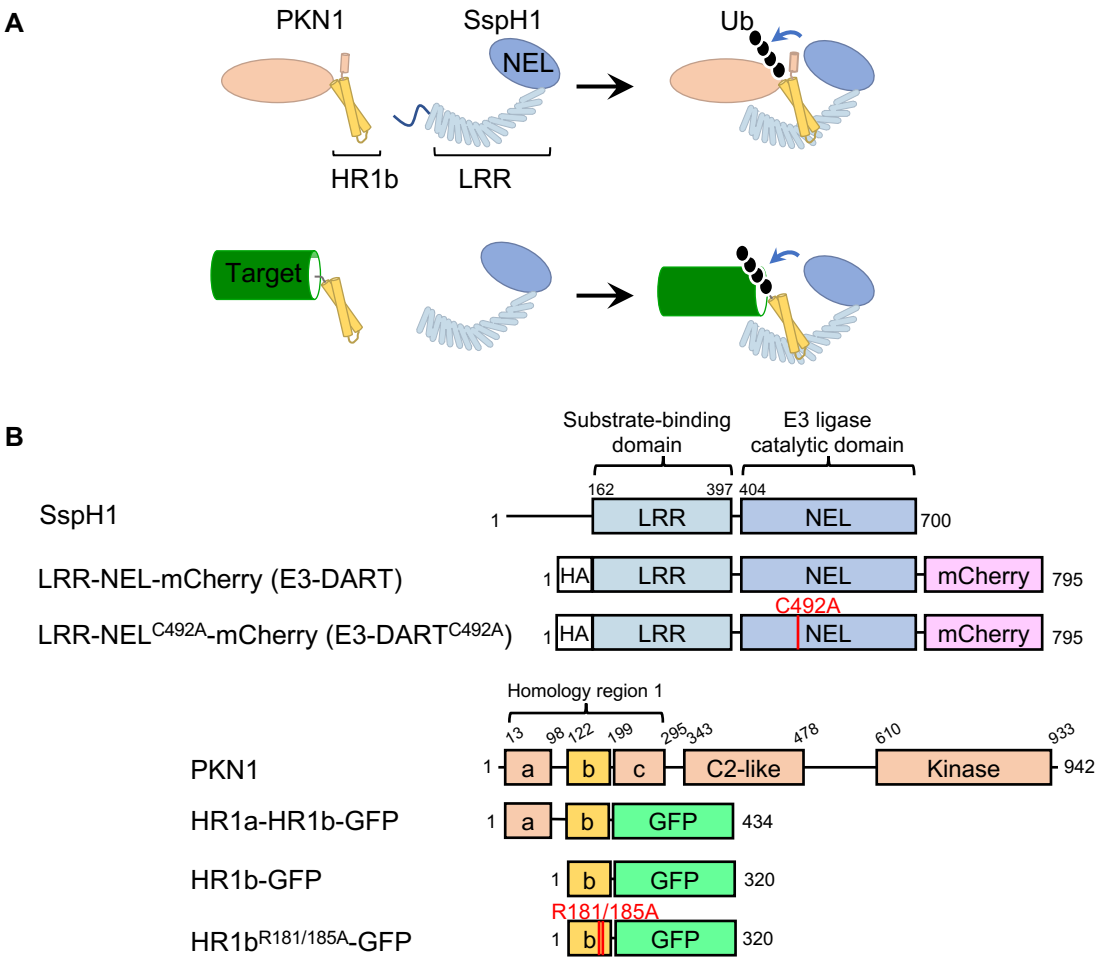
A
PKN1
SspH1
NEL
Ub
HR1b
LRR
Target
B
Substrate-binding domain
E3 ligase catalytic domain
SspH1
LRR-NEL-mCherry (E3-DART)
LRR-NEL^C492A-mCherry (E3-DART^C492A)
HA
LRR
NEL
mCherry
C492A
Homology region 1
PKN1
HR1a-HR1b-GFP
HR1b-GFP
HR1b^R181/185A-GFP
a
b
c
C2-like
Kinase
GFP
R181/185A
C
LRR-NEL-mCh
merged
GFP
HR1b-GFP
HR1a-1b-GFP
GFP-HR1b
GFP-HR1a-1b

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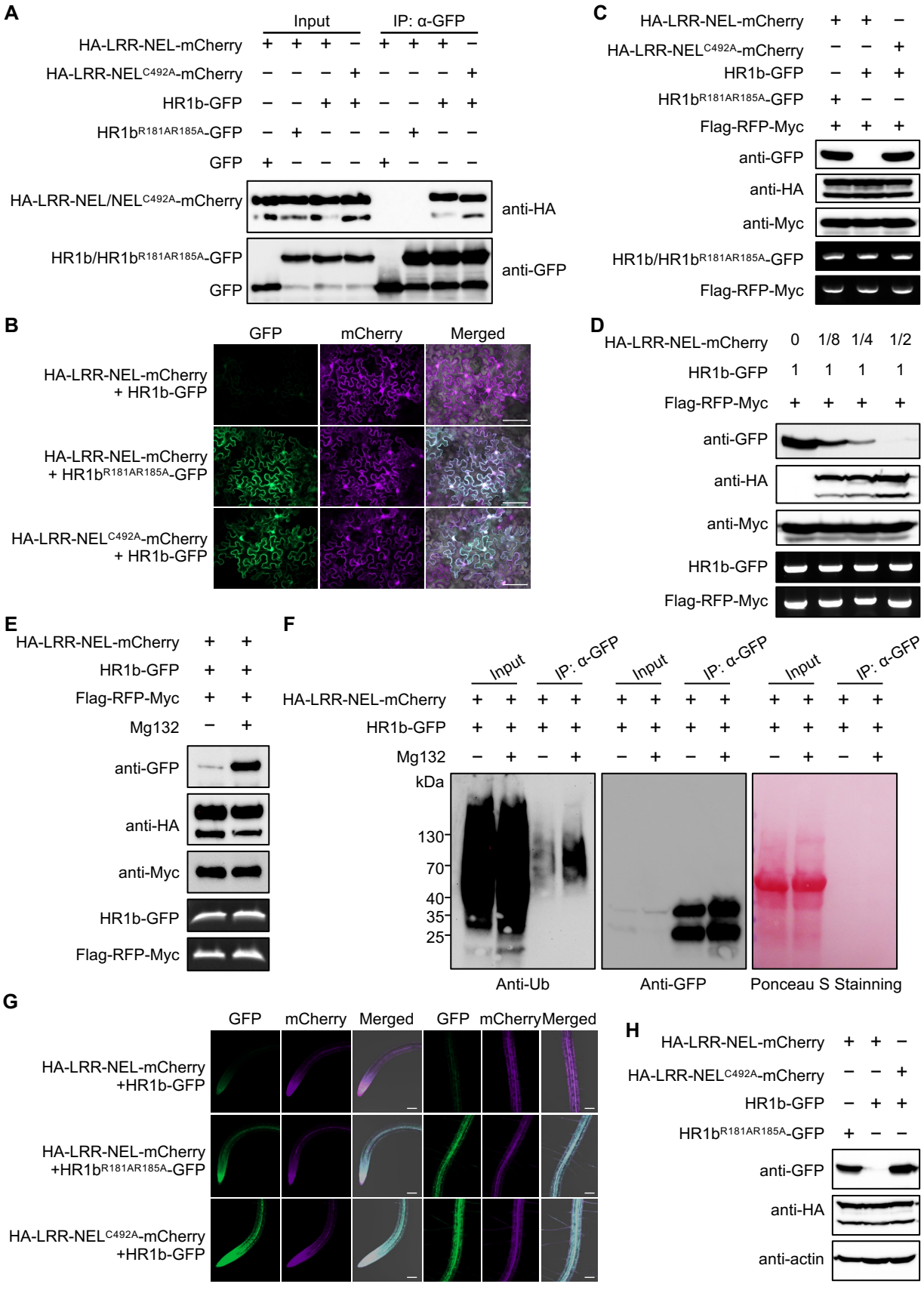
A
Input
IP: α-GFP
HA-LRR-NEL-mCherry
HA-LRR-NEL$^{C492A}$-mCherry
HR1b-GFP
HR1b$^{R181AR185A}$-GFP
GFP
HA-LRR-NEL/NEL$^{C492A}$-mCherry
anti-HA
HR1b/HR1b$^{R181AR185A}$-GFP
GFP
anti-GFP
B
GFP
mCherry
Merged
HA-LRR-NEL-mCherry + HR1b-GFP
HA-LRR-NEL-mCherry + HR1b$^{R181AR185A}$-GFP
HA-LRR-NEL$^{C492A}$-mCherry + HR1b-GFP
C
HA-LRR-NEL-mCherry
HA-LRR-NEL$^{C492A}$-mCherry
HR1b-GFP
HR1b$^{R181AR185A}$-GFP
Flag-RFP-Myc
anti-GFP
anti-HA
anti-Myc
HR1b/HR1b$^{R181AR185A}$-GFP
Flag-RFP-Myc
D
HA-LRR-NEL-mCherry 0 1/8 1/4 1/2
HR1b-GFP 1 1 1 1
Flag-RFP-Myc
anti-GFP
anti-HA
anti-Myc
HR1b-GFP
Flag-RFP-Myc
E
HA-LRR-NEL-mCherry
HR1b-GFP
Flag-RFP-Myc
Mg132
anti-GFP
anti-HA
anti-Myc
HR1b-GFP
Flag-RFP-Myc
F
Input
IP: α-GFP
HA-LRR-NEL-mCherry
HR1b-GFP
Mg132
kDa
130
70
40
35
25
Anti-Ub
Anti-GFP
Ponceau S Stainning
G
GFP
mCherry
Merged
HA-LRR-NEL-mCherry +HR1b-GFP
HA-LRR-NEL-mCherry +HR1b$^{R181AR185A}$-GFP
HA-LRR-NEL$^{C492A}$-mCherry +HR1b-GFP
H
HA-LRR-NEL-mCherry
HA-LRR-NEL$^{C492A}$-mCherry
HR1b-GFP
HR1b$^{R181AR185A}$-GFP
anti-GFP
anti-HA
anti-actin

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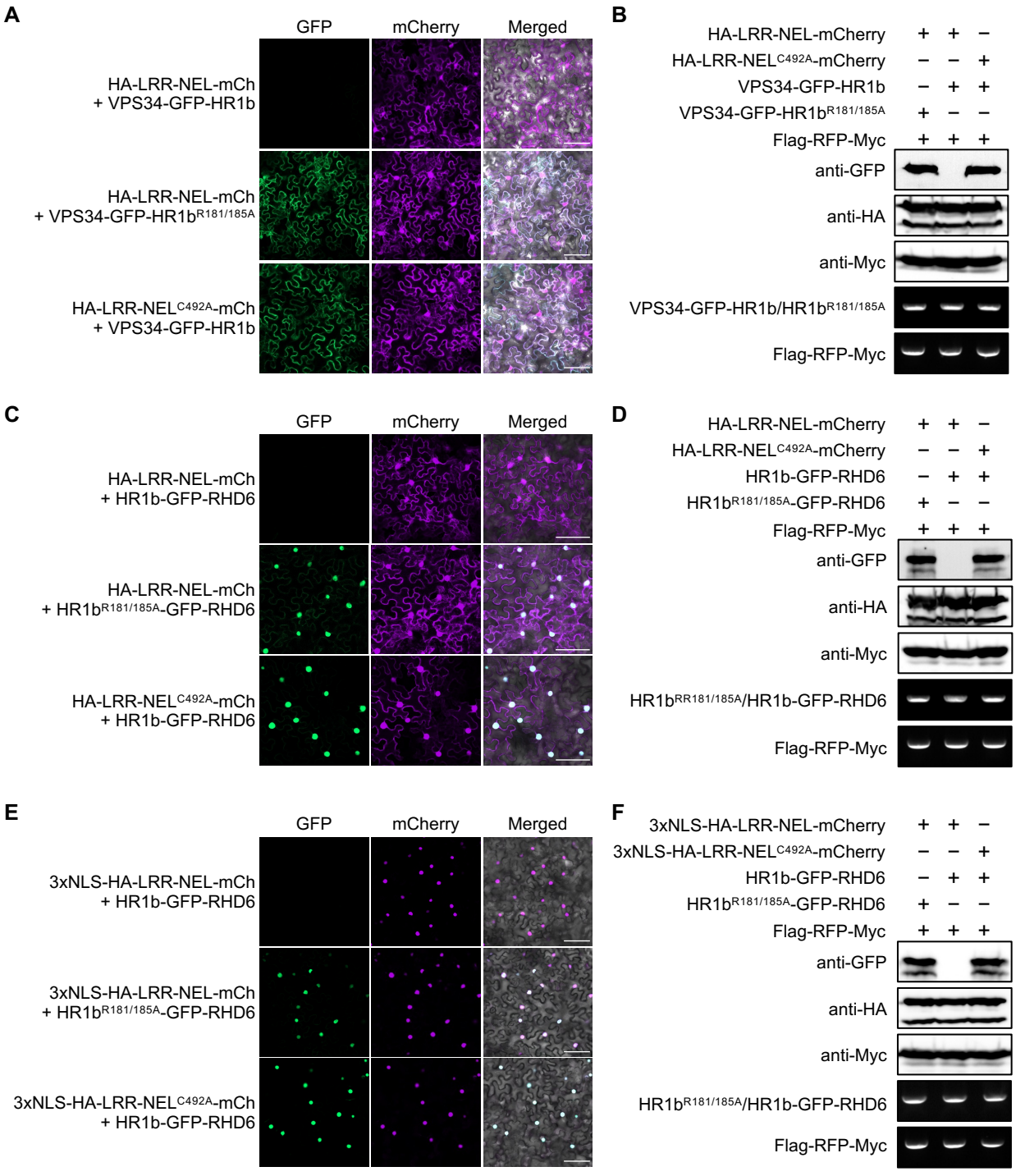
A
GFP
mCherry
Merged
HA-LRR-NEL-mCh
+ VPS34-GFP-HR1b
HA-LRR-NEL-mCh
+ VPS34-GFP-HR1b$^{R181/185A}$
HA-LRR-NEL$^{C492A}$-mCh
+ VPS34-GFP-HR1b
B
HA-LRR-NEL-mCherry + + −
HA-LRR-NEL$^{C492A}$-mCherry − − +
VPS34-GFP-HR1b − + +
VPS34-GFP-HR1b$^{R181/185A}$ + − −
Flag-RFP-Myc + + +
anti-GFP
anti-HA
anti-Myc
VPS34-GFP-HR1b/HR1b$^{R181/185A}$
Flag-RFP-Myc
C
GFP
mCherry
Merged
HA-LRR-NEL-mCh
+ HR1b-GFP-RHD6
HA-LRR-NEL-mCh
+ HR1b$^{R181/185A}$-GFP-RHD6
HA-LRR-NEL$^{C492A}$-mCh
+ HR1b-GFP-RHD6
D
HA-LRR-NEL-mCherry + + −
HA-LRR-NEL$^{C492A}$-mCherry − − +
HR1b-GFP-RHD6 − + +
HR1b$^{R181/185A}$-GFP-RHD6 + − −
Flag-RFP-Myc + + +
anti-GFP
anti-HA
anti-Myc
HR1b$^{RR181/185A}$/HR1b-GFP-RHD6
Flag-RFP-Myc
E
GFP
mCherry
Merged
3xNLS-HA-LRR-NEL-mCh
+ HR1b-GFP-RHD6
3xNLS-HA-LRR-NEL-mCh
+ HR1b$^{R181/185A}$-GFP-RHD6
3xNLS-HA-LRR-NEL$^{C492A}$-mCh
+ HR1b-GFP-RHD6
F
3xNLS-HA-LRR-NEL-mCherry + + −
3xNLS-HA-LRR-NEL$^{C492A}$-mCherry − − +
HR1b-GFP-RHD6 − + +
HR1b$^{R181/185A}$-GFP-RHD6 + − −
Flag-RFP-Myc + + +
anti-GFP
anti-HA
anti-Myc
HR1b$^{R181/185A}$/HR1b-GFP-RHD6
Flag-RFP-Myc

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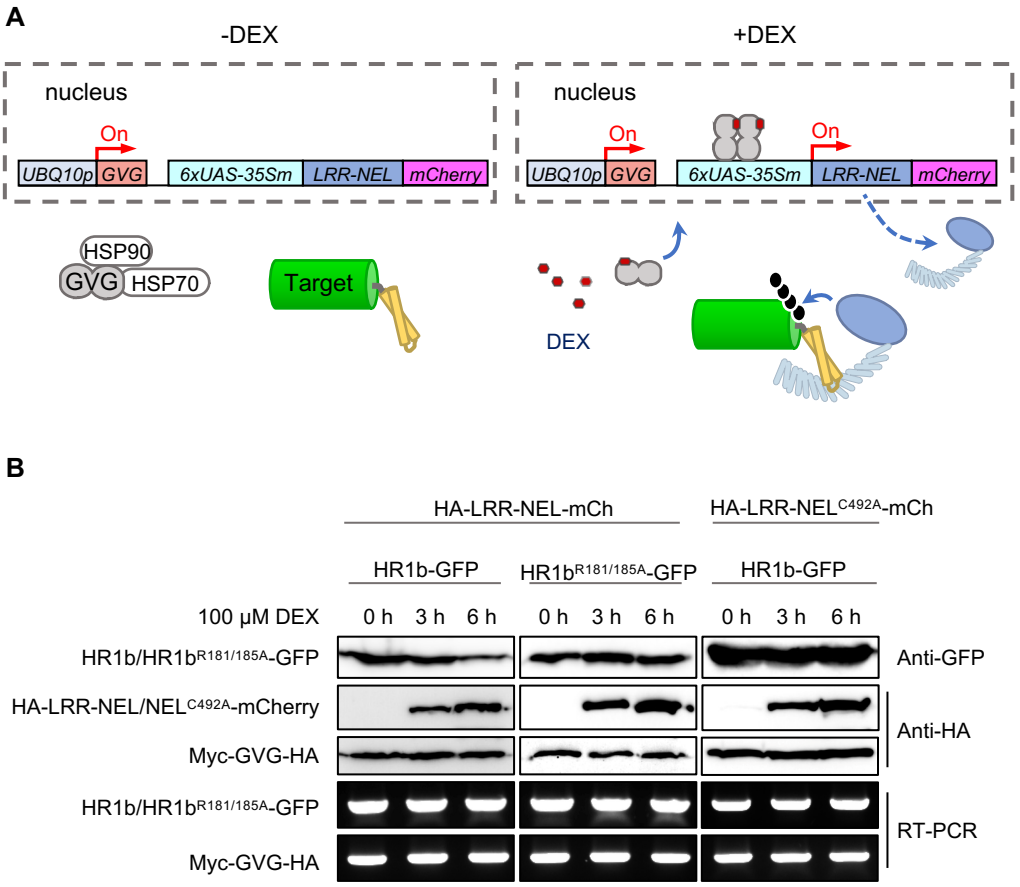


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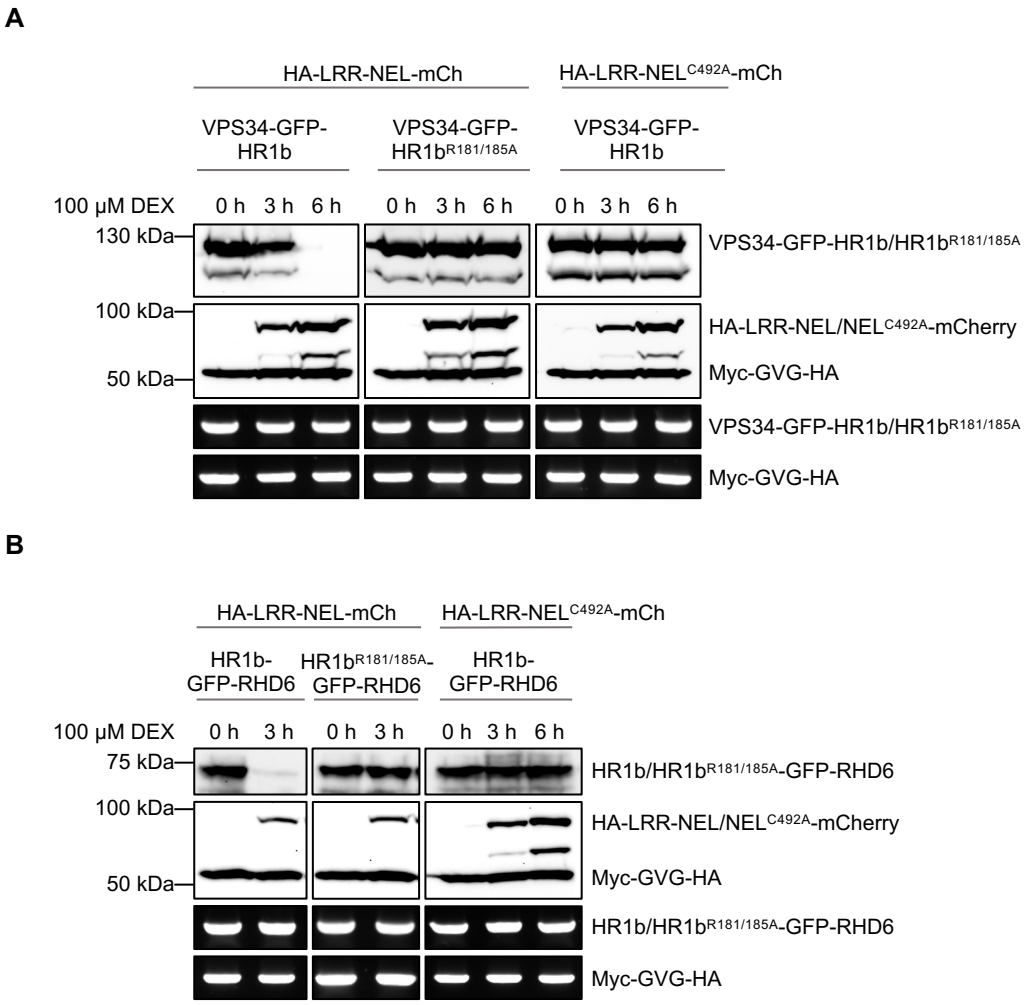


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