CHAPTER

5 Applying the auxin-inducible degradation system for rapid protein depletion in mammalian cells

Bramwell G. Lambrus[2], Tyler C. Moyer[2], Andrew J. Holland[1]
Johns Hopkins University School of Medicine, Baltimore, MD, United States
[1]Corresponding author: e-mail address: aholland@jhmi.edu

CHAPTER OUTLINE

1 Introduction 108
1.1 Hijacking the SCF Complex 109
1.2 The AID 113
1.3 Future Optimization of the AID System 113
1.4 Tagging Approaches 114
2 Materials Required 115
2.1 Recipes 115
3 Methods 116
3.1 Designing Reagents for Site-Specific AID Integration 116
3.1.1 Choice of Cas9/sgRNA Delivery System 118
3.1.2 Designing a Guide RNA for sequence-specific DNA Cleavage by SpCas9 118
3.1.3 Cloning Oligonucleotides Into the PX459 Vector 119
3.1.4 Design of a Repair Template 121
3.2 Transfection and Screening of AID-Tagged Clonal Lines 123
3.2.1 Isolation of Clonal Lines 124
3.2.2 Genomic DNA Extraction 124
3.2.3 Screening 125
3.2.4 Sequencing Clones 128
3.3 Generation of An OsTIR1 Cell Line 128
3.3.1 Production of OsTIR1 Retrovirus 128
3.3.2 Viral Transduction of OsTIR1 Into Cells 128
3.3.3 Selection and Isolation of OsTIR1-Expressing Cells 129
3.3.4 Screening for high-expressing OsTIR1 Clones 129

[2]These authors contributed equally to this work.

Methods in Cell Biology, Volume 144, ISSN 0091-679X, https://doi.org/10.1016/bs.mcb.2018.03.004

4 Functional Analysis 129
4.1 Validation of mAID-Fusion Protein in Cells 129
4.2 Testing Inducible Depletion of mAID-Tagged Protein 130
5 Useful Tips 130
5.1 Biallelic Tagging 130
5.2 IAA Reagent 131
6 Troubleshooting 131
6.1 Lack of Edited Clones 131
6.2 AID-Tagged Protein is Not Degraded Upon Induction with Auxin 131
6.3 AID-Tagged Protein is Unstable 132
7 Conclusion 132
References 132

Abstract

The ability to deplete a protein of interest is critical for dissecting cellular processes. Traditional methods of protein depletion are often slow acting, which can be problematic when characterizing a cellular process that occurs within a short period of time, such as mitosis. Furthermore, these methods are usually not reversible. Recent advances to achieve protein depletion function by inducibly trafficking proteins of interest to an endogenous E3 ubiquitin ligase complex to promote ubiquitination and subsequent degradation by the proteasome. One of these systems, the auxin-inducible degron (AID) system, has been shown to permit rapid and inducible degradation of AID-tagged target proteins in mammalian cells. The AID system can control the abundance of a diverse set of cellular proteins, including those contained within protein complexes, and is active in all phases of the cell cycle. Here we discuss considerations for the successful implementation of the AID system and describe a protocol using CRISPR/Cas9 to achieve biallelic insertion of an AID in human cells. This method can also be adapted to insert other tags, such as fluorescent proteins, at defined genomic locations.

1 INTRODUCTION

The ability to deplete proteins from mammalian cells is critical for studying their role in biological processes. Traditional methods of disrupting protein function include DNA editing to produce a gene knockout (Sauer & Henderson, 1988) or RNA interference (RNAi) to downregulate mRNA (Elbashir et al., 2001). While these methods are staples of a cell biologist's toolkit, in both cases protein depletion is indirect and the rate of protein loss depends on the stability of the protein. Furthermore, neither gene knockout nor RNAi is easily reversible, and RNAi can suffer from incomplete silencing and off-target effects (Bartlett & Davis, 2006).

To overcome the slow protein depletion achieved with genetic manipulation, researchers have turned to modulating protein function with cell permeable, small

molecules. While fast acting, small-molecule probes are challenging to develop and frequently limited by specificity. However, combining pharmacologic manipulation with genetic engineering expands the possibilities for achieving rapid and specific modulation of protein function. One powerful example is the analog-sensitive (AS) kinase method pioneered by Bishop et al. (1998) and Shah, Liu, Deirmengian, and Shokat (1997). In this approach, mutation of conserved residues in the ATP-binding pocket of a kinase allows the kinase to accommodate, and be specifically inhibited by, bulky nonhydrolyzable ATP analogs. Importantly, wild-type kinases are resistant to such inhibition. The AS kinase approach achieves specificity and reversibility, but is so far limited to manipulating proteins in the kinase family. More recent advances in chemical genetics have opened the possibility of regulating classically nondruggable targets by tagging proteins of interest with degrons (Banaszynski, Chen, Maynard-Smith, Ooi, & Wandless, 2006; Iwamoto, Bjorklund, Lundberg, Kirik, & Wandless, 2010). In these cases, proteins can be stabilized or targeted for degradation through the introduction of small molecules. A comparison of the protein degradation systems currently available is shown in Table 1.

1.1 HIJACKING THE SCF COMPLEX

Several systems have achieved protein degradation by ectopically targeting proteins to endogenous E3 ubiquitin ligase complexes, including the VHL, MDM2, cIAP, CRBN, and SCF complexes (Lu et al., 2015; Nishimura et al., 2009; Sakamoto et al., 2001; Schneekloth, Pucheault, Tae, & Crews, 2008; Sekine et al., 2008; Winter et al., 2015). The SCF (Skp1, Cul1, and F-box) complex is a member of a family of cullin-RING-ligase E3 ubiquitin ligases (Skaar, Pagan, & Pagano, 2013). The Cul1 subunit acts as the major scaffold, bringing together Skp1 and RBX1. RBX1 is responsible for recruiting the E2 ubiquitin-conjugating enzyme to the complex, while Skp1 associates with an F-box protein that interacts with substrates containing a degradation motif (Fig. 1A). The human genome encodes nearly 70 unique F-box proteins that each interact with a distinct subset of substrates, allowing the SCF complex to serve as a versatile tool for controlling the proteome (Kipreos & Pagano, 2000).

The SCF complex is highly conserved among eukaryotes (Skaar et al., 2013), making it possible to transplant F-box proteins from one organism into another, to form a functional SCF E3 ligase complex that can instruct the degradation of proteins tagged with a cognate degron. One such F-box/degron system that has been transplanted across kingdoms is the auxin-inducible degron (AID) system (Nishimura et al., 2009) (Fig. 1B). In the presence of auxin (a class of structurally similar plant hormones, of which indole-3-acetic acid (IAA) is the most common), the plant-specific F-box protein transport inhibitor response 1 (TIR1) associates with the auxin or indole-3-acetic acid (AUX/IAA) family of transcription factors. This brings the AUX/IAA transcription factors to the SCF complex for their ubiquitination and subsequent degradation by the proteasome (Dharmasiri, Dharmasiri, & Estelle, 2005; Kepinski & Leyser, 2005; Tan et al., 2007).

Table 1 Methods for Protein Depletion in Mammalian Cells

Name	Explanation	Advantages	Disadvantages	References
RNAi	Small interfering RNAs (siRNAs) targeting the mRNA of a protein of interest are introduced to the cell to activate an intrinsic RNA degradation pathway. Degradation of mRNA leads to subsequent protein loss	• Only requires commercially available siRNAs	• Incomplete protein depletion • Slow acting. Relies on turnover of the endogenous protein • siRNAs can silence off-target mRNAs • Not readily reversible	
Gene knockout	Genomic loci are mutated through a variety of methods. Mutations lead to the introduction of premature stop codons or the generation of a nonfunctional protein	• Complete loss of the protein • Gene specific	• Slow acting. Relies on turnover of the endogenous protein • Not readily reversible	
Halo-Tag Degron	Protein of interest is tagged with a Halo tag, which can be bound by a hydrophobic, cell-permeable ligand. This ligand mimics an unfolded protein and targets the Halo-tagged protein for degradation	• Gene specific • Can be tuned with dosage of the synthetic ligand	• Slower degradation kinetics than the AID system • Addition of the Halo tag (33 kDa) may disrupt protein function	Neklesa et al. (2011)
(DD)-FKBP12	Protein of interest is tagged with the destabilizing domain (DD) of the FK506- and rapamycin-binding protein (FKBP12). Protein is constitutively degraded until a synthetic ligand, Shield-1, is provided that protects the protein from degradation	• Gene specific • Reversible • Can be tuned with the dosage of Shield-1	• Slower degradation kinetics than the AID system • Expression of normal protein levels requires the constant presence of a synthetic ligand (i.e., Drug “On”) • Addition of the DD domain (12 kDa) may disrupt protein function	Banaszynski et al. (2006)

(DD)-DHFR	Protein of interest is tagged with the intrinsically unstable modified dihydrofolate reductase (DHFR) from *Escherichia coli*. Protein is constitutively degraded until the ligand, trimethoprim, is added	• Gene specific • Reversible • Can be tuned with the dosage of synthetic ligand	• Slower degradation kinetics than the AID system • Expression of normal protein levels requires the constant presence of trimethoprim (i.e., Drug "On")	Iwamoto et al. (2010)
LID domain	Protein of interest is tagged with a ligand-induced degradation (LID) domain. Addition of a small molecule, Shield-1, changes the conformation of the degron, leading to its recognition by an E3 ubiquitin ligase and subsequent degradation	• Reversible • Gene specific • Can be tuned with the dosage of Shield-1	• Slower degradation kinetics than the AID system • Degradation of the LID-tagged protein is often incomplete • Addition of the LID domain (14 kDa) may disrupt protein function	Bonger, Chen, Liu, and Wandless (2011)
deGradFP	Protein of interest is tagged with GFP. Expression of an F-box protein fused to a GFP-nanobody causes the protein of interest to be trafficked to the SCF E3 ligase complex for ubiquitination and subsequent degradation	• Gene specific • When introduced in a transgenic organism, the nanobody-tagged F-box protein can be placed under a tissue-specific promoter	• Degradation kinetics are dependent upon expression level of the nanobody/F-box protein fusion • Degradation is not inducible with a small molecule • Addition of the GFP (27 kDa) may disrupt protein function	Caussinus, Kanca, and Affolter (2011)

Continued

Table 1 Methods for Protein Depletion in Mammalian Cells—cont'd

Name	Explanation	Advantages	Disadvantages	References
Auxin-inducible degron (AID)	Protein of interest is tagged with the AID. In the presence of the plant hormone auxin, the AID interacts with the F-box protein OsTIR1, bringing the protein of interest to the SCF E3 ubiquitin ligase complex for ubiquitination and subsequent degradation by the proteasome	• Rapid kinetics of protein degradation • Reversible • Gene specific • Can be tuned with dosage of auxin • Inexpensive ligand (auxin)	• Two-component system requiring addition of the AID and expression of the F-box protein OsTIR1 • Addition of the mAID (5 kDa) may disrupt protein function	Nishimura, Fukagawa, Takisawa, Kakimoto, and Kanemaki (2009)
Jasmonate-inducible degron (JID)	Protein of interest is tagged with the JAZ degron. In the presence of isoleucine-conjugated jasmonate (JA-Ile), the JAZ degron interacts with the F-box protein COI1, bringing the protein of interest to the SCF E3 ubiquitin ligase complex for ubiquitination and subsequent degradation by the proteasome	• Fast kinetics of protein degradation • Reversible • Gene specific • Can be tuned with dosage of JA-Ile	• Two-component system requiring addition of the JAZ degron and expression of the F-box protein (COI1) • Kinetics of degradation are slower than the AID system • Addition of the JAZ degron (3 kDa) may disrupt protein function	Brosh et al. (2016)
PROTAC	PROTAC: *Pro*teolysis *ta*rgeting *c*himeric molecules. Bifunctional molecules are introduced that bind to an E3 ubiquitin ligase complex in addition to a protein of interest. The protein of interest is brought to the E3 ubiquitin ligase complex, ubiquitinated, and degraded by the proteasome	• No genetic modification required • Reversible • Gene specific • Can be tuned with dosage of specific PROTAC	• Applicable only to proteins that have known small molecules that bind to them • Varying kinetics depending on binding affinity of the protein and the small molecule	Collins, Wang, Caldwell, and Chopra (2017), Lu et al. (2015), and Winter et al. (2015)

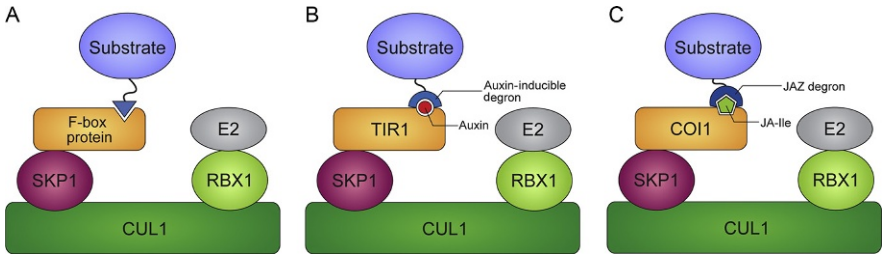

FIG. 1

SCF E3 ubiquitin ligase complexes. (A) Substrates are brought to the SCF complex through an interaction with a specific F-box protein. Owing to the conserved nature of the SCF complex, F-box proteins from one kingdom can be introduced into other species to produce a functional SCF complex. (B) In the AID system, the interaction between the F-box protein TIR1 and an AID-containing substrate is facilitated through the hormone Auxin. (C) In the JAZ degron system, the interaction between the F-box protein COI1 and a JAZ degron-containing substrate is facilitated through the hormone jasmonate-isoleucine (Ja-Ile).

1.2 THE AID

Nishimura and colleagues first demonstrated the plant-specific F-box protein TIR1 could form a functional SCF^{TIR1} complex and facilitate the conditional degradation of proteins tagged with an AID in budding yeast and human cells (Nishimura et al., 2009). In the presence of auxin, cells expressing TIR1 can degrade an AID-tagged green fluorescent protein (GFP) to completion within 1 h. The AID system has subsequently been used in fission yeast, *Caenorhabditis elegans* and *Drosophila melanogaster* (Kanke et al., 2011; Trost, Blattner, & Lehner, 2016; Zhang, Ward, Cheng, & Dernburg, 2015). Thus, the AID system can be used in a variety of organisms to manipulate protein function with acute temporal precision.

The AID system has additionally been shown to be active against a diverse set of cellular proteins, including those contained within macromolecular complexes, and is active in all phases of the cell cycle (Holland, Fachinetti, Han, & Cleveland, 2012). Importantly, the system is rapidly reversible, allowing AID-tagged proteins to begin reaccumulating within minutes of auxin removal. Nevertheless, a complete restoration of protein levels can take several hours and depends on the protein synthesis rate and half-life (Holland et al., 2012). In addition to the AID system, another plant-based degradation system, the JAZ degron system, has recently been transplanted into human cells (Brosh et al., 2016) (Fig. 1C and Table 1). While the JAZ system has slower kinetics of protein degradation than the AID system, it nevertheless allows for the use of two orthogonal, inducible degradation systems in the same cell.

1.3 FUTURE OPTIMIZATION OF THE AID SYSTEM

The AID system has been used to inducibly degrade a wide variety of proteins in human cells (Brosh et al., 2016; Fachinetti et al., 2015; Holland et al., 2012; Lambrus et al., 2015; McKinley et al., 2015; Natsume, Kiyomitsu, Saga, & Kanemaki, 2016;

Nishimura et al., 2009). However, while many AID-tagged substrates are degraded in <1 h, the destruction of some abundant substrates can take longer to reach completion (Holland et al., 2012). This raises the possibility of optimizing the efficiency of AID system to achieve more rapid and complete protein degradation. One approach is to screen for mutations in TIR1 or the AID that increases the affinity of the auxin bound complex and enhances the rates of protein degradation (Yu et al., 2013). An additional possibility is to exploit the diversity of TIR1 receptors and AUX/IAA substrates across the plant kingdom. In this case, distinct TIR1 and AUX/IAA degron pairs could be adapted to tune the kinetics of protein degradation. Indeed, substrate degradation rates in yeast have been shown to vary widely depending on the specific TIR1 receptor of AUX/IAA degron motif used (Havens et al., 2012). It is worth noting that while *Arabidopsis thaliana* TIR1 (*At*TIR1) could promote the degradation of AID-tagged proteins in yeast, it was not able to promote degradation of proteins in human cells (Nishimura et al., 2009). This is likely due to instability of *At*TIR1 at 37°C, driving the need to use the thermostable TIR1 cloned from rice *Oryza sativa* to achieve efficient protein destruction in human cells (Nishimura et al., 2009).

Like other inducible degradation systems, the AID system has some capacity for "leaky" protein destruction in the absence of auxin (Natsume et al., 2016). Crystallography has shown that auxin fills a hydrophobic cavity between TIR1 and the AID to generate a stable trimeric complex interacting through a continuous hydrophobic core (Dharmasiri et al., 2005; Kepinski & Leyser, 2005; Tan et al., 2007). However, TIR1 and the AID can also weakly associate in the absence of auxin. In principle, one way to reduce "leaky" protein degradation would be to introduce mutations that further destabilize the TIR1–AID complex in the absence of auxin. An additional approach is to inducibly express the TIR1 receptor immediately prior to auxin treatment (Natsume et al., 2016).

1.4 TAGGING APPROACHES

Several methods have been used to achieve inducible protein degradation with the AID system in mammalian cells. One approach involves the knockout or knockdown of an endogenous protein and functional replacement with an AID-tagged transgene (Brosh et al., 2016; Fachinetti et al., 2015; Holland et al., 2012). While powerful, this approach has some drawbacks, including incomplete depletion of the endogenous protein, a lack of representation of splice isoforms, and difficulty in achieving endogenous protein expression levels using a transgene. An alternative strategy that overcomes these limitations is to introduce the AID at the endogenous locus. This was first achieved in mammalian cells using adeno-associated virus-mediated gene targeting (Lambrus et al., 2015), but recently CRISPR/Cas9 genome editing has been used to more efficiently introduce the AID tag onto endogenous proteins (McKinley et al., 2015; Natsume et al., 2016).

Given the short window of time in which mitosis takes place, the ability to rapidly deplete proteins of interest is of considerable value to researchers aiming to understand pathways and macromolecular complexes that control mitotic progression. Indeed, the AID system has been used to provide insights into the function of the

spindle assembly checkpoint (SAC) (Han et al., 2013; Kumar, Dhali, Srikanth, Ghosh, & Srivastava, 2014; Rodriguez-Bravo et al., 2014), the kinetochore (McKinley et al., 2015; Wood et al., 2016), centromere (Fachinetti et al., 2015), cohesin (Natsume et al., 2016), and the Ran GTP/GDP gradient (Furuta, Hori, & Fukagawa, 2016). Here, we outline a CRISPR/Cas9-based method for the generation of AID-tagged target proteins in human cell lines and describe important considerations for the successful implementation of the AID system. While we specifically focus on the use of the AID system, the scheme outlined below can also be applied to modifying endogenous loci with any tag of interest, such as fluorescent proteins, epitope tags, and subcellular localization signals.

2 MATERIALS REQUIRED

- Expression plasmid for OsTIR1
  - pBabe Neo osTIR1-9Myc (Addgene #80072)
  - pBabe Blast osTIR1-9Myc (Addgene #80073)
  - pBabe Puro osTIR1-9Myc (Addgene #80074)
- PX459 plasmid encoding single-guide RNA (sgRNA) targeting desired genomic locus
  - PX459 V2.0 (Addgene #62988)
  - PX459 VQR (Addgene #101715)
  - PX459 VRER (Addgene #101716)
  - PX459 EQR (Addgene #101732)
  - PX458 (Addgene # 48138)
  - PX458 VQR (Addgene #101727)
  - PX458 VRER (Addgene #101728)
  - PX458 EQR (Addgene #101731)
  - PX330 (Addgene # 42230)
  - PX330 VQR (Addgene #101730)
  - PX330 VRER (Addgene #101729)
  - PX330 EQR (Addgene #101733)
- Plasmid template containing the mini AID tag
  - pcDNA5/FRT miniAID-EGFP (Addgene #101713)
  - pcDNA5/FRT EGFP-miniAID (Addgene #101714)
- Cells of choice for validation experiments and growth media for those cells
- Antibodies specific to the epitope tag of the OsTIR1 (i.e., Myc)
- Standard immunofluorescence and immunoblotting reagents
- PCR purification/concentration kit
- Transfection or nucleofection reagents

2.1 RECIPES

- Polyethylenimine (PEI) (1 mg/mL)
  - Dissolve PEI powder (25 kDa, linear) to a concentration of 1 mg/mL in water which has been heated to 80°C.

  - Allow solution to cool to room temperature. Adjust pH to 7.0 with 5 M HCl.
  - Filter–sterilize the solution through a 0.22 μm membrane.
  - Freeze aliquots at −80°C. Once thawed, keep at 4°C (stable for 2 months).
- Polybrene (10 mg/mL)
  - Dissolve to a concentration of 10 mg/mL in water.
  - Filter–sterilize the solution and freeze aliquots at −80°C. Once thawed, keep at 4°C (stable for 2 months).
- IAA sodium salt (500 mM)
  - Dissolve IAA sodium salt (Sigma # I5148) at a concentration of 500 mM in water.
  - Filter–sterilize the solution and freeze aliquots in opaque tubes at −20°C. Stable at 4°C for a few weeks. Keep protected from light.

3 METHODS

Two modifications are required to achieve an inducible loss of protein function with the AID system: (1) expression of the OsTIR1 F-box protein and (2) integration of the AID tag into both alleles of the gene of interest. These modifications can be made in either order, though we recommend to first generate a cell line with the endogenously AID-tagged protein of interest and then to integrate the OsTIR1 transgene. High OsTIR1 expression is critical for rapid and complete degradation of AID-tagged target proteins; however, for some target proteins, very high levels of OsTIR1 expression may promote "leaky" protein degradation in the absence of auxin. Integrating the OsTIR1 transgene after AID tagging the protein of interest offers more flexibility in identifying the optimal level of OsTIR1 expression for a specific target.

3.1 DESIGNING REAGENTS FOR SITE-SPECIFIC AID INTEGRATION

The CRISPR/Cas9 system is comprised of an RNA-guided nuclease that can be easily programmed to generate targeted DSBs in the mammalian genome. Here, we describe how to use the well characterized, two-component *Streptococcus pyogenes* (Sp) CRISPR/Cas9 system with engineered sgRNA for site-specific AID targeting. The specificity of SpCas9 targeting is determined by (1) Watson–Crick base pairing between the sgRNA and the genomic target sequence and (2) direct interactions between the SpCas9 protein and a short protospacer adjacent motif (PAM) in the genomic target sequence (Fig. 2A) (Mojica, Diez-Villasenor, Garcia-Martinez, & Almendros, 2009; Shah, Erdmann, Mojica, & Garrett, 2013). Reprogramming the SpCas9 to target a site is as simple as changing its guide RNA sequence. Upon recognizing a target sequence, SpCas9 cleaves the DNA 3-nt upstream of the PAM to produce a blunt-ended DSB (Fig. 2A), which is then repaired either by error-prone nonhomologous end-joining (NHEJ) or by homology-directed repair (HDR). For the purposes of integrating an AID tag at a specific genomic locus, we require cells to undergo HDR using a provided repair template. In the following sections, we describe how to design the guide RNA and the repair template.

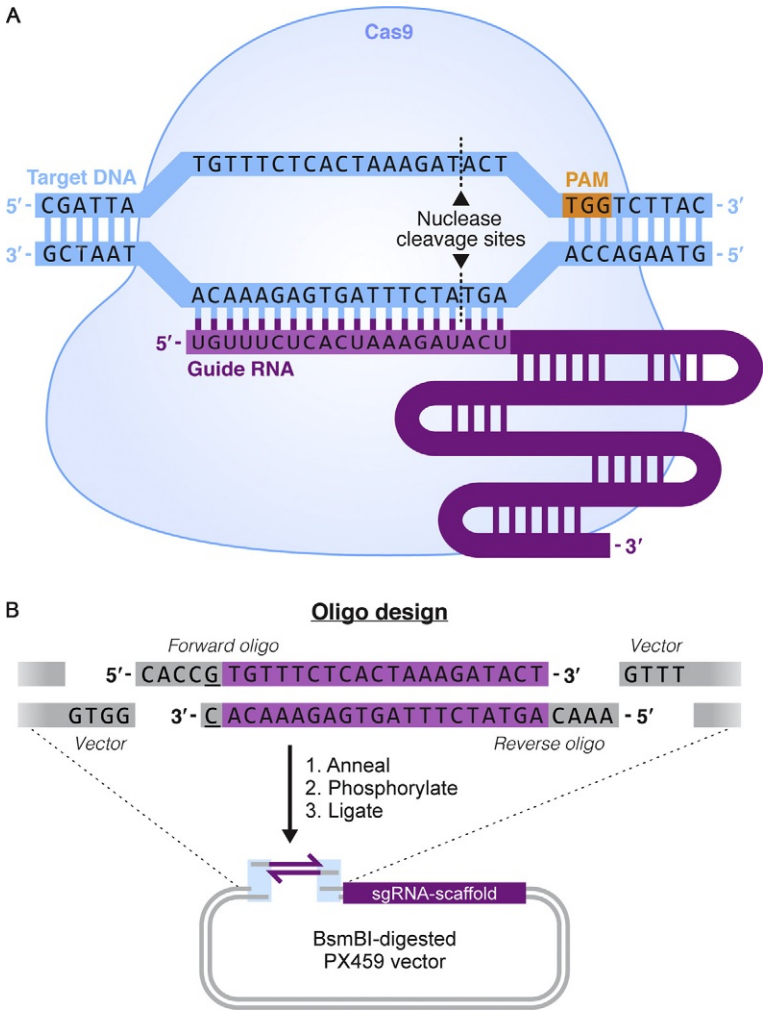

FIG. 2

CRISPR/Cas9 system and sgRNA cloning strategy. (A) Schematic of Cas9 in complex with a sgRNA, targeted to its complementary DNA sequence. Relative locations of the PAM motif (shown in *orange*) and nuclease cleavage sites are indicated. (B) Diagram of oligonucleotide design for sgRNA cloning into PX459. A 20-nt oligonucleotide sequence is chosen that targets the desired genomic sequence. If this sequence does not begin with a "G," then a G should be appended to maximize expression from the U6 promoter (shown *underlined*). Overhangs must then be added to the oligos, with sequences complementary to those left by BsmBI digest of the PX459 vector. Oligos are then resuspended, annealed, and phosphorylated, after which they are ready for ligation into BsmBI-digested PX459.

3.1.1 Choice of Cas9/sgRNA delivery system

The SpCas9 and sgRNA can either be expressed from a plasmid or introduced into cells as a complex of purified SpCas9 protein and sgRNA (Lin, Staahl, Alla, & Doudna, 2014; Paix, Folkmann, Rasoloson, & Seydoux, 2015). Here we describe the plasmid approach, as it is the most accessible method and offers a simple means of selecting cells expressing the SpCas9 protein.

A variety of plasmids are available for SpCas9/gRNA expression in mammalian cells. Here we use the PX459 v2.0 vector (available from Addgene, #62988), which expresses SpCas9, a user-specified sgRNA and a puromycin resistance gene.

3.1.2 Designing a guide RNA for sequence-specific DNA cleavage by SpCas9

The first step of sgRNA design is to determine the location of the AID tag. It is important that the tag does not impair normal localization, stability, or function of the target protein. The minimal degron, called the mini AID (mAID) is only 5 kDa, and is normally appended to the N- or C-terminus of the protein (Brosh et al., 2016). In our experience, proteins that are functional when tagged with GFP are also functional when tagged at the same site with mAID, though this should be empirically determined for each protein. To increase the probability of obtaining a functional AID-tagged protein, we recommend designing a strategy for tagging both the N- and C-termini of the protein.

Once the location of the AID tag has been determined, follow the steps below to design a sgRNA to direct cleavage at the specific genomic site.

1. Download the genomic sequence of the target gene from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/gene).
2. Identify the position of desired AID insertion and copy the sequence of 50 nucleotides on either side of this site.
3. Visit http://crispor.tefor.net/ (Haeussler et al., 2016) and paste the copied sequence into the query box. Next, select the appropriate genome from the drop-down menu (e.g., *Homo sapiens*) and select the PAM motif for the SpCas9 that is to be used (e.g., 20 bp-NGG-Sp Cas9). Hit submit.
4. The results will show a list of the potential genomic targets for sgRNA recognition, ranked by their computed "specificity score." This score reflects the likelihood of off-target activity against other regions of the genome and is based on the number and position of mismatches in the sgRNA (Hsu et al., 2013). High specificity scores are shown in green. While a high-scoring sgRNA is desirable to reduce the likelihood of off-target mutations, another key consideration is that HDR efficiency decreases with increasing distance from the cut site. Therefore, it is important to minimize the distance between the cut site and the desired insertion site. Taking these two parameters into account, we recommend choosing a sgRNA that has at least four base pair mismatches to any other sequence in the genome and promotes cutting at <20 nt from the site where the tag is introduced. For some genomic target sequences, it is not possible to achieve these parameters, in which case we select the highest scoring sgRNA

that directs cutting within 20 nt on either side of where the AID tag is to be inserted. In cases where an "NGG" PAM sequence is not available close to the site of the desired insertion, it may be possible to use PX459 expressing a modified version of SpCas9 with altered PAM specificity (SpCas9 variants available from Addgene: VQR #101715, VRER #101716, EQR #101732) (Kleinstiver et al., 2015).

5. If multiple sgRNA sequences are available for use, we recommend selecting 2–3 sgRNAs and testing each for cleavage efficiency using the SURVEYOR® Mutation Detection Kit (Transgenomic), see Ran et al. (2013).
6. To determine cloning primers for the chosen sgRNA, click "Cloning/PCR primers" under the desired guide sequence in the CRISPOR web interface. Then select the destination Addgene plasmid (PX330 and derivatives) to view the sequences necessary for cloning into PX459. Alternatively, manual oligo design is described here:
   a. Copy the 20-nt sequence of the chosen genomic target sequence. The PX459 vector uses a U6 promoter to transcribe the sgRNA and this requires that a G be the first nucleotide in the transcript. In cases where the genomic target sequence does not begin with a G, append an extra G at the 5′ end of the sgRNA (Fig. 2B).
   b. Generate the reverse complement of the genomic target sequence (including the 5′ G if it was added) (Fig. 2B).
   c. Add the overhang sequence 5′-CACC-3′ to the 5′ end of genomic target and the sequence 5′-AAAC-3′ to the 5′ end of the reverse complement (Fig. 2B). These sequences will produce the correct overhangs for cloning into the PX459 vector.
7. Order single-stranded DNA (ssDNA) oligonucleotides for the two sequences generated in step 6.

3.1.3 Cloning oligonucleotides into the PX459 vector

The PX459 vector contains two BbsI cleavage sites that allow for the insertion of annealed oligonucleotides containing the sgRNA target sequence. BbsI cleaves DNA outside of its recognition site to produce overhangs complementary to those added in step 8 above. Below we describe how to clone the sgRNA into the PX459 expression vector.

Vector preparation

1. Digest 1 μg of the PX459 vector with BbsI at 37°C for 2 h.
2. To remove terminal phosphates, add 0.1 μL of calf intestinal phosphatase to the reaction and incubate at 37°C for 30 min.
   - Since the two overhangs produced following BbsI digestion are not complementary, this step is not required, but usually reduces background.
3. Purify the cut vector using a standard PCR cleanup kit. The purified linear vector can be stored at −20°C until ready for use.
   - Note that BbsI digestion of PX459 produces a small 22-nt fragment that will pass through the column, leaving a 9153-nt, linear piece of vector DNA.

Oligonucleotide annealing

4. Combine:
   - 43 µL molecular biology grade H_2O
   - 1 µL of each oligonucleotide from a 100 µM stock
   - 5 µL of New England Biolabs Buffer 3 (www.neb.com)
5. Anneal oligonucleotides in a thermocycler with the following protocol:
   (a) 4 min at 95°C
   (b) 10 min at 70°C
   (c) Cool to 4°C at 1°C/min

Annealed oligonucleotides can be stored at −20°C until ready for use.

Oligonucleotide phosphorylation

We use T4 PNK from New England Biolabs to add terminal phosphates to the annealed oligonucleotides.

6. Combine:
   - 5 µL molecular biology grade H_2O
   - 2 µL of the annealed oligonucleotides from above
   - 1 µL T4 PNK buffer (www.neb.com)
   - 1 µL ATP (10 mM stock)
   - 1 µL T4 PolyNucleotide Kinase (PNK) (www.neb.com)
7. Allow reaction to proceed in a thermocycler with the following protocol:
   (a) 30 min at 37°C
   (b) 10 min at 70°C (to inactivate PNK)
   (c) Quick cool to 4°C

Phosphorylated oligonucleotides can be stored at −20°C until ready for use.

Ligation and transformation

We use a 2X stock of Takara T4 DNA Ligase (DNA ligation kit, Version 2.1) and homemade TOP10 competent cells.

In a 0.5 mL tube, combine the following:
- 1 µL BbsI digested vector
- 4 µL phosphorylated annealed oligonucleotides
- 5 µL 2X Takara T4 Ligase

8. As a control, set up the same reaction as above but substitute 4 µL molecular biology grade H_2O for the oligonucleotides.
9. Allow ligation to proceed for 1 h at room temperature.
10. Hand thaw a frozen aliquot of competent bacteria and keep on ice.
11. Add the entire 10 µL reaction mixture to the competent bacteria and incubate on ice for 20–30 min.
12. Heat shock the bacteria for 1 min at 42°C and return to ice for at least 1 min.
13. Plate the bacteria on prewarmed ampicillin (or carbenicillin) agar plates and incubate at 37°C for 16 h. A successful ligation should produce few (if any)

colonies on a control (vector alone) plate and many fold more colonies on an experimental (vector with insert) plate.

14. Select a colony from the experimental plate and prepare a 1–5 mL culture in LB containing ampicillin. Shake at 37°C for at least 16 h and perform a standard plasmid purification.
15. To check for correct oligonucleotide insertion, sequence the plasmid using the U6 forward primer (5′-ACTATCATATGCTTACCGTAAC-3′).
16. PX459 plasmid DNA containing a correctly cloned sgRNA can be stored at −20°C until ready for use.

3.1.4 Design of a repair template

To integrate AID into the site-directed DSBs generated by SpCas9, cells must undergo HDR, using a repair template containing the mAID tag flanked by homology arms to the adjacent genome sequence (Fig. 3A). Repair templates can be ssDNA or double-stranded DNA (dsDNA) with homology arms flanking the cut site. The optimal length of the homology arms is an area of ongoing study; we have had success with ~500 bp homology arms, but recent work suggests that short, 35-bp homology arms may be sufficient (Paix et al., 2017). In this case, a dsDNA repair template can be generated by PCR-amplification of the mAID tag from a plasmid template with primers that include the homology arms (Fig. 3B). We recommend include a ~10 aa flexible linker sequence between the terminus of the gene and the mAID tag to reduce the possibility of the tag disrupting protein function (Chen, Zaro, & Shen, 2013).

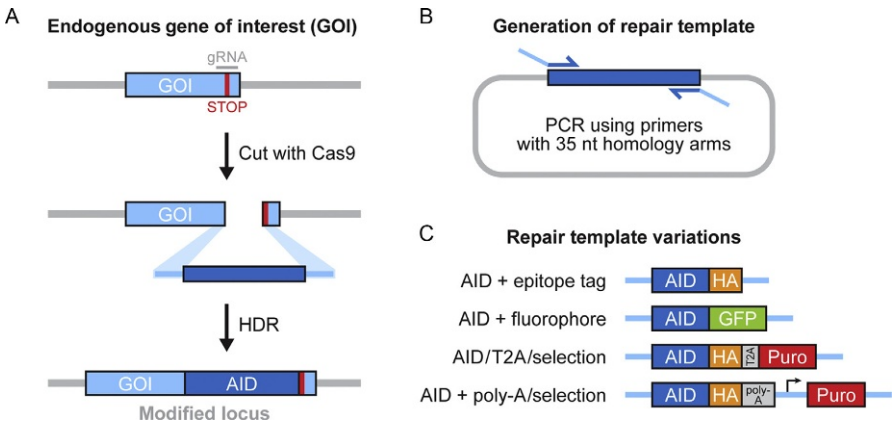

FIG. 3

HDR and design of repair templates. (A) An overview of Cas9-facilitated homology-directed repair (HDR). (B) A schematic showing how HDR repair templates are easily generated by PCR, using custom oligos that encode 35-nt homology arms to the gene of interest. (C) Various tags and selection cassettes can be used in combination with the AID, offering different options for isolating edited cells and detecting tagged endogenous protein.

A further consideration at this point is the addition of fluorophores, tags, or antibiotic resistant cassettes for downstream detection of positive clones. These tags can be appended to the AID tag by cloning the tag into the AID template plasmid prior to PCR amplification of the tag cassette. We recommend integrating an EGFP-mAID or mAID-EGFP tag, to streamline downstream identification of positive clones by fluorescence-activated cell sorting (FACS). The EGFP-mAID and mAID-EGFP template plasmid are available on Addgene (#101714 and #101713). An additional option is to include a T2A self-cleaving peptide and promoter-less antibiotic resistance gene in the repair construct, so that the tagged target protein and the selectable marker are expressed from the same transcript under the control of the endogenous gene promoter. Correctly targeted clones can then by identified by antibiotic selection. Some examples of repair constructs are shown in Fig. 3C.

It is critical that the repair template should carry a mutated PAM site to prevent cutting by SpCas9 after HDR. Mutation of the "NGG" sequence is a robust way to prevent recognition by SpCas9. If it is not possible to make silent mutations that disrupt the PAM, an alternative approach is to make at least four silent mutations within the guide RNA-recognition sequence, preferably close to the PAM (Hsu et al., 2013). Finally, depending on the position of the cut site, tag integration itself may disrupt the PAM or sgRNA-binding site to prevent further SpCas9 recognition.

In the example below, we outline the steps for designing the PCR-amplified dsDNA repair template for introducing an N-terminal EGFP-mAID tag.

Designing primers containing homology arms

1. Identify the specific site of cleavage by SpCas9 in the genomic target sequence. This will be 3-nt's upstream of the PAM sequence (Fig. 2).
2. Select 35 nucleotides on either side of the cut site to act as the homology for the repair template.
3. Design primers to amplify EGFP-mAID and append the homology arms to the primers. For example, the forward primer should consist of (5′-3′): 35-nt of the 5′ homology arm, then the EGFP-amplifying sequence (Fig. 3B).

Mutation of the PAM site

4. Identify the PAM in the repair template and replace one of the two G bases with a C or T to create a silent mutation. For SpCas9, the "NGG" PAM sequence can be mutated to anything other than "NAG" to prevent SpCas9 cleavage (Hsu et al., 2013; Jiang, Bikard, Cox, Zhang, & Marraffini, 2013).

PCR amplification of HDR template

5. Order the finalized ssDNA primers from IDT (http://www.idtdna.com/site).
6. Follow standard PCR procedures to amplify the EGFP-mAID tag using the primers designed earlier and purify the product using a PCR clean-up kit.
   - Note that to achieve a highly concentrated PCR product, we combine the products from eight separate PCR reactions together and purify them using the MiniElute PCR purification kit from Qiagen.

3.2 TRANSFECTION AND SCREENING OF AID-TAGGED CLONAL LINES

For genome-editing experiments, we recommend selecting a stably diploid cell line as this simplifies the process of achieving homozygous gene targeting. While we have successfully targeted aneuploid cell lines using the CRISPR/Cas9 system, determining the genotype of the resulting clones is more complex. Since puromycin is used to achieve rapid killing of cells that do not receive the PX459 expression vector, it is important that the chosen cell line is also puromycin sensitive. An alternative approach is to use the PX458 expression vector (available from Addgene #48138) that coexpresses SpCas9, sgRNA, and GFP, allowing fluorescent transfected cells to be directly sorted into individual wells of a 96-well plate.

There are several methods for delivering DNA to mammalian cells in culture. Here, we describe a method using Roche's X-tremeGENE 9 transfection reagent. Depending on the cell line to be used, other DNA delivery methods (e.g., nucleofection) may be required.

Day 1

1. Seed cells for transfection at 2×10^5 cells/well in 2 mL of media in a 6-well plate. Seed at least two wells per transfection, with one of these wells serving as a nontransfected control. Transfections are carried out in the presence of serum and, if desired, antibiotics.

Day 2

2. Change media on cells 30 min prior to transfection.
3. In a 1.5 mL tube prepare the following for each transfected well in the order written below:
   - 100 μL serum-free media at room temperature
   - 3 μL X-tremeGENE 9 transfection reagent
   - 1 μg of PX459 plasmid
   - ≥20:1 M ratio of purified repair dsDNA template:PX459 plasmid
4. Flick tube gently 10× to mix.
5. Incubate at room temperature for 15–20 min.
6. Add the transfection mixture dropwise to cells and return cells to the incubator.

Day 4

7. Change media on all cells (including controls) with fresh media containing puromycin (1–5 μg/mL is usually sufficient). Return cells to the incubator.
   - The PX459 plasmid contains a puromycin resistance marker to select for transfected cells.
8. Cells in the untransfected control wells should all die within 1–2 days in puromycin. Once control cells have died, proceed to isolate clones, described below.
   - Cells should not remain in puromycin for more than 2–3 days, as the PX459 plasmid does not integrate into the genome and resistance to puromycin is lost over time.

3.2.1 Isolation of clonal lines

After puromycin selection, single cell clones should be isolated by cell sorting or limiting dilution. If inserting EGFP-mAID, sorting for green fluorescence by FACS will greatly reduce the number of colonies to screen. For nonfluorescent tags, obtain clonal lines by either cell sorting or limiting dilution. Below we outline how to obtain single clones using dilution cloning.

Cells are diluted across 96-well plates to obtain wells containing single cells. Since the clonogenic survival of cell lines varies greatly, we recommend seeding multiple 96-well plates with varying cell densities (1, 3, 10, and 30 cells/well). A 96-well plate that has growth in 10% of the wells will have a ~90% probability of a given well having a single colony.

1. Add 15 mL of puromycin-free media to a sterile reagent reservoir.
2. Add the desired number of cells to the media. For example, to achieve 10 cells/well, 1000 cells would be added to the media.
3. Mix the cells in the media by pipetting up and down 5 × with a 10 mL pipette.
4. Using a multichannel pipette, add 150 μL of the cell suspension to each well of the 96-well plate.
5. Repeat steps 1–4 for each cell density.
6. Wrap the 96-well plates in plastic film and return to the incubator. Allow 2–3 weeks for colonies to grow.
   a. At ~10 days, colonies will be easily identifiable. We recommend to visually screen the wells for single colonies at this early stage, to be confident of clonality. At later stages, multiple colonies merge and become indistinguishable from single clones.

Allow 2–3 weeks for single cells to grow into colonies, then expand into larger wells for immunoblot analysis or genomic DNA extraction for PCR analysis (described below). If analyzing by immunoblot, correctly targeted clones can be identified by a band shift in the endogenous protein corresponding to the size of the integrated mAID tag. Alternatively, clones that have integrated the mAID tag at the correct location can by identified by PCR analysis. In both cases, it is important to identify clones with homozygous targeting of the mAID tag. The process of extracting genomic DNA for PCR analysis is described below.

3.2.2 Genomic DNA extraction

To extract genomic DNA from a small number of clones, we use the Sigma GenElute Mammalian Genomic DNA Miniprep Kit (G1N350). For extracting genomic DNA from larger numbers of clones we use a protocol adapted to use with 96-well plates and outlined below.

1. Using a tissue culture microscope, identify wells containing a single colony. Transfer 96 individual clones into 24-well plates. Return cells to the incubator.
2. When the clones are confluent, trypsinize cells in 200 μL of 0.05% trypsin.

3. Remove 160 µL of the trypsinized cell suspension and place into a single well of a 96-well plate with U-bottom wells.
4. Add 1 mL of media to the cells remaining in the 24-well plates and return plates to the incubator.
5. Spin the 96-well plate at 2000 RPM in a swinging-bucket rotor for 10 min to pellet cells.
6. To remove supernatant, quickly invert the plate to remove media and remove excess liquid by blotting on paper towels.
7. Resuspend cells in each well with 150 µL PBS and spin at 2000 RPM for 10 min. Remove PBS as in step 6.
8. To lyse cells, add 50 µL of lysis buffer (10 mM Tris–HCL pH 7.5, 10 mM EDTA, 0.5% SDS, 10 mM NaCl, 1 mg/mL Proteinase K) and seal the plate with parafilm. Place the plate into a humidified chamber at 60°C overnight. A humidified chamber can be created by placing a few inches of water in a small plastic container with a sealable lid. The 96-well plate is placed in the sealed container on top of a test tube rack so that it rests above the water level.
9. The next day, remove the plate from the humidified chamber and cool to room temperature.
10. Add 100 µL ice-cold EtOH/NaCl mix (75 mM NaCl in ~100% EtOH; forms a cloudy solution) to precipitate DNA and mix well.
11. Incubate at room temperature for 30 min.
12. Spin at 4000 RPM in a swinging-bucket rotor for 20 min to pellet precipitated DNA.
13. Decant liquid as in step 6.
14. Rinse pellet with 150 µL cold 70% EtOH and spin for 10 min at 4000 RPM.
15. Decant liquid as in step 6.
16. Repeat washing step (#14–15) and air-dry DNA for 10 min.
17. Add 50 µL TE pH 8.0 (10 mM Tris–HCl pH 8.0, 1 mM EDTA) to genomic DNA pellet.
18. Cover plate with parafilm and incubate at 50°C for 2 h.

Genomic DNA is ready for further screening or may be stored at 4°C until ready for use.

3.2.3 Screening

HDR can be tracked by PCR amplification of genomic DNA. Forward and reverse genomic primers are designed to bind to regions of the genome outside of the homology arms of the repair template to amplify a region of 250–500 nt. Primers are designed using the Primer3 program (biotools.umassmed.edu). We recommend screening at least three different forward and reverse genomic primers in all possible combinations to identify a primer pair that yields a strong and specific PCR product from low quantities of DNA. The genomic primer pair will amplify the untagged allele, and can also amplify a modified locus that incorporated a small tag

(e.g., mAID alone or mAID-HA), which would appear as a band of increased size. If the incorporated insert is large, however, a third tag primer should also be designed that will bind to a sequence contained within the mAID tag. In combination with the opposing genomic primer, this primer will amplify a band specific for the mAID-targeted allele. The following sequence has worked for us in the past with the mAID tag: 5′-CCGCTAGACTTCTGACAGG-3′. When selecting primers, keep in mind that the tag-specific primer pair should amplify a different-sized product to that amplified from the untagged allele with the genomic primer pair (Fig. 4A). In this way, homozygous untagged, heterozygous mAID-tagged, and homozygous mAID-tagged clones can be readily distinguished.

In most cases, the PCR protocol outlined below produces good results. However, PCR optimization may be required.

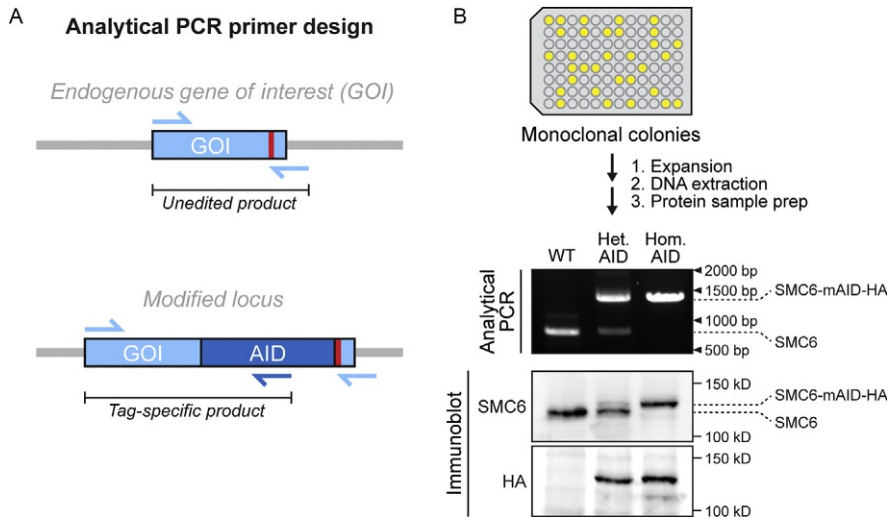

FIG. 4

Analysis of edited cell lines. (A) Analytical primers are designed to amplify a small region around the unedited, endogenous site. These primers may also be sufficient to detect modified loci that incorporate small tags (e.g., AID alone or AID-HA), which would be observed as a product of larger size. To detect the recombination of larger inserts, however, it is recommended to design a primer within the tag itself, which would yield a tag-specific product only from edited cells. (B) After expanding monoclonal colonies to enable collection of DNA and protein sample, clones can be analyzed by analytical PCR and immunoblot. The example shown here depicts results from successful editing of endogenous SMC6. PCR amplification using primers that amplify a small region around the edited site results in expected products for WT, heterozygous (Het.), and homozygous (Hom.) clones, where inclusion of the mAID-HA tag yields a larger PCR product. The SMC6 immunoblot likewise shows increased protein size from fusion of the mAID-HA tag, and the HA immunoblot shows successful tagging with HA epitope.

PCR amplification

1. Prepare a master mix (100 reactions per plate) as follows:

• Molecular biology grade H_2O	1060 µL
• 5X GC buffer (www.neb.com)	400 µL
• Forward genomic primer (10 µM stock)	100 µL
• Reverse genomic primer (10 µM stock)	100 µL
• Forward or reverse tag primer (10 µM stock)	100 µL
• dNTPs (stock with each dNTP at 10 mM)	40 µL
• Phusion polymerase (www.neb.com)	40 µL

2. Dispense 18 µL of the master mix to each well of a 96-well plate.
3. Add 2 µL of genomic DNA from individual clones to each well of the 96-well plate.
4. Cover the plate with a piece of sealing film and perform a PCR reaction using the following parameters:

(a) 98°C	30 s
(b) 98°C	10 s
(c) 57°C	15 s
(d) 72°C	15 s
(e) Repeat steps (b)–(d)	34X
(f) 72°C	5 min
(g) 12°C	hold

5. Run products on an agarose gel and image on a gel documentation system. Identify positive clones by the relative size of the PCR amplicons (Fig. 4B).

There are three possible results for each PCR reaction.

1. Band corresponding to the size of the PCR product amplified by the untagged allele only. This reflects the size of the endogenous sequence, which did not integrate the mAID tag. It is possible that NHEJ occurred, resulting in the creation of insertions or deletions (InDels) that prevent further cutting by SpCas9.
2. Band corresponding to the size of the PCR product amplified by the tagged allele only. This clone is homozygous for insertion of the mAID tag.
3. PCR products amplified by both the tagged and untagged allele. There are two explanations for this result. (1) The clone is heterozygous. One allele integrated the tag by HDR and the other did not. In most cases the untagged allele will have undergone NHEJ, which may generate frameshift mutations. If you are adding an amino terminal, mAID tag and the guide RNA was designed to cut in the coding sequence of the target gene, it is possible for cells to carry a tagged allele and a frameshifted null allele. In this case, all the endogenous protein

carries the mAID tag and the clone may be suitable for further analysis. (2) An alternative possibility is the cells are polyclonal. This may occur when two or more parental cells grow in a single well.

3.2.4 Sequencing clones

To verify insertion of the desired mutation, the PCR product can be cloned into a vector for sequencing. We recommend using the ZeroBlunt® TOPO® Cloning Kit from Invitrogen, which allows for easy cloning of blunt end PCR products into the pCR-Blunt II-TOPO vector. We usually sequence ~10 clones to ensure sequence coverage of both alleles.

Clones that are homozygous for the mAID tag can be taken forward for further analysis. Heterozygous clones that are tagged in one allele, but carry a functional second allele, may serve as useful controls.

3.3 GENERATION OF AN OsTIR1 CELL LINE

Once a cell line has been produced in which both endogenous alleles are tagged with a mAID, we then integrate the OsTIR1 protein and isolate monoclonal cell lines. An important consideration is that low expression of OsTIR1 can be rate limiting to the degradation of AID-tagged targets, and therefore it is recommended to screen several clones by immunoblot and select a fast-growing and high-expressing OsTIR1 clone. Plasmids encoding osTIR1 are available from Addgene (Neo #80072, Blast #80073, Puro #80074).

3.3.1 Production of OsTIR1 retrovirus

1. Coat a 10 cm dish with poly-L-lysine. Incubate for 30 min at room temp. Remove and rinse once with 1 × PBS.
2. Seed 3×10^6 HEK293GP cells. Allow to settle overnight.
3. 24 h later, prepare the following transfection cocktail:
   - 600 μL Opti-MEM
   - 35 μL (10 mg/mL) PEI
   - 4.5 μg pBabe OsTIR1 retrovirus plasmid
   - 2–3 μg VSV-G plasmid
4. Mix gently and incubate 20 min at RT.
5. Add dropwise to existing media on HEK293GP cells.
6. Next day, replace with fresh medium.
7. Incubate for 48 h.
8. Collect virus-containing media and filter through a 0.45 μm filter to remove cells.
9. Viral supernatant may be saved as 1 mL aliquots and snap frozen for later use.

3.3.2 Viral transduction of OsTIR1 into cells

1. Seed two wells of a 6-well plate with 2×10^5 cycling cells. One of these wells will serve as a control for antibiotic selection. Allow cells to settle overnight.
   - Retroviral integration requires nuclear envelope breakdown; ensure cells are cycling at the time of transduction.
2. The next day, aspirate media and add back 1 mL complete media.

3. Add 1 mL of viral supernatant to the well. To the selection control, add 1 mL complete media. Add polybrene to a final concentration of 10 μg/mL, and swirl to mix. Place back in incubator and allow 2 days for expression.
   - Some cell lines are sensitive to polybrene. To avoid toxicity, it is recommended to replace with fresh media after overnight incubation with virus.

3.3.3 Selection and Isolation of OsTIR1-Expressing cells

Two days after viral transduction, cells are ready to undergo antibiotic selection.

1. Add antibiotic to transduced and control wells, and maintain selection until all control cells have died (this will take several days, depending on cell line and antibiotic used).
2. The surviving cells should express OsTIR1. If cells are sparse, provide fresh media to allow for recovery and allow several days to grow to reasonable density.

Following selection, single cell clones should be isolated and screened to identify cells expressing high levels of OsTIR1. Either single cell sorting or dilution cloning (see above) may be used to isolate single cells.

3.3.4 Screening for high-expressing OsTIR1 clones

Once clonal OsTIR1 lines have grown, they must be expanded and screened to identify a high-expressing clone. We use the pBabe OsTIR1 construct with C-terminal 9 × Myc, and immunoblot against Myc to compare expression. We describe our procedure for harvesting samples, below.

1. Identify ~6–24 of the fast-growing monoclonal lines from the 96-well plates, and expand them into 12-well plates. Allow them to grow to confluence.
2. Once the 12 wells are confluent, trypsinize the cells, resuspend in 1 mL media, and transfer 700 μL of the suspension to microfuge tubes.
3. Centrifuge at 500 *g* for 5 min to pellet cells. Remove the supernatant and resuspend the sample in an appropriate volume of 2 × sample buffer.
4. Boil the sample at 95°C for 10 min.
5. Run lysates on an SDS-PAGE gel, transfer onto nitrocellulose, then block and blot the membrane with primary antibody against Myc (Millipore Sigma; Anti-Myc Tag clone 4A6; 05-724). Once expression of OsTIR1 is visualized, select the highest expressing clone to expand for AID tagging.
   - Very high expression of OsTIR1 may interfere with the degradation of endogenous SCF substrates and slow cell cycle progression. For this reason, we isolate only the fast-growing monoclonal cell lines.

4 FUNCTIONAL ANALYSIS

4.1 VALIDATION OF mAID-FUSION PROTEIN IN CELLS

As the goal of AID-tagged degradation of proteins is to test the function of the endogenous protein in its normal context, the expression, localization, and function of the mAID-tagged protein should be validated. Expression of a protein with the

correct molecular weight can be validated by immunoblotting, and protein abundance can be compared to endogenous levels. Localization can be tested by standard immunofluorescence techniques. Function of the mAID-tagged protein can be evaluated by comparison to known loss-of-function phenotypes. For example, to analyze the in vivo requirements of BubR1 and Mad2, two SAC components, Han and colleagues suppresses endogenous Mad2 or BubR1 with siRNA and expressed physiological levels of an AID-tagged transgene (Han et al., 2013). The AID-tagged transgenes supported the functionality of the SAC, but led to an inducible null phenotype rapidly after addition of auxin.

4.2 TESTING INDUCIBLE DEPLETION OF mAID-TAGGED PROTEIN

Responsiveness of mAID-fusion proteins to auxin induction can be assayed by immunofluorescence and immunoblotting. Recommended assay conditions are described below.

Immunofluorescence assay

1. Prepare 6× glass coverslips in a 12-well plate.
2. Seed mAID-tagged OsTIR1 cells onto coverslips. Allow cells to attach overnight.
3. Next day, prepare an auxin (IAA) timecourse: add in 500 μM IAA to treat cells for a total of 5 min, 10 min, 30 min, 1 h, and 2 h. Leave one coverslip as the untreated control.
4. At the end of the timecourse, rinse cells with 1× PBS, and fix for 10 min at room temperature in 4% formaldehyde (FA) in 1× PBST.
5. Proceed with standard immunostaining procedures. Probe for either endogenous protein or epitope tag, and quantify signal across conditions.

Immunoblotting assay

1. Seed mAID-tagged OsTIR1 cells in six wells of a 12-well plate, such that cells are close to confluent the next day. Allow cells to attach overnight.
2. Next day, prepare an auxin (IAA) timecourse: spike in 500 μM IAA to treat cells for a total of 5 min, 10 min, 30 min, 1 h, and 2 h. Leave one well as the untreated control.
3. At the end of the timecourse, add 100 μL 2× SB to each well to harvest lysates for immunoblotting.
4. Boil samples at 95°C for 10 min.
5. Proceed to run samples following usual SDS-PAGE procedures. Blot for either endogenous protein or epitope tag, and quantify signal across conditions.
   a. Protein abundance and degradation kinetics can be tuned by altering the concentration of IAA used (Lambrus et al., 2015).

5 USEFUL TIPS

5.1 BIALLELIC TAGGING

- If only heterozygous clones are obtained, in which case usually the nontagged allele has been repaired by NHEJ (and is resistant to cutting by the original

sgRNA), it may be possible to design an updated sgRNA to target the nontagged allele. Electroporating the heterozygous line with the PX459 vector encoding the new sgRNA provides another opportunity to undergo HDR integration of the mAID at the nontagged allele.
- To enrich for biallelic tagging, it is possible to supply two repair templates, such as AID-EGFP and AID-mCherry. A fraction of cells will incorporate AID-EGFP at one locus and AID-mCherry at the other, and these double-positive cells can be identified and isolated by FACS.

5.2 IAA REAGENT

- We recommend using IAA sodium salt (Sigma I5148) as it can be directly dissolved in water to a working stock of 500 mM. The nonsodium salt requires initial solubilization with ethanol and NaOH.
- IAA is light-sensitive, and stock solutions should be kept protected from light. The 500 mM aqueous stock is normally a light-tan solution and darkening of this color indicates deterioration of the stock.

6 TROUBLESHOOTING

6.1 LACK OF EDITED CLONES

- Ensure transfection or electroporation protocol is effective by testing with a GFP-expressing control plasmid.
- The tagged version of the protein may not function normally and therefore cannot support viability. Perform an RNAi and add back using the tagged version of the protein to test if it can rescue a known phenotype(s).
- Editing frequency varies by cell line; one could have to screen several 100 clones to observe editing. To improve odds, HDR can be promoted by maximizing the likelihood that cuts are generated in S/G2 phase, as NHEJ is the default repair mechanism in G1. Variations of SpCas9 plasmids are available that provide cell cycle-specific expression of Cas9 or cells can be synchronized prior to electroporation (Gutschner, Haemmerle, Genovese, Draetta, & Chin, 2016; Lin et al., 2014).
- Use the SURVEYOR® assay to ensure the sgRNA promotes efficient cutting of the target locus.

6.2 AID-TAGGED PROTEIN IS NOT DEGRADED UPON INDUCTION WITH AUXIN

- Identify and test a high-expressing OsTIR1 clone. Higher expression of OsTIR1 may be required, especially if the AID-tagged protein is of high abundance.
- Test if higher concentrations of IAA will improve responsiveness.
- This is sometimes observed when a mAID-tagged transgene is expressed from a plasmid using transient transfection. Transient transfections often produce

extremely high levels of protein expression that can overwhelm the SCF^{OsTIR1} complex. We recommend using stable expression of the mAID-tagged transgene.

6.3 AID-TAGGED PROTEIN IS UNSTABLE

- Test if the protein is stable when tagged at the targeted terminus with a GFP tag. It is possible that tagging prevents appropriate folding or stabilizing interactions with binding partners. If so, increasing linker length may help.
- High levels of OsTIR1 expression may cause leaky degradation of AID-tagged proteins. Test a clone with lower levels of OsTIR1 or consider using a doxycycline-inducible OsTIR1 expression system.

7 CONCLUSION

The AID system offers a rapid, inducible, and reversible system to achieve protein depletion across a range of organisms and cell types. Several groups have successfully implemented the AID system to uncover novel insights into pathways and protein complexes that control progression through mitosis (Fachinetti et al., 2015; Furuta et al., 2016; Han et al., 2013; Kumar et al., 2014; McKinley et al., 2015; Natsume et al., 2016; Rodriguez-Bravo et al., 2014; Wood et al., 2016). Here we described a CRISPR/Cas9-based method for the generation of biallelic, AID-tagged genes in mammalian cells. While this method is specific to the AID, the repair strategy can be applied to other tags of interest, including fluorescent proteins or epitope tags. A current limitation of the approach outlined here is the relatively low frequency of HDR compared to NHEJ in most established cell lines. Future advances in genome engineering are likely to offer methodologies for achieving higher efficiencies of HDR and biallelic tagging.

REFERENCES

Banaszynski, L. A., Chen, L. C., Maynard-Smith, L. A., Ooi, A. G., & Wandless, T. J. (2006). A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. *Cell*, *126*(5), 995–1004. https://doi.org/10.1016/j.cell.2006.07.025.

Bartlett, D. W., & Davis, M. E. (2006). Insights into the kinetics of siRNA-mediated gene silencing from live-cell and live-animal bioluminescent imaging. *Nucleic Acids Research*, *34*(1), 322–333. https://doi.org/10.1093/nar/gkj439.

Bishop, A. C., Shah, K., Liu, Y., Witucki, L., Kung, C., & Shokat, K. M. (1998). Design of allele-specific inhibitors to probe protein kinase signaling. *Current Biology*, *8*(5), 257–266.

Bonger, K. M., Chen, L. C., Liu, C. W., & Wandless, T. J. (2011). Small-molecule displacement of a cryptic degron causes conditional protein degradation. *Nature Chemical Biology*, *7*(8), 531–537. https://doi.org/10.1038/nchembio.598.

Brosh, R., Hrynyk, I., Shen, J., Waghray, A., Zheng, N., & Lemischka, I. R. (2016). A dual molecular analogue tuner for dissecting protein function in mammalian cells. *Nature Communications, 7*, 11742. https://doi.org/10.1038/ncomms11742.

Caussinus, E., Kanca, O., & Affolter, M. (2011). Fluorescent fusion protein knockout mediated by anti-GFP nanobody. *Nature Structural & Molecular Biology*, *19*(1), 117–121. https://doi.org/10.1038/nsmb.2180.

Chen, X., Zaro, J. L., & Shen, W. C. (2013). Fusion protein linkers: Property, design and functionality. *Advanced Drug Delivery Reviews*, *65*(10), 1357–1369. https://doi.org/10.1016/j.addr.2012.09.039.

Collins, I., Wang, H., Caldwell, J. J., & Chopra, R. (2017). Chemical approaches to targeted protein degradation through modulation of the ubiquitin-proteasome pathway. *The Biochemical Journal*, *474*(7), 1127–1147. https://doi.org/10.1042/BCJ20160762.

Dharmasiri, N., Dharmasiri, S., & Estelle, M. (2005). The F-box protein TIR1 is an auxin receptor. *Nature*, *435*(7041), 441–445. https://doi.org/10.1038/nature03543.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., & Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*, *411*(6836), 494–498. https://doi.org/10.1038/35078107.

Fachinetti, D., Han, J. S., McMahon, M. A., Ly, P., Abdullah, A., Wong, A. J., et al. (2015). DNA sequence-specific binding of CENP-B enhances the fidelity of human centromere function. *Developmental Cell*, *33*(3), 314–327. https://doi.org/10.1016/j.devcel.2015.03.020.

Furuta, M., Hori, T., & Fukagawa, T. (2016). Chromatin binding of RCC1 during mitosis is important for its nuclear localization in interphase. *Molecular Biology of the Cell*, *27*(2), 371–381. https://doi.org/10.1091/mbc.E15-07-0497.

Gutschner, T., Haemmerle, M., Genovese, G., Draetta, G. F., & Chin, L. (2016). Post-translational regulation of Cas9 during G1 enhances homology-directed repair. *Cell Reports*, *14*(6), 1555–1566. https://doi.org/10.1016/j.celrep.2016.01.019.

Haeussler, M., Schonig, K., Eckert, H., Eschstruth, A., Mianne, J., Renaud, J. B., et al. (2016). Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. *Genome Biology*, *17*(1), 148. https://doi.org/10.1186/s13059-016-1012-2.

Han, J. S., Holland, A. J., Fachinetti, D., Kulukian, A., Cetin, B., & Cleveland, D. W. (2013). Catalytic assembly of the mitotic checkpoint inhibitor BubR1-Cdc20 by a Mad2-induced functional switch in Cdc20. *Molecular Cell*, *51*(1), 92–104. https://doi.org/10.1016/j.molcel.2013.05.019.

Havens, K. A., Guseman, J. M., Jang, S. S., Pierre-Jerome, E., Bolten, N., Klavins, E., et al. (2012). A synthetic approach reveals extensive tunability of auxin signaling. *Plant Physiology*, *160*(1), 135–142. https://doi.org/10.1104/pp.112.202184.

Holland, A. J., Fachinetti, D., Han, J. S., & Cleveland, D. W. (2012). Inducible, reversible system for the rapid and complete degradation of proteins in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(49), E3350–3357. https://doi.org/10.1073/pnas.1216880109.

Hsu, P. D., Scott, D. A., Weinstein, J. A., Ran, F. A., Konermann, S., Agarwala, V., et al. (2013). DNA targeting specificity of RNA-guided Cas9 nucleases. *Nature Biotechnology*, *31*(9), 827–832. https://doi.org/10.1038/nbt.2647.

Iwamoto, M., Bjorklund, T., Lundberg, C., Kirik, D., & Wandless, T. J. (2010). A general chemical method to regulate protein stability in the mammalian central nervous system. *Chemistry & Biology*, *17*(9), 981–988. https://doi.org/10.1016/j.chembiol.2010.07.009.

Jiang, W., Bikard, D., Cox, D., Zhang, F., & Marraffini, L. A. (2013). RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nature Biotechnology*, *31*(3), 233–239. https://doi.org/10.1038/nbt.2508.

Kanke, M., Nishimura, K., Kanemaki, M., Kakimoto, T., Takahashi, T. S., Nakagawa, T., et al. (2011). Auxin-inducible protein depletion system in fission yeast. *BMC Cell Biology*, *12*, 8. https://doi.org/10.1186/1471-2121-12-8.

Kepinski, S., & Leyser, O. (2005). The Arabidopsis F-box protein TIR1 is an auxin receptor. *Nature*, *435*(7041), 446–451. https://doi.org/10.1038/nature03542.

Kipreos, E. T., & Pagano, M. (2000). The F-box protein family. *Genome Biology, 1*(5), Reviews3002. https://doi.org/10.1186/gb-2000-1-5-reviews3002.

Kleinstiver, B. P., Prew, M. S., Tsai, S. Q., Topkar, V. V., Nguyen, N. T., Zheng, Z., et al. (2015). Engineered CRISPR–Cas9 nucleases with altered PAM specificities. *Nature*, *523*(7561), 481–485. https://doi.org/10.1038/nature14592.

Kumar, R., Dhali, S., Srikanth, R., Ghosh, S. K., & Srivastava, S. (2014). Comparative proteomics of mitosis and meiosis in Saccharomyces cerevisiae. *Journal of Proteomics*, *109*, 1–15. https://doi.org/10.1016/j.jprot.2014.06.006.

Lambrus, B. G., Uetake, Y., Clutario, K. M., Daggubati, V., Snyder, M., Sluder, G., et al. (2015). p53 protects against genome instability following centriole duplication failure. *The Journal of Cell Biology*, *210*(1), 63–77. https://doi.org/10.1083/jcb.201502089.

Lin, S., Staahl, B. T., Alla, R. K., & Doudna, J. A. (2014). Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. *eLife, 3*, e04766. https://doi.org/10.7554/eLife.04766.

Lu, J., Qian, Y., Altieri, M., Dong, H., Wang, J., Raina, K., et al. (2015). Hijacking the E3 ubiquitin ligase cereblon to efficiently target BRD4. *Chemistry & Biology*, *22*(6), 755–763. https://doi.org/10.1016/j.chembiol.2015.05.009.

McKinley, K. L., Sekulic, N., Guo, L. Y., Tsinman, T., Black, B. E., & Cheeseman, I. M. (2015). The CENP-L-N complex forms a critical node in an integrated meshwork of interactions at the centromere–kinetochore interface. *Molecular Cell*, *60*(6), 886–898. https://doi.org/10.1016/j.molcel.2015.10.027.

Mojica, F. J., Diez-Villasenor, C., Garcia-Martinez, J., & Almendros, C. (2009). Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology*, *155*(Pt 3), 733–740. https://doi.org/10.1099/mic.0.023960-0.

Natsume, T., Kiyomitsu, T., Saga, Y., & Kanemaki, M. T. (2016). Rapid protein depletion in human cells by auxin-inducible degron tagging with short homology donors. *Cell Reports*, *15*(1), 210–218. https://doi.org/10.1016/j.celrep.2016.03.001.

Neklesa, T. K., Tae, H. S., Schneekloth, A. R., Stulberg, M. J., Corson, T. W., Sundberg, T. B., et al. (2011). Small-molecule hydrophobic tagging-induced degradation of HaloTag fusion proteins. *Nature Chemical Biology*, *7*(8), 538–543. https://doi.org/10.1038/nchembio.597.

Nishimura, K., Fukagawa, T., Takisawa, H., Kakimoto, T., & Kanemaki, M. (2009). An auxin-based degron system for the rapid depletion of proteins in nonplant cells. *Nature Methods*, *6*(12), 917–922. https://doi.org/10.1038/nmeth.1401.

Paix, A., Folkmann, A., Goldman, D. H., Kulaga, H., Grzelak, M. J., Rasoloson, D., et al. (2017). Precision genome editing using synthesis-dependent repair of Cas9-induced DNA breaks. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(50), E10745–E10754. https://doi.org/10.1073/pnas.1711979114.

Paix, A., Folkmann, A., Rasoloson, D., & Seydoux, G. (2015). High efficiency, homology-directed genome editing in Caenorhabditis elegans using CRISPR–Cas9 ribonucleoprotein complexes. *Genetics*, *201*(1), 47–54. https://doi.org/10.1534/genetics.115.179382.

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., & Zhang, F. (2013). Genome engineering using the CRISPR–Cas9 system. *Nature Protocols*, *8*(11), 2281–2308. https://doi.org/10.1038/nprot.2013.143.

Rodriguez-Bravo, V., Maciejowski, J., Corona, J., Buch, H. K., Collin, P., Kanemaki, M. T., et al. (2014). Nuclear pores protect genome integrity by assembling a premitotic and

Mad1-dependent anaphase inhibitor. *Cell*, *156*(5), 1017–1031. https://doi.org/10.1016/j.cell.2014.01.010.

Sakamoto, K. M., Kim, K. B., Kumagai, A., Mercurio, F., Crews, C. M., & Deshaies, R. J. (2001). Protacs: Chimeric molecules that target proteins to the Skp1–Cullin–F box complex for ubiquitination and degradation. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(15), 8554–8559. https://doi.org/10.1073/pnas.141230798.

Sauer, B., & Henderson, N. (1988). Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proceedings of the National Academy of Sciences of the United States of America*, *85*(14), 5166–5170.

Schneekloth, A. R., Pucheault, M., Tae, H. S., & Crews, C. M. (2008). Targeted intracellular protein degradation induced by a small molecule: En route to chemical proteomics. *Bioorganic & Medicinal Chemistry Letters*, *18*(22), 5904–5908. https://doi.org/10.1016/j.bmcl.2008.07.114.

Sekine, K., Takubo, K., Kikuchi, R., Nishimoto, M., Kitagawa, M., Abe, F., et al. (2008). Small molecules destabilize cIAP1 by activating auto-ubiquitylation. *The Journal of Biological Chemistry*, *283*(14), 8961–8968. https://doi.org/10.1074/jbc.M709525200.

Shah, S. A., Erdmann, S., Mojica, F. J., & Garrett, R. A. (2013). Protospacer recognition motifs: Mixed identities and functional diversity. *RNA Biology*, *10*(5), 891–899. https://doi.org/10.4161/rna.23764.

Shah, K., Liu, Y., Deirmengian, C., & Shokat, K. M. (1997). Engineering unnatural nucleotide specificity for Rous sarcoma virus tyrosine kinase to uniquely label its direct substrates. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(8), 3565–3570.

Skaar, J. R., Pagan, J. K., & Pagano, M. (2013). Mechanisms and function of substrate recruitment by F-box proteins. *Nature Reviews. Molecular Cell Biology*, *14*(6), 369–381. https://doi.org/10.1038/nrm3582.

Tan, X., Calderon-Villalobos, L. I., Sharon, M., Zheng, C., Robinson, C. V., Estelle, M., et al. (2007). Mechanism of auxin perception by the TIR1 ubiquitin ligase. *Nature*, *446*(7136), 640–645. https://doi.org/10.1038/nature05731.

Trost, M., Blattner, A. C., & Lehner, C. F. (2016). Regulated protein depletion by the auxin-inducible degradation system in Drosophila melanogaster. *Fly (Austin)*, *10*(1), 35–46. https://doi.org/10.1080/19336934.2016.1168552.

Winter, G. E., Buckley, D. L., Paulk, J., Roberts, J. M., Souza, A., Dhe-Paganon, S., et al. (2015). Drug development. Phthalimide conjugation as a strategy for in vivo target protein degradation. *Science*, *348*(6241), 1376–1381. https://doi.org/10.1126/science.aab1433.

Wood, L., Booth, D. G., Vargiu, G., Ohta, S., deLima Alves, F., Samejima, K., et al. (2016). Auxin/AID versus conventional knockouts: Distinguishing the roles of CENP-T/W in mitotic kinetochore assembly and stability. *Open Biology*, *6*(1), 150230. https://doi.org/10.1098/rsob.150230.

Yu, H., Moss, B. L., Jang, S. S., Prigge, M., Klavins, E., Nemhauser, J. L., et al. (2013). Mutations in the TIR1 auxin receptor that increase affinity for auxin/indole-3-acetic acid proteins result in auxin hypersensitivity. *Plant Physiology*, *162*(1), 295–303. https://doi.org/10.1104/pp.113.215582.

Zhang, L., Ward, J. D., Cheng, Z., & Dernburg, A. F. (2015). The auxin-inducible degradation (AID) system enables versatile conditional protein depletion in C. elegans. *Development*, *142*(24), 4374–4384. https://doi.org/10.1242/dev.129635.