One-step multiplex toolkit for efficient generation of conditional gene silencing human cell lines

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ABSTRACT **Loss-of-function analysis is one of the major arsenals we have for understanding gene functions in mammalian cells. For analysis of essential genes, the major challenge is to develop simple methodologies for tight and rapid inducible gene inactivation. One approach involves CRISPR-Cas9-mediated disruption of the endogenous locus in conjunction with the expression of a rescue construct, which can subsequently be turned off to produce a gene inactivation effect. Here we describe the development of a set of Sleeping Beauty transposon-based vectors for expressing auxin-inducible degron (AID)-tagged genes under the regulation of a tetracycline-controlled promoter. The dual transcriptional and degron-mediated post-translational regulation allows rapid and tight silencing of protein expression in mammalian cells. We demonstrated that both non-essential and essential genes could be targeted in human cell lines using a one-step transfection method. Moreover, multiple genes could be simultaneously or sequentially targeted, allowing inducible inactivation of multiple genes. These resources enable highly efficient generation of conditional gene silencing cell lines to facilitate functional studies of essential genes.**

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INTRODUCTION

Loss-of-function analysis is a powerful approach for characterizing gene functions in mammalian cells. Various whole genome studies have estimated that there are 1,500 to over 2,000 essential genes in human cell lines (Hart *et al.*, 2015; Bertomeu *et al.*, 2018; Wang *et al.*, 2015). While gene disruption represents the most direct approach in silencing a gene, it is generally irreversible and cannot be used for essential genes. To study essential genes, tactics involving conditional inactivation or depletion are generally required.

One contemporary approach for analysis of essential genes involves CRISPR-Cas9-mediated disruption of the endogenous locus in conjunction with the expression of a rescue construct (itself resistant to the CRISPR-Cas9), which can subsequently be turned off to produce a gene inactivation effect. Nonetheless, major obstacles of this approach include the specificity of gene depletion as well as the kinetics and tightness of the suppression of the rescue construct.

Recently we have demonstrated that combining transcriptional control and degron-mediated post-translational regulation allows rapid and tight control of protein expression in mammalian cells (Ng *et al.*, 2019). Transcription is controlled by a widely used tetracycline-controlled promoter (Tet-Off) system (Gossen and Bujard, 1992). In the absence of tetracycline, tetracycline-controlled transcriptional activator (tTA) dimers bind the tetracycline response element (TRE) and activate the expression of the downstream transgene. The presence of tetracycline or derivatives such as doxycycline (Dox) induces a conformational change in the tTA and prevents binding to TRE, thereby turning off gene expression. Concurrently, the stability of the protein is controlled using an auxin-inducible degron (AID). When expressed in mammalian cells, a plant F-box protein called TIR1 can form a complex with endogenous cullin, RBX1, SKP1 to form a SCF-type ubiquitin ligase (Hayashi and Karlseder, 2013). In the presence of auxin (indole-3-acetic acid; IAA), the

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Abbreviations used: AID, auxin-inducible degron; CDK, cyclin-dependent kinase; Dox, doxycycline; IAA, indole-3-acetic acid; ITR, inverted terminal repeats; mAID, minimal functional AID; SB, sleeping beauty; TRE, tetracycline response element; tTA, tetracycline-controlled transcriptional activator.

TIR1-containing SCF complex targets AID-containing proteins for ubiquitylation and subsequent proteasome-mediated degradation (Nishimura *et al.*, 2009). The integrated approach addresses some of the shortcomings of the TRE system and AID system, including the "leakiness" and relatively slow responses of the tetracycline-controlled promoters as well as the presence of residue levels of AID-fusion proteins after IAA treatment (Ng *et al.*, 2019).

When used in conjunction with CRISPR-Cas9, the TRE-AID system facilitates functional analysis by allowing rapid and tight conditional inactivation of genes. Advantages of this strategy in comparison to approaches that tag a degron onto an endogenous gene include that the TRE-controlled AID-tagged gene does not needed to integrate into the endogenous loci using homologous recombination, which is in general inefficient. This is particularly useful for studies involving cancer cell lines because they frequently contain multiple gene copies. Moreover, as this is already a rescue system by design, the effects after the AID-tagged gene is turned off can be confidently attributed to the functions of the gene rather than off-target effects of the CRISPR-Cas9.

A limitation of the above TRE-AID system is that multiple plasmids are required to be transfected (those expressing CRISPR-Cas9, AID-fusion protein, TIR1, and tTA). This can become highly inefficient when multiple genes are targeted at the same time. Here we refined the technology by constructing a series of vectors for expressing AID-tagged proteins under the control of TRE promoter. Moreover, by putting the sequences as a genetic cargo in Sleeping Beauty transposon vectors, cell lines with conditional silencing of single and multiple genes can be generated in a one-step procedure with high efficiency.

RESULTS

A system for generating conditional silencing cell lines for essential genes

Figure 1A summarizes the concept of this conditional deficiency system. Concurrent with the disruption of the gene of interest with CRISPR-Cas9, an AID-tagged cDNA of the gene (containing silence mutations rendering it resistant to the CRISPR-Cas9) under the control of a Tet-Off promoter is integrated randomly into the genome using Sleeping Beauty (SB) transposons. On the one hand, expression of tTA in the cells enables turning off of the transcription of the AID-tagged cDNA using doxycycline (Dox). On the other hand, the presence of TIR1 facilitates rapid degradation of the AID-tagged proteins using indole-3-acetic acid (IAA). A minimal functional AID (mAID) could be used as a smaller degron compared with AID (Yesbolatova *et al.*, 2019; Kubota *et al.*, 2013; Brosh *et al.*, 2016; Morawska and Ulrich, 2013).

To enhance the ease of use of the system, we explored whether it is possible to use a single transfection step to generate stable cell lines with conditional silencing of one or more genes. A series of SB-based vectors for expressing AID- or mAID-tagged proteins were constructed (pUHD-SB-AID and pUHD-SB-mAID respectively) (Figure 1B). TRE-driven AID/mAID was placed within the inverted terminal repeats (ITRs) of SB. The cDNA of the gene of interest can be cloned after the AID/mAID. The use of an engineered hyperactive SB transposase SB100X enables stable transfer of the genetic cargo into genomes of mammalian cells with high efficiency (Mátés *et al.*, 2009). Additional versions of the pUHD-SB-mAID plasmid containing different antibiotic resistance genes (blasticidin, hygromycin, or zeocin) driven by a constitutive promoter enable selection in mammalian cells. To generate cell lines lacking the endogenous gene while expressing a mAID-fusion rescue construct, four plasmids were transfected together, including (1) pUHD-SB-mAID; (2) a SB-based plasmid expressing TIR1 and tTA; (3) a plasmid expressing the CRISPR-Cas9; and (4) a plasmid expressing SB transposase. The availability of different antibiotic resistance genes in the AID and TIR1/tTA plasmids allowed selection of cells containing both cassettes in the genome (Figure 1B). As the CRISPR-Cas9 and SB transposase plasmids required only to be transiently expressed, no antibiotic was required for their selection.

We first characterized the system by targeting an essential gene that has a relatively slow KO effect in HeLa cells. SGT1 is an interactor with both the chaperone HSP90 and the SCF ubiquitin ligase component SKP1 and is implicated in various processes including kinetochore assembly (Kitagawa *et al.*, 1999; Davies and Kaplan, 2010). Tet-Off cells were transfected with plasmids carrying CRISPR-Cas against SGT1, ^{mAID}SGT1 (in pUHD-SB-AID/Hyg), TIR1 (in pSBbi-TIR1/Pur), and SB transposase. After selection with hygromycin and/or puromycin for 2 wk, the surviving cells (^{mAID}SGT1^{KO}SGT1) were analyzed with immunoblotting (Figure 2A). Due to the high efficiency of SB transposons, both ^{mAID}SGT1 and TIR1 could be detected even when the cells were selected with only one of the antibiotics on the two plasmids (albeit enrichment of expression of ^{mAID}SGT1 or TIR1 could be observed when the cassette was selected with the antibiotic). No endogenous SGT1 was detected, indicating that it was efficiently disrupted with CRISPR-Cas9. As anticipated, the expression of ^{mAID}SGT1 could be turned off by incubation with Dox and IAA (Figure 2B). Note that some residual ^{mAID}SGT1 could be detected when the cells were treated with IAA alone in the mixed population.

Single colonies were then isolated and tested for their expression SGT1 (Figure 2C). ^{mAID}SGT1 was detected in 87% of colonies (20/23); endogenous SGT1 expression was disrupted in 91% (21/23); and TIR1 in all the colonies (100%). Overall, >80% (19/23) of colonies lacked endogenous SGT1 and expressed ^{mAID}SGT1. Treatment of a purified clone of ^{mAID}SGT1^{KO}SGT1 with Dox and/or IAA, confirmed that ^{mAID}SGT1 was depleted more efficiently in the presence of Dox and IAA together than the individual chemicals alone (Figure 2D). Although depletion of SGT1 did not have an impact on the cell cycle after 24 h (Figure 2E), clonogenic survival assay indicated that SGT1 was essential for long-term survival (Figure 2F).

We next generated conditional silencing cell lines targeting an essential gene that has a rapid KO effect. Cyclin-dependent kinase 1 (CDK1) is the catalytic subunit of the main mitotic engine (cyclin B–CDK1) in eukaryotes (Malumbres, 2014). After transfection and selection, the mixed population of ^{AID}CDK1^{KO}CDK1 contained very low levels of endogenous CDK1 and expressed AID-CDK1, which could be turned off with Dox and IAA (Figure 3A). Addition of both Dox and IAA together triggered a more complete suppression of ^{AID}CDK1 than the individual chemicals alone. Consistent with the pivotal functions of CDK1 in G_2–M, inactivation of CDK1 promoted a G_2/M delay as revealed by flow cytometry (Figure 3B). The detailed effects of CDK1 inactivation are unexpected and will be described in another study (manuscript in preparation).

To assess if the ratio of different transfection components affects the generation of conditional silencing cells, the amount of each plasmid was reduced individually. Figure 3C shows that reducing the amount of ^{AID}CDK1-expressing plasmid diminished the relative expression of ^{AID}CDK1 after selection. Likewise, decreasing the amount of CRISPR-Cas reduced the knockout of endogenous CDK1. These results indicate that while the system is robust, optimal ratios of different plasmids should be empirically determined to increase the number of desirable colonies. Single colony analysis indicated that the efficiency of generating cells lacking endogenous CDK1

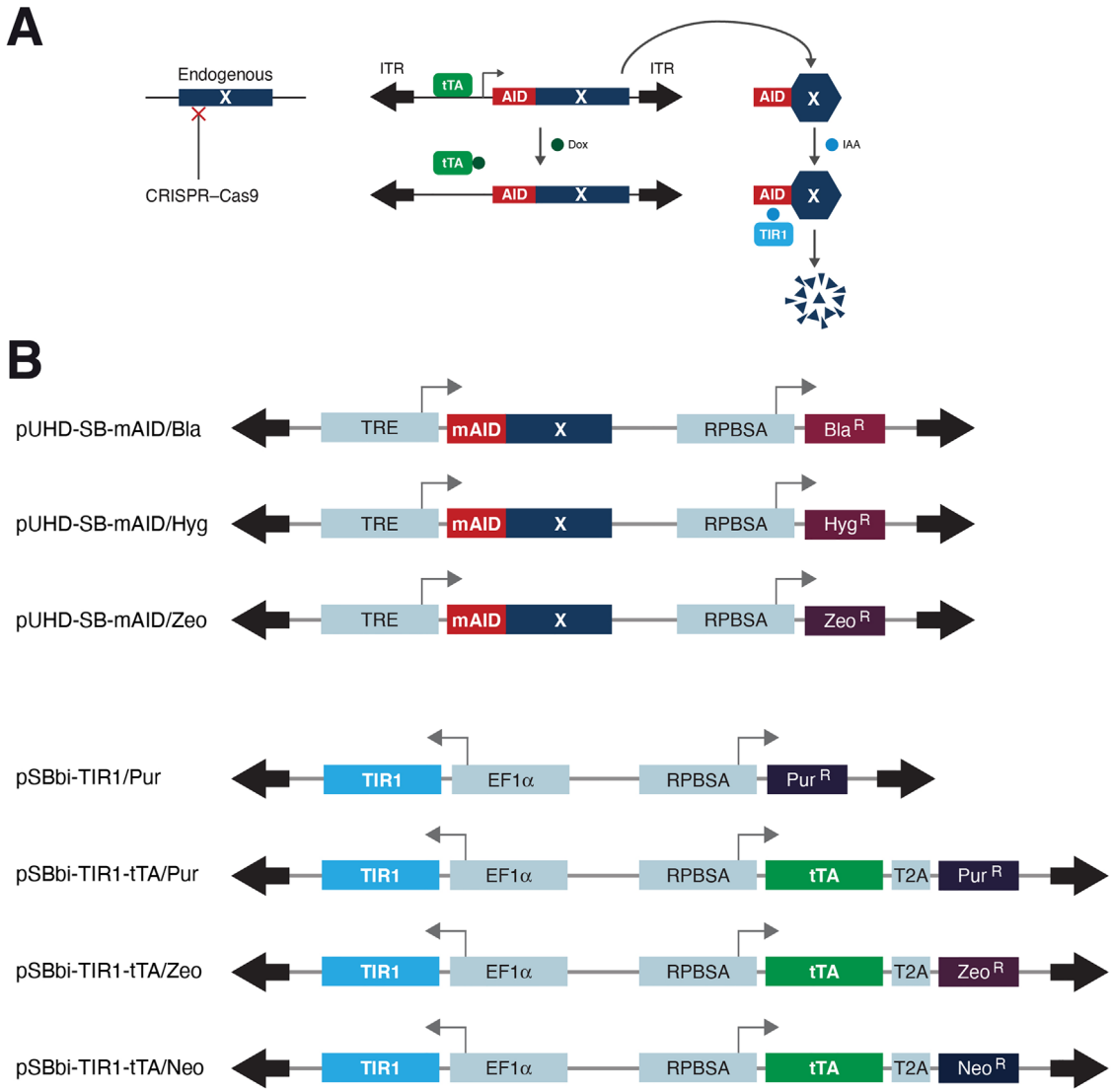

FIGURE 1: Strategy and constructs. (A) Conditional gene silencing. The endogenous locus of a gene (X) is disrupted using CRISPR-Cas9 (note that cancer cell lines may contain multiple copies of the gene). The cDNA of the gene is put inside a Sleeping Beauty (SB) cassette and delivered to the genome to rescue the knockout effects (ITR: inverted terminal repeats). Silence mutations (changing 3-4 bases) are introduced into the cDNA to render the cDNA resistant to the CRISPR-Cas9. The tetracycline-controlled transcriptional activator (tTA) binds to the TRE in the promoter and activates the transcription of AID/mAID-tagged cDNA in the absence of Dox. Addition of Dox turns off the transcription of the promoter. In response to IAA, AID/mAID-fusion protein is rapidly targeted for degradation in cells expressing the ubiquitin ligase SCF^{TIR1}. Combining transcriptional and degron control allows both rapid and tight switching off of the rescue construct. (B) Sleeping Beauty constructs for generating conditional gene silencing cell lines. The pUHD-SB-mAID series contain TRE-driven mAID-fusion cDNA (X) placed in between the ITRs of SB transposon. Similar vectors containing AID instead of mAID are also available (see Supplemental vector information S1–S5). The pSBbi-TIR1-tTA vectors contain TIR1 driven by a constitutive E2F1α promoter and tTA driven by a constitutive RPBSA promoter within the ITRs. A version (pSBbi-TIR1/Pur) contains TIR1 without tTA. Different versions of the vectors contain different antibiotic resistance genes for blasticidin (Bla^R), hygromycin (Hyg^R), puromycin (Pur^R), or zeocin (Zeo^R) driven by a RPBSA promoter (or co-expression via T2A).

and expressing ^{AID}CDK1 was >80% (15/18) (Figure 3D). As expected, ^{AID}CDK1 in ^{AID}CDK1^{KO}CDK1 cells could be turned off in the presence of Dox and IAA to produce a CDK1-null effect (Figure 3E).

We further repeated the above experiments using a different cell line (H1299). As H1299 was not a Tet-Off cell line, both TIR1 and tTA were delivered together in the same SB plasmid (Figure 1B). As with HeLa cells, ^{mAID}SGT1 expression could be turned off in the presence of DI in a SGT1-knockout background (Supplemental Figure S1A). Likewise, ^{AID}CDK1^{KO}CDK1 cells could readily be generated in H1299 using a similar one-step procedure (Figure S1B). As in HeLa cells, Dox and IAA together achieved a significantly tighter suppression of ^{AID}CDK1 than the individual chemicals alone. Single colony analysis indicated that the efficiency of generating cells lacking endogenous CDK1 and expressing ^{AID}CDK1 was 100% (15/15) (Figure S1C). All the clones contained TIR1 and could degrade ^{AID}CDK1 in the presence of DI. These studies indicate that conditional deficiency can be generating with a one-step transfection in different cell lines.

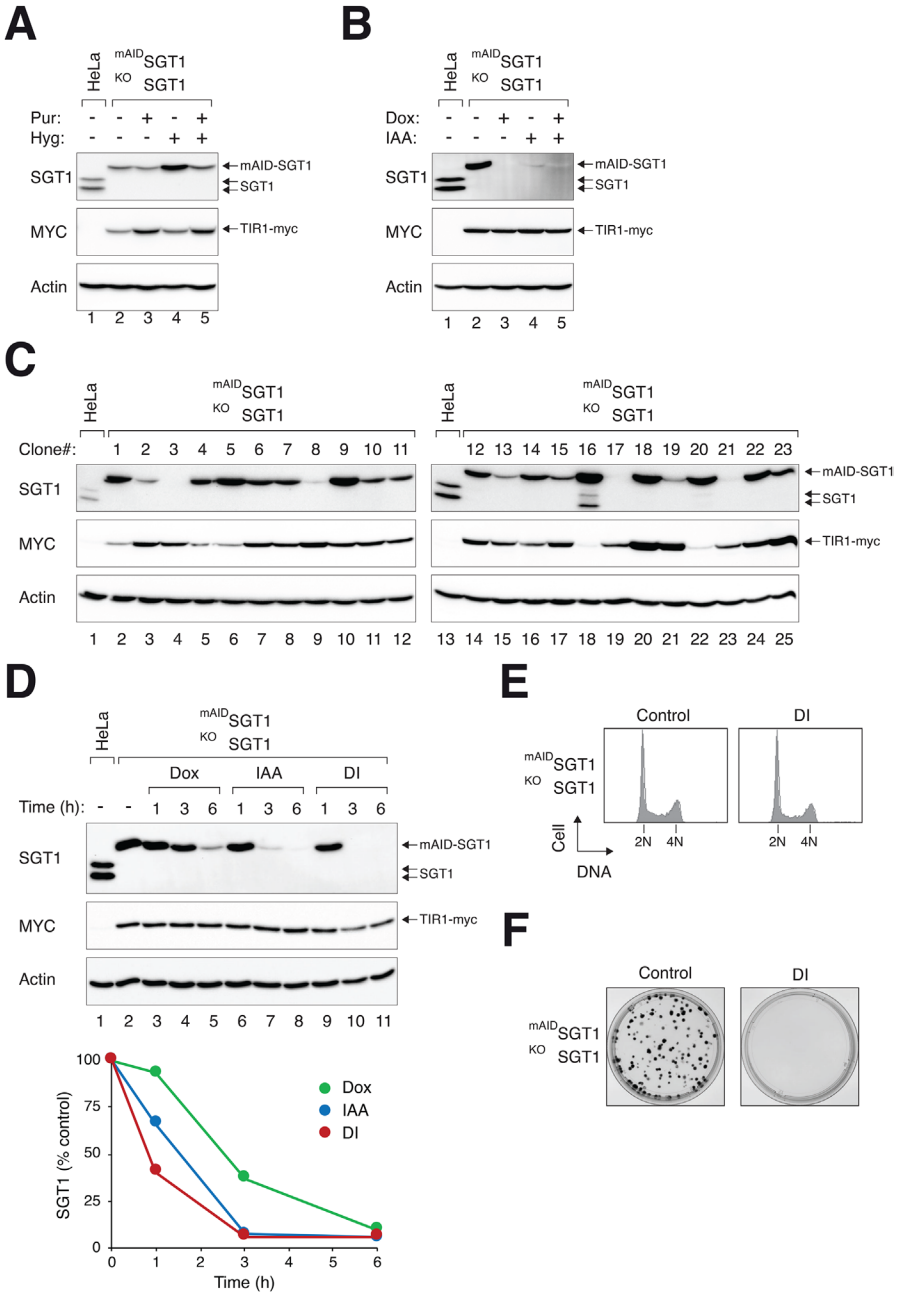

FIGURE 2: Conditional silencing of a relatively slow-acting essential gene. (A) Generation of SGT1 conditional silencing cell lines. HeLa Tet-Off cells (already expressing tTA) were transfected with plasmids carrying ^{mAID}SGT1 (in pUHD-SB-mAID/Hyg), TIR1 (pSBbi-TIR1/Pur), CRISPR-Cas against SGT1, and SB transposase. After selection with hygromycin and/or puromycin for 2 wk, lysates were prepared and analyzed with immunoblotting. Lysates from HeLa cells were loaded as controls to indicate the position of SGT1. Note that endogenous SGT1 contained two bands corresponding to isoforms A (smaller) and B (larger). ^{mAID}SGT1 was generated from isoform A. Equal loading of lysates was confirmed by immunoblotting for actin. (B) Transcription repression and degron-mediated proteolysis of SGT1. ^{mAID}SGT1^{KO}SGT1 cells were generated by transfection and selection with hygromycin B and puromycin as described in panel A. The cells were treated with Dox and/or IAA for 24 h before harvested for immunoblotting analysis. (C) Isolation of ^{mAID}SGT1^{KO}SGT1 colonies. Cells transfected and selected as described in panel A were plated at low density. After 2 wk, individual colonies were isolated and expanded. Lysates were prepared and analyzed with immunoblotting. Overall, >80% (19/23) of colonies lacked endogenous SGT1 and expressed ^{mAID}SGT1. (D) Rapid and complete depletion of ^{mAID}SGT1 after incubation with Dox and IAA. ^{mAID}SGT1^{KO}SGT1 cells (clone #2) were treated with Dox, IAA, or both chemicals together (DI) and harvested at different time points for immunoblotting analysis. The ^{mAID}SGT1 signals were quantified using densitometry analysis with serially diluted samples from lane 2 as standard curves (bottom panel). (E) Depletion of SGT1 does not affect short-term cell cycle progression. ^{mAID}SGT1^{KO}SGT1 cells were untreated or incubated with DI. After 24 h, the cells were analyzed with flow cytometry. The positions of 2N and 4N DNA contents are indicated. (F) Depletion of SGT1 abolishes long-term survival. ^{mAID}SGT1^{KO}SGT1 cells (200) were seeded and with either untreated or DI. After 14 d, colonies were fixed and stained. Representative plates are shown.

Collectively, these data indicate that using a single step transfection of four plasmids, conditional silencing cell lines can be produced with high efficiency for essential genes.

Simultaneous targeting of multiple genes

The ability to conditionally silence more than one gene simultaneously is a powerful strategy in functional studies. We next targeted both CDK1 and CDK2 as a proof of concept that conditional silencing of multiple genes can also be achieved using a one-step transfection. Although highly related to CDK1, CDK2 is mainly involved in cell cycle control other than mitosis (Malumbres, 2014). In general, two antibiotic resistance genes are used for selecting integration of the two AID-expressing constructs. As one of the limiting factors of targeting multiple genes is the number of antibiotic resistance markers that can be used for selection, we placed both ^{AID}CDK1 and ^{AID}CDK2 in plasmids contain the same antibiotic resistance gene to test if this could affect the efficiency adversely. Figure 4A shows that following antibiotic selection, the transfected cells contained very low levels of CDK1 and CDK2, while expressing both ^{AID}CDK1 and ^{AID}CDK2. Isolation and analysis of individual colonies revealed clones expressed both AID constructs while lacking both endogenous CDK1 and CDK2. Not surprisingly, the efficiency of successfully targeting both CDK1 and CDK2 was lower than targeting single genes (Figure 4B). Overall, 40% (10/25) of the clones expressed ^{AID}CDK1, ^{AID}CDK2, TIR1, and without endogenous CDK1 and CDK2. Treatment of a single clone of ^{AID}CDK1,2^{KO}CDK1,2 cells with DI resulted in rapid disappearance of both ^{AID}CDK1 and ^{AID}CDK2 (Figure 4C). These results demonstrated that conditional silencing of more than one gene can be achieved with a similar timeline as targeting single genes.

Sequential targeting of multiple genes

The ability to target multiple genes sequentially is useful for many functional studies. A possible concern, however, is that the expression of SB transposases may affect the expression of SB cargos from previous rounds of targeting. To test this possibility, we first

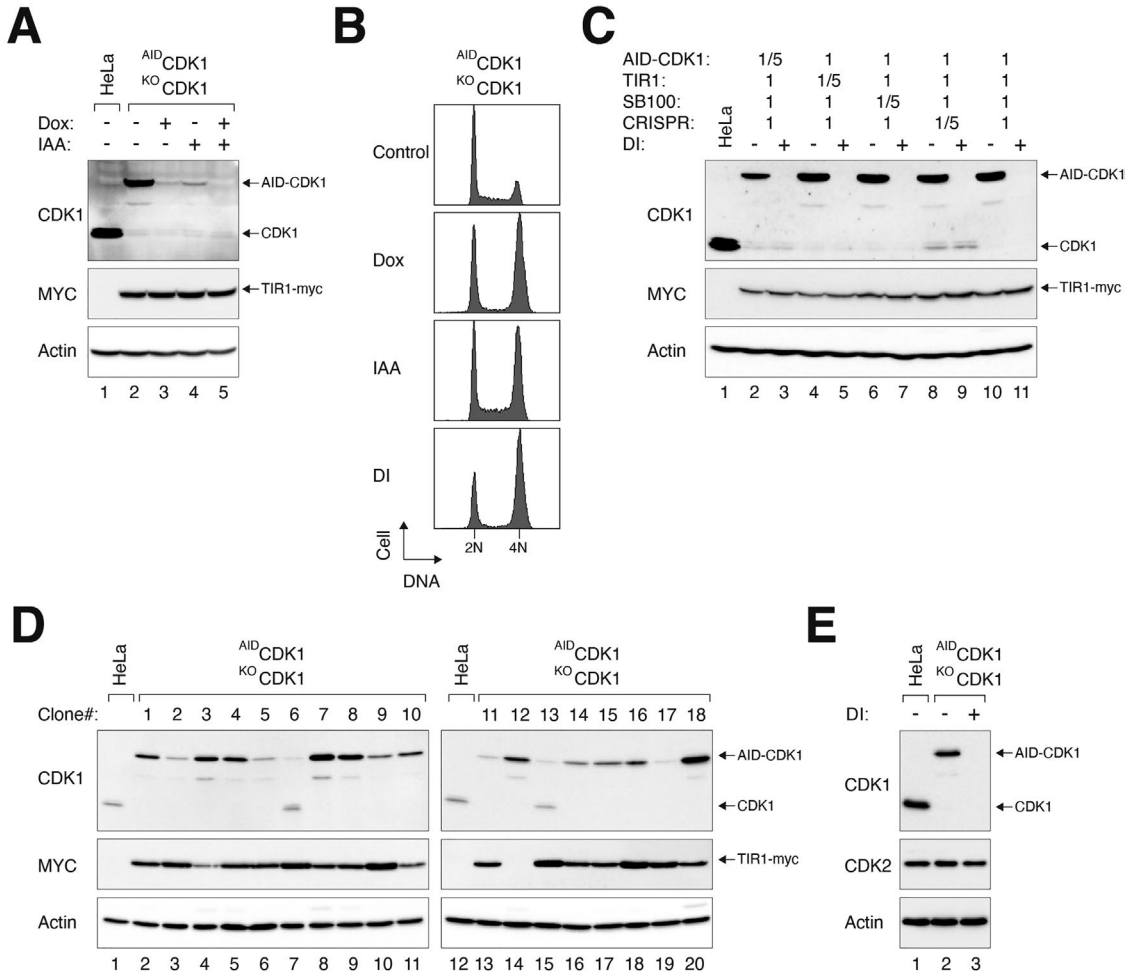

FIGURE 3: Conditional silencing of the essential mitotic kinase CDK1. (A) Generation of CDK1 conditional silencing cell lines (^{AID}CDK1^{KO}CDK1). HeLa Tet-Off cells were transfected with plasmids carrying ^{AID}CDK1 (in pUHD-SB-AID/Hyg), TIR1 (pSBbi-TIR1/Pur), CRISPR-Cas against CDK1, and SB transposase. After selection with hygromycin B and puromycin for 2 wk, the cells were treated with Dox and/or IAA for 24 h. Lysates were prepared and analyzed with immunoblotting. The expression of endogenous CDK1 is shown in the parental HeLa cells. Actin analysis was included to assess protein loading and transfer. (B) Loss of CDK1 induces a G_2/M cell cycle arrest. ^{AID}CDK1^{KO}CDK1 cells were incubated with Dox and/or IAA as described in panel A. The DNA contents of the cells were analyzed with flow cytometry. (C) The impact of different amount of transfection components on generation of stable cell lines. Cells were transfected using either the normal concentration ("1") or 1/5 the concentration of the 4 plasmids used to generate inducible cell lines. After 2 wk of selection, the cells were treated with Dox and IAA (DI) for 24 h. Protein expression was analyzed with immunoblotting. Lysates from the parental HeLa cells were loaded as controls. (D) Isolation of ^{AID}CDK1^{KO}CDK1 colonies. Cells transfected and selected as described in panel A were plated at low density. After 2 wk, individual colonies were isolated and expanded. Lysates were prepared and analyzed with immunoblotting. ^{AID}CDK1 and TIR1 expression was found in 100% (18/18) and 94% (17/18) of the colonies, respectively. Knockout of endogenous CDK1 was achieved in 89% (16/18) of the colonies. Overall, >80% (15/18) of colonies lacked endogenous CDK1 and expressed ^{AID}CDK1. (E) Conditional silencing of CDK1. ^{AID}CDK1^{KO}CDK1 cells derived from a colony (#14) were treated with either buffer or DI for 24 h. The expression of CDK1 was detected with immunoblotting. The expression of CDK2 and actin was unaffected by the treatment.

generated stable EGFP-expressing cells using a SB construct and SB transposase. The cells were then transfected with a SB construct carrying mRFP (carrying a blasticidin-resistance gene) and SB transposase before selecting with blasticidin. If transfection of SB transposase could remove SB cargos in the genome, we expect a decrease of cells expressing EGFP (Figure S2A). As expected, the second transfection resulted in mRFP expression in all the cells. Importantly, the EGFP signals were not affected by the SB transposase (Figure S2B). Similar results were obtained when the second mRFP construct was delivered using a different transposon system. The minimal Tol2 transposon (mTol2) can integrate payloads larger than that of SB into human cells (Balciunas *et al.*, 2006). Integration of mRFP in a plasmid containing mTol2's ITRs with Tol2 transposase did not affect EGFP expression (Figure S2B). These results suggested that subsequent expression of transposase does not affect the expression of previously integrated SB transposons.

To generate conditional gene silencing using a second round of transfection, ^{AID}CDK1,2^{KO}CDK1,2 produced as described above was used as a parent to further target CDK4 and CDK6. Figure 5A shows that ^{AID}CDK4 and ^{AID}CDK6 could be detected after selection with antibiotics. Decrease in endogenous CDK4 and CDK6 could also be detected in these mixed population of cells. Importantly, there is no significant change in the expression of ^{AID}CDK1, ^{AID}CDK2, and TIR1, indicating that a second round of SB transposase expression did not

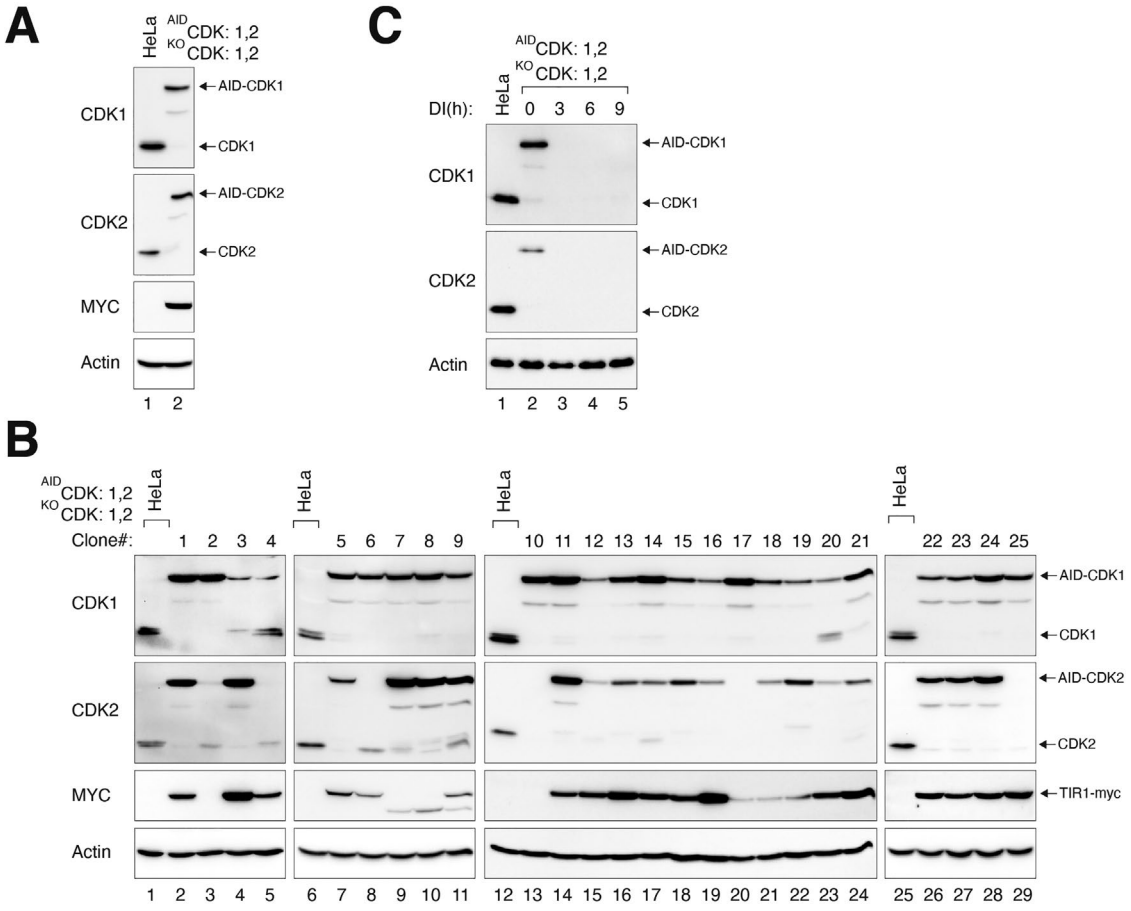

FIGURE 4: Conditional silencing of both CDK1 and CDK2. (A) Generation of ^{AID}CDK1,2^{KO}CDK1,2 conditional silencing cell lines. HeLa Tet-Off cells were transfected with plasmids carrying ^{AID}CDK1 (in pUHD-SB-AID/Hyg), ^{AID}CDK2 (in pUHD-SB-AID/Hyg), TIR1 (pSBbi-TIR1/Pur), CRISPR-Cas against CDK1 and CDK2 (two separate plasmids), and SB transposase. After selection with hygromycin B and puromycin for 2 wk, the cells were harvested and analyzed with immunoblotting. (B) Isolation of ^{AID}CDK1,2^{KO}CDK1,2 colonies. Cells transfected and selected as described in panel A were plated at low density. After 2 wk, individual colonies were isolated and expanded. Lysates were prepared and analyzed with immunoblotting. ^{AID}CDK1 and ^{AID}CDK2 were expressed in 100% (25/25) and 80% (20/25) of the clones, respectively. Knockout of both endogenous CDK1 or CDK2 was achieved in 52% (13/25) of clones. TIR1 expression was found in 84% (21/25) of the clones. Overall, 40% (10/25) of the clones expressed ^{AID}CDK1, ^{AID}CDK2, TIR1, and without endogenous CDK1 and CDK2. (C) Concerted inactivation of CDK1 and CDK2. ^{AID}CDK1,2^{KO}CDK1,2 cells derived from a colony (#16) were treated with DI and harvested at different time points. The expression of AID- and endogenous CDK1/CDK2 was detected with immunoblotting.

excise SB inserts from the genome. Single colonies isolation and analysis indicated ~28% (10/35) of the clones expressed all the AID-tagged CDKs as well as lacking all the endogenous CDKs (^{AID}CDK1,2,4,6^{KO}CDK1,2,4,6) (Figure 5B). Importantly, all colonies retained the expression of ^{AID}CDK1, ^{AID}CDK2, and TIR1. Treatment of a single clone with DI resulted in rapid disappearance of all AID-tagged CDKs (CDK1, CDK2, CDK4, CDK6), resulting in cells that lacked all four CDKs (Figure 5C). Collectively, these results demonstrated that the possibility of using multiple rounds of this system to generate conditional silencing cell lines.

DISCUSSION

Here we have devised a set of vectors for cloning and expressing AID-tagged proteins under the control of Tet-Off promoters. As disruption of a large subset of genes in the genome affects fitness of the cell (Hart *et al.*, 2015; Bertomeu *et al.*, 2018; Wang *et al.*, 2015), rapid conditional silencing will be a powerful approach that can reveal the functions of these essential genes. While RNA interference can be used to downregulate gene expression at the level of mRNA stability and/or translation, caveats including off-target effects and slow responses limit their use in many studies. The combination of transcriptional and degron control allow both rapid and tight silencing of the AID-fusion rescue construct (Ng *et al.*, 2019). The addition of SB transposons and multiple antibiotic resistance genes to the system described here will facilitate one-step generation of conditional silencing cell lines. A short protocol is provided for easy reference (Supplemental Materials).

To conditionally silence a gene in a cell line, four plasmids were transfected that expressed (1) AID/mAID-fusion protein; (2) TIR1 and tTA; (3) CRISPR-Cas9; (4) SB transposase. While the system was robust, the relative amount of the plasmids used did affect the efficacy of knockout or expression of the different components (Figure 3C). The optimized ratio of the plasmids for targeting different genes will likely require empirical determination.

In this methodology, one of the limiting factors for targeting multiple genes is the number of antibiotics that can be used for selection. Vectors expressing different antibiotic resistance genes for blasticidin, hygromycin, puromycin, or zeocin were generated (Figure 1B). We also find that it is possible to express two AID-tagged genes with the same antibiotic selection marker (Figure 3),

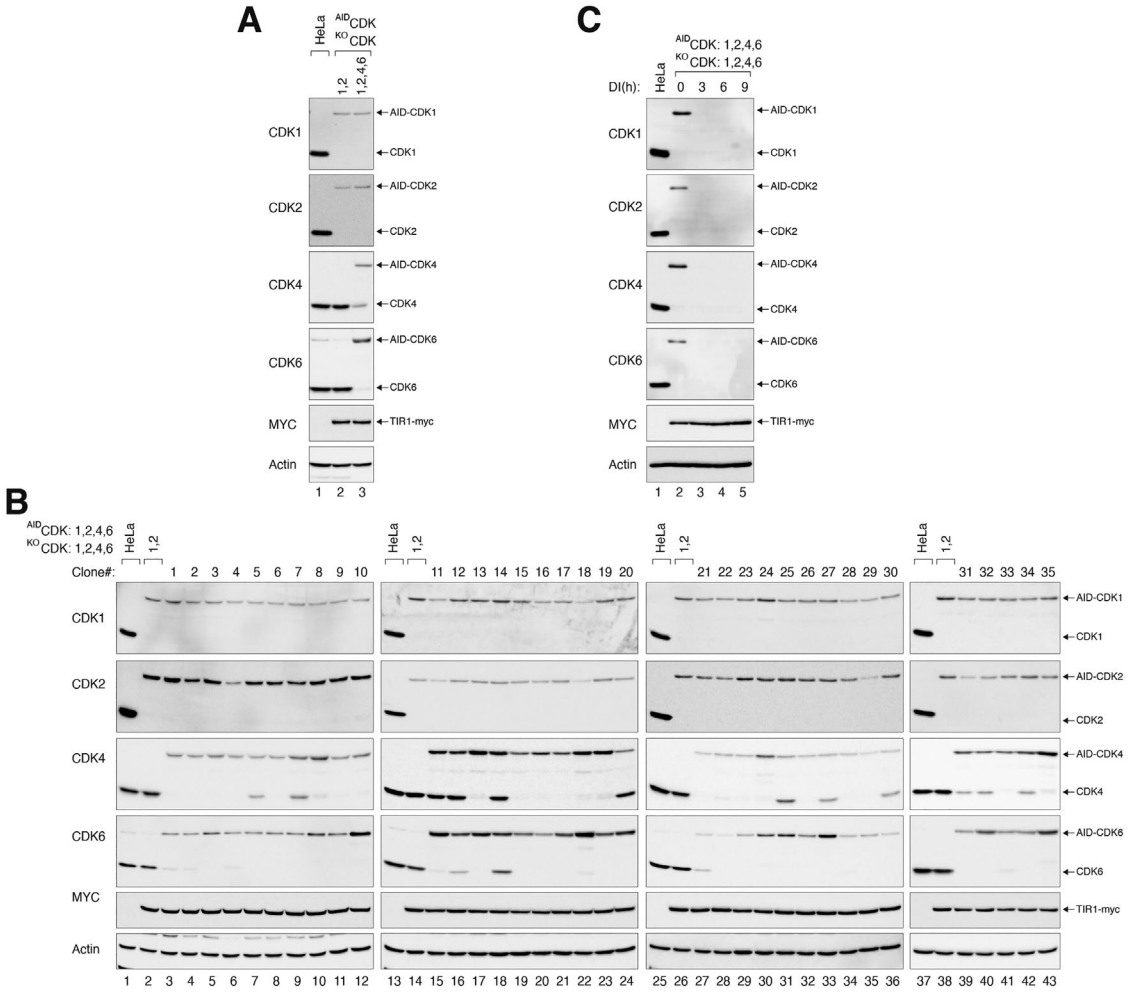

FIGURE 5: Conditional silencing of CDK1, CDK2, CDK4, and CDK6 using multiple rounds of transfection. (A) Generation of ^{AID}CDK1,2,4,6^{KO}CDK1,2,4,6 conditional silencing cell lines. ^{AID}CDK1,2^{KO}CDK1,2 cells were transfected with plasmids carrying ^{AID}CDK4 (in pUHD-SB-AID/Bla) and ^{AID}CDK6 (in pUHD-SB-AID/Zeo), CRISPR-Cas against CDK4 and CDK6 (two separate plasmids), and SB transposase. After selection with blasticidin and zeocin for 2 wk, the cells were harvested and analyzed with immunoblotting. (B) Isolation of ^{AID}CDK1,2,4,6^{KO}CDK1,2,4,6 colonies. Cells transfected and selected as described in panel A were plated at low density. After 2 wk, individual colonies were isolated and expanded. Lysates were prepared and analyzed with immunoblotting. ^{AID}CDK1, ^{AID}CDK2, and TIR1 were expressed in 100% (35/35) of the clones. Knockout of endogenous CDK4 and CDK6 was achieved in 49% (17/35) and 74% (26/35) of clones, respectively. Both ^{AID}CDK4 and ^{AID}CDK6 were expressed in 100% (35/35) of the clones. Overall, ~28% (10/35) of the clones expressed all the AID-tagged CDKs as well as lacking all the endogenous CDKs (^{AID}CDK1,2,4,6^{KO}CDK1,2,4,6). (C) Concerted inactivation of CDK1, CDK2, CDK4, and CDK6. ^{AID}CDK1,2,4,6^{KO}CDK1,2,4,6 cells derived from a colony (#3) were treated with DI and harvested at different time points. The expression of AID- and endogenous CDK1/CDK2/CDK4/CDK6 was detected with immunoblotting.

probably because of the high efficiency of SB-mediated integration. Nonetheless, we found that the SB transposons could be used for a second round without affecting the expression of cDNAs previously integrated with SB transposons (Figures S2 and 5).

We have generated vectors for both AID and mAID. The smaller size of the mAID is likely to be an advantage over the full-length AID for tagging proteins. Although we have not performed extensive side-by-side comparison, no major differences in the degradation kinetics between AID and mAID had been observed (our unpublished observations). Recent improvements of the AID system, such as the use of a TIR1 inhibitor (Yesbolatova *et al.*, 2019) or the use of AID2 (involving a TIR1(F74G) mutant and a ligand 5-Ph-IAA) (Yesbolatova *et al.*, 2020) can probably be used to further enhance the tightness and response kinetics of our system.

One limitation of the system described here is that since the AID-tagged gene is put under the control of an inducible promoter, its transcriptional control is likely to differ from that of the endogenous loci. This could be a critical factor for genes of which functions are highly dependent on transcriptional regulation. On the other hand, the integration of an inducible promoter offers several advantages over tagging AID into the endogenous loci. These include the ability of the system to complement weaknesses of using the AID system alone, such as the presence of background degradation in the absence of IAA and the incomplete degradation of AID-fusion proteins after IAA is added (Ng *et al.*, 2019). High efficiency of generating cell lines can also be achieved due to the fact that the AID-tagged gene does not needed to integrate into the endogenous loci (frequently with multiple copies in cancer cell lines).

MATERIALS AND METHODS

New vectors

Vector	Name	Purpose	Methods
1	pSBtet(TetOnΔ)	Intermediate	Insertion of two PCR products ((1) primers:1-2; template: pRevTRE2.2 (Ng *et al.*, 2019); then cut with SalI-NdeI; (2) primers: 3-4; template: pSBtet-Hyg (Addgene #60508); then cut with XbaI-XhoI) into AvrII-NdeI-cut pSBtet-Hyg.(Ng *et al.*, 2019)
2	pSBtet-Hyg(TetOnΔ)	Intermediate	Ligation of BglII-cut PCR product (primers: 5-6; template: pSBtet-Hyg) into BamHI-cut Vector 1.
3	pUHD-SB/Hyg	Tet-Off vector with SB ITRs	Ligation of a PCR product (primers: 7-8; template: modified pUHD-P3 (Huang *et al.*, 2016); then cut with AvrII-ClaI) and ClaI-SphI-cut Vector 2 into XbaI(partial)-SphI-cut Vector 2.
4	pUHD-SB-AID/Hyg	Tet-Off vector with SB ITRs for expressing AID-tagged proteins; hygromycin resistance	Insertion of NheI-BamHI fragment from pRevTRE-AID (Ng *et al.*, 2019) into Vector 3.
5	pUHD-SB-AID/Bla	Tet-Off vector with SB ITRs for expressing AID-tagged proteins; blasticidin resistance	Insertion of BamHI-XhoI-cut PCR product (primers 3-9; template: Vector 4) and SalI-HindIII-cut PCR product (primers 10-11; template: pcDNA6/V5-HisA (Life Technologies (Carlsbad, CA, USA)) into BamHI-HindIII-cut Vector 4.
6	pUHD-SB-mAID/Hyg	Tet-Off vector with SB ITRs for expressing mAID-tagged proteins; hygromycin resistance	Insertion of NheI-BamHI fragment from pREVTRE2.2-mAID (Lok *et al.*, 2020) into Vector 3.
7	pUHD-SB-mAID/Bla	Tet-Off vector with SB ITRs for expressing mAID-tagged proteins; blasticidin resistance	Insertion of BamHI-HindIII fragment from Vector 5 into Vector 6.
8	pUHD-SB-mAID/Zeo	Tet-Off vector with SB ITRs for expressing mAID-tagged proteins; zeocin resistance	Insertion of MscI-HindIII-cut PCR product (primers 12-13; template: pVgRXR, a gift from Dong-Yan Jin, The University of Hong Kong) into Vector 7.
9	pSBbi-TIR1/Pur	Vector with SB ITRs for expressing TIR1; puromycin resistance	Ligating of a NcoI-HindIII-cut double PCR product (first PCRs: primers 14-15; template: Rosa26-OsTIR1-myc (a gift from Helfrid Hochegger, University of Sussex, UK) (Hégarat *et al.*, 2020); primers 16-17; template: TIR1-9myc in pBabe-puro (a retroviral construct containing TIR1-myc (Ma and Poon, 2018)); second PCR: primers 14-17) into NcoI-HindIII-cut pSBbi-Pur (Addgene #60523).
10	pIRESpuro3-T2A/Bla	Intermediate	Ligation of two PCR products ((1) primers 18-19; template: Rosa26-OsTIR1-myc; then cut with NdeI-BamHI; (2) primers 20-21; template: pLenti_dCAS9-VP64_Blast (GenScript, Piscataway, NJ, USA); then cut with BglII-XbaI) into NdeI-XbaI-cut pIRESpuro3 (Takara Bio, Kusatsu, Japan).
11	ptTA-VP64-T2A/Bla	Intermediate	Ligation of NdeI(partial) fragment from pUHD15-1 (Gossen and Bujard, 1992) into NdeI-cut Vector 10.
12	pSBbi-TIR1-tTA/Pur	Vector with SB ITRs for expressing TIR1 and tTA; puromycin resistance	Ligation of NcoI and XhoI(partial)-cut PCR product (primers 3-22; template: Vector 9), SalI-ClaI-cut PCR product (primers 23-24; template: Vector 11), and ClaI-EcoRI-cut PCR product (primers 25-26; template: Vector 9) into NcoI-EcoRI-cut Vector 9.
13	pSBbi-TIR1-tTA/Neo	Vector with SB ITRs for expressing TIR1 and tTA; neomycin resistance	Ligation of ClaI-EcoRI-cut Vector 12 with ClaI-EcoRI-digested PCR product (primers 27-28; template: pcDNA3.1(-)).
14	pSBbi-TIR1-tTA/Zeo	Vector with SB ITRs for expressing TIR1 and tTA; zeocin resistance	Insertion of a ClaI-EcoRI-cut PCR product (primers 12-29; template: pVgRXR, a gift from Dong-Yan Jin, The University of Hong Kong) into Vector 12.
15	pUHD-SB-EGFP/Hyg	Tet-Off vector with SB ITRs for expressing EGFP; blasticidin resistance	Ligation of BamHI-NheI fragment from EGFP in pUHD-P3 (Ma *et al.*, 2009) into Vector 3.

(*Continues*)

16	pUHD-SB-mRFP/Bla	Tet-Off vector with SB ITRs for expressing mRFP; blasticidin resistance	Ligation of BamHI-NheI fragment from mRFP1 in pUHD-P3T (Ma *et al.*, 2009) into Vector 7.
17	pUHD-mTol2-mAID/Bla	Tet-Off vector with mTol2 ITRs for expressing mAID-tagged proteins; blasticidin resistance	Use Seamless Ligation Cloning Extract (SLiCE) (Motohashi, 2015) to insert the two PCR products ((1) primers 30-31; template: Vector 7; (2) primers 32-33; template: Vector 7) into BglII-EcoRI-cut pminiTol2 (Addgene #31829).
18	pUHD-mTo2-mRFP/Bla	Tet-Off vector with mTol2 ITRs for expressing mRFP; blasticidin resistance	Insertion of BamHI-NheI fragment from mRFP1 in pUHD-P3T (Ma *et al.*, 2009) into Vector 17.

The above vectors (except cloning intermediates) will be deposited to Addgene (Watertown, MA, USA).

CDK constructs

CDK1 CRISPR-Cas9 targeting TACTTTGTTTCAGGTACCTA was prepared by ligating the annealed product of primers 34 and 35 to BbsI-cut pX330 (Addgene #42230). To generate ^{AID}CDK1 in pUHD-SB-AID/Hyg, the EcoRI fragment from CDK1 (a gift from Tony Hunter, the Salk Institute) was ligated into EcoRI-cut pUHD-SB-AID/Hyg. ^{AID}CDK1 was not targeted by the CRISPR-Cas9 because the targeting region spanned the UTR and ORF. CRISPR-Cas9 targeting CDK2 was generated as described (Ng *et al.*, 2019). ^{AID}CDK2 in pUHD-SB-AID/Hyg was generated by ligating the EcoRI-NcoI fragment from FLAG-CDK2 in pUHD-P1 (Yam *et al.*, 2000) into pUHD-SB-AID/Hyg. CDK4 CRISPR-Cas9 targeting GTGCCACATCCCGAACTGAC was prepared by ligating the annealed product of primers 36 and 37 to BbsI-cut pX330. To generate CRISPR-resistant CDK4, silence mutations were introduced by a double PCR procedure (first PCRs: primers 38-39 and 40-41, respectively; template: FLAG-CDK4-HA in pUHD-P1; second PCR: primers 38-41; templates: products of the first PCRs). ^{AID}CDK4 (containing silence mutations) in pRevTRE-AID was generated by inserting NcoI-BamHI-cut double PCR product into pRevTRE-AID. The NcoI-BamHI fragment from ^{AID}CDK4 in pRevTRE-AID was ligated into pUHD-SB-AID/Hyg to generate ^{AID}CDK4 in pUHD-SB-AID/Hyg. ^{AID}CDK4 in pUHD-SB-AID/Bla was generated by using a Seamless Ligation Cloning Extract (SLiCE) cloning method to insert a PCR product (primers 42-43; template: ^{AID}CDK4 in pUHD-SB-AID/Hyg) into NheI-BamHI-cut pUHD-SB-mAID/Bla. CDK6 CRISPR-Cas9 targeting GGAAACTATAGATGCGGGCA was prepared by ligating the annealed product of primers 44 and 45 to BbsI-cut pX330. CRISPR-resistant CDK6 was prepared by double PCR procedure (first PCRs: primers 46-47 and 48-49, respectively; template: CDK6 (a gift from Godon Peters); second PCR: primers 46-49). ^{AID}CDK6 in pRevTRE-AID was generated by inserting the NcoI-BamHI-cut double PCR product into pRevTRE-AID. The NheI-BamHI fragment from the above plasmid was inserted into pUHD-SB-mAID/Zeo to generate ^{AID}CDK6 in pUHD-SB-AID/Zeo.

SGT1 constructs

SGT1 CRISPR-Cas9 targeting GCGACTACGAGGGATGGCGG (spanning the UTR and ORF) was prepared by ligating the annealed product of primers 50 and 51 to BbsI-cut pX330 (Addgene #42230). ^{mAID}SGT1 in pUHD-SB-mAID/Hyg was generated by ligating the NcoI-EcoRI-digested PCR product (primers 52-53; template: SGT1A in pT7T3D-Pac mod1 (from German Human Genome Project DHGP) into pUHD-SB-mAID/Hyg.

Oligonucleotides

Primers
1: 5′-TAGTCGACATGGATAGATCCGGAAA-3′
2: 5′-ACCTACAGGTGGGGTCTTTCATTCCC-3′
3: 5′-GCCTCGAGTGCAGAGGTTTCTAC-3′
4: 5′-TATCTAGACGAGACCCTGTCTCA-3′
5: 5′-AGAGATCTGCTTCCTCGCTCACTG-3′
6: 5′-CGCTAACAAGATCTTAACGCTTAC-3′
7: 5′-CGTATCCCTAGGCCCTTTCGTC-3′
8: 5′-CTCATCAATCGATCTTATCATGTCTGG-3′
9: 5′-CCGGATCCAGACATGATAAGATCG-3′
10: 5′-TAGTCGACATGGCCAAGCCTTTGT-3′
11: 5′-CATAAGCTTCGGCCACGAAGTGCT-3′
12: 5′-ACTAATCGATGGCCAAGTTGACC-3′
13: 5′-GACAAGCTTCAGTCCTGCTCC-3′
14: 5′-CACCATGGCATACTTTCCTGA-3′
15: 5′-GTACACATATAATTTCTCCACCTTT-3′
16: 5′-TGGAGAAATTATATGTGTACCGC-3′
17: 5′-GATCGTTAAGCTTTTTAGCTAGTGG-3′
18: 5′-GGAACTAATCATATGTGGCCTGG-3′
19: 5′-GGAGATGGATCCGGGGAGCA-3′
20: 5′-CAAGATCTGGAGAGGGCAGAGGAA-3′
21: 5′-CGTCTAGACCTCCCACACATAAC-3′
22: 5′-AAGTATGCCATGGTGGCCTCAGA-3′
23: 5′-CCGTCGACATATGTCTAGATTAGATAAA-3′
24: 5′-TTGCGGCCGCATCGATGGGCCAGGATTCTCC-3′
25: 5′-GAGGCATCGATGACCGAGTACAAG-3′
26: 5′-GAGTGAATTCACGACAGGC-3′
27: 5′-CAGAATTCCGCTCAGAAGAACT-3′
28: 5′-CGCTATCGATGATTGAACAAGATGG-3′
29: 5′-TCGAATTCTCGTAGCACGTGT-3′
30: 5′-ATGCCCAGTTTAATTTAAATATCTAGGCCC-3′
31: 5′-CAATGTATCTTATCATGTCTATCGATC-3′
32: 5′-GATCGATAGACATGATAAGATACATTG-3′

(*Continues*)

33: 5′-GAAAACTAGAGATTCTTGTTTAGGCCCAGC-3′
34: 5′-CACCGTACTTTGTTTCAGGTACCTA-3′
35: 5′-AAACTAGGTACCTGAAACAAAGTAC-3′
36: 5′-CACCGTGCCACATCCCGAACTGAC-3′
37: 5′-AAACGTCAGTTCGGGATGTGGCAC-3′
38: 5′-AGCTCGTTTAGTGAACCGTCAGATCG-3′
39: 5′-TCTGTCGGTCCTCGATGTGGCA-3′
40: 5′-CGAGGACCGACAGAGAGATCAA-3′
41: 5′-TTGGATCCTACTCCGGATTACC-3′
42: 5′-ACCGATCCAGCCTCCGCGGG-3′
43: 5′-CATGTCTATCGATCTTATCATGTCTG-3′
44: 5′-CACCGGAAACTATAGATGCGGGCA-3′
45: 5′-AAACTGCCCGCATCTATAGTTTCC-3′
46: 5′-AAAGGCGCCATGGAGAAGGACG-3′
47: 5′-TGCGTGCCAATCCGAAGTCAGC-3′
48: 5′-CGGATTGGCACGCATCTAT-3′
49: 5′-CTGGATCCTCAGGCTGTATTCA-3′
50: 5′-CACCGCGACTACGAGGGATGGCGG-3′
51: 5′-AAACCCGCCATCCCTCGTAGTCGC-3′
52: 5′-GAGCCATGGCGGCGGCTGCAGCAGG-3′
53: 5′-TAGAATTCTTAGTACTTTTTCCATT-3′

Cell lines

HeLa (cervical carcinoma) used in this study was a clone expressing the tTA tetracycline transactivator. H1299 (non-small cell lung carcinoma) was obtained from American Type Culture Collection (Manassas, VA, USA).

Cells expressing different combination of ^{AID}CDK1 and/or ^{AID}CDK2 in ^{KO}CDK1 and/or ^{KO}CDK2 background were generated by transfecting HeLa with a mixture of plasmids expressing ^{AID}CDK1, ^{AID}CDK2, CDK1 CRISPR-Cas9, CDK2 CRISPR-Cas9, TIR1, and SB transposase (pCMV(CAT)T7-SB100; Addgene, #34879) as appropriate. A plasmid expressing blasticidin-resistant gene was also co-transfected. Transfected cells were enriched with blasticidin selection for 36 h, followed by selection with hygromycin B and puromycin for 2 wk.

^{AID}CDK1^{KO}CDK1 cells in H1299 were generated by transfecting cells with ^{AID}CDK1 in pUHD-SB-AID/Hyg, pSBbi-TIR1-tTA/Pur, CDK1 CRISPR-Cas9, and SB transposase, then selected with hygromycin B and puromycin for 2 wk.

^{AID}CDK1,2,4,6^{KO}CDK1,2,4,6 cells were generated by transfecting ^{AID}CDK1,2^{KO}CDK1,2 HeLa cells with plasmids expressing ^{AID}CDK4, ^{AID}CDK6, CDK4 CRISPR-Cas9, CDK6 CRISPR-Cas9, and SB transposase. Cells were allowed to recovery for 72 h after transfection, followed by selection with blasticidin and zeocin for 2 wk.

^{mAID}SGT1^{KO}SGT1 HeLa and H1299 cells were generated with an identical procedure as ^{AID}CDK1^{KO}CDK1 in H1299, except that the ^{mAID}SGT1, pSBbi-TIR1/Pur, and SGT1 CRISPR constructs were used.

Stable EGFP-expressing cells were generated by transfecting HeLa cells with plasmids expressing EGFP and SB transposase. A plasmid expressing blasticidin-resistant gene (Ma and Poon, 2018) was also co-transfected. Transfected cells were enriched with blasticidin selection for 36 h, followed by selection with hygromycin B for 2 wk. Stable EGFP and mRFP-expressing cells were generated by transfecting EGFP-expressing cells with plasmids expressing mRFP and SB transposase, or mRFP and mTol2 transposase. A plasmid expressing puromycin-resistant gene (Ma *et al*., 2009) was also co-transfected. Transfected cells were enriched with puromycin selection for 36 h, followed by selection with blasticidin for 2 wk.

Cell culture

Cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) calf serum (for HeLa) or fetal bovine serum (for H1299) and 50 U/ml penicillin streptomycin (Life Technologies, Carlsbad, CA, USA). Cells were treated with the following reagents at the indicated final concentration: blasticidin (Thermo Fisher; 3.75 μg/ml for transient selection; 2 μg/ml for stable selection), doxycycline (Dox) (Sigma-Aldrich; 2 μg/ml), G418 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 0.75 mg/ml), hygromycin B (Life Technologies; 0.5 mg/ml), indole-3-acetic acid (IAA) (50 μg/ml), (Fermentas, Vilnius, Lithuania), puromycin (Sigma-Aldrich, St. Louis, MO, USA; 0.75 μg/ml for transient selection; 0.3 μg/ml for stable selection) and zeocin (Life Technologies; 0.04 mg/ml). Cells were transfected using a calcium phosphate precipitation method (Ausubel *et al*., 1995). For colony formation assay, 200 cells were seeded onto 60-mm plates and treated with the indicated chemicals. After 14 d, colonies were fixed with methanol:acetic acid (2:1 vol/vol) and stained with 2% (wt/vol) crystal violet for visualization.

Flow Cytometry

Flow cytometry analysis after propidium iodide staining was performed as previously described (Mak *et al*., 2020).

Antibodies and immunological methods

Immunoblotting was performed as previously described (Ng *et al*., 2019). The following antibodies were obtained from the indicated sources: beta-actin (Sigma-Aldrich), CDK1 (sc-54, Santa Cruz Biotechnology, Santa Cruz, CA, USA), CDK2 (sc-53220 or sc-6248, Santa Cruz Biotechnology; or ab32147, Abcam, Cambridge, UK) CDK4 (mAb#12790, Cell Signaling Technology), CDK6 (sc-53638, Santa Cruz Biotechnology), MYC (sc-40, Santa Cruz Biotechnology), and SGT1 (sc-398625, Santa Cruz Biotechnology).

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