A novel auxin-inducible degron system for rapid, cell cycle-specific targeted proteolysis

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Abstract

The OsTIR1/auxin-inducible degron (AID) system allows selective protein degradation upon exposure to the phytohormone auxin. However, this technology does not allow to study the effect of acute protein depletion selectively in one phase of the cell cycle. Here, we report a new AID system to Regulate OsTIR1 Levels based on the Cell Cycle Status (ROLECCS) for phase-specific target proteolysis. Finally, we applied the ROLECCS technology to show that the tumor suppressor TP53 plays a S/G2-specific role in suppression of micronuclei accumulation. This new tool allows the analysis of different protein functions during cell cycle progression with unprecedented temporal resolution.

Introduction

The cell-division cycle, also known as cell cycle, is the fundamental, precise and complex process at the basis of life and physiological processes such as development, tissue growth, homeostasis, regeneration, and aging in multicellular organisms[1,2]. In mitotic cells, the division into two daughter cells (cytokinesis) occurs after the parental cell undergoes the semiconservative synthesis (S phase) of a new copy of its entire genome, followed by the mitotic chromosomal segregation (M phase). Two gap phases, G_0/G_1 and G_2, precede the S and M phases, respectively[1–3].

The mechanisms leading to and controlling DNA replication and segregation are historically among the most studied and understood processes happening throughout cell division[4]. Critical molecular players involved in cell cycle regulation and control have been identified based on the effect that their mutation, overexpression, or silencing have on genome replication, either in physiological conditions or in response to DNA damaging agents[3,5,6] .

However, genomic DNA is not the only cellular component undergoing dramatic changes during cell cycle progression. Proteins, organelles, and cellular membranes experience profound modifications to allow the appropriate segregation of all the required materials in the daughter cells[7,8]. One obvious paradigm is constituted by the nuclear membrane, which disassembles immediately before cells enter mitosis to be promptly re-assembled at the completion of the cell division cycle[7–9]. During this process, the nuclear content and proteins of the nuclear pore complexes are released in the open cytoplasm, and novel protein-protein interactions can take place[10–12]. Hence, it could be assumed that virtually any cellular protein might become part of alternative multi-protein complexes and perform different biological tasks, such as preserving DNA integrity[13] or cytoskeletal dynamics[14].

To date, existing technical limitations have prevented an appropriate discrimination of phase-specific protein functions, especially in physiological conditions. Insights into the cell cycle regulatory networks were initially obtained by analyzing cells synchronized in specific phases of the cell cycle. However synchronization is routinely achieved by exposing cells to stress conditions, such as serum starvation, inhibition of DNA synthesis, or by disrupting microtubule dynamics[15,16].

To assess the cell cycle phase-specific functions of a protein of interest, one option is to take advantage of “cell cycle tags”, cell cycle-dependent protein degrons that can restrict protein expression to a specific phase of the cell cycle[17–19]. However, these approaches suffer from intrinsic limitations, as cell cycle degrons do not respond to external stimuli to regulate their functions. Therefore, previously reported cell cycle tags are constitutively functioning and not tunable.

A major advancement in the field of cell cycle tags was represented by the development of the FUCCI (Fluorescent Ubiquitination-based Cell Cycle Indicator) system[20–22]. This technology is based on the enzymatic activity of two E3 ubiquitin ligases, APC^{Cdh1} and SCF^{Skp2} [22–25], involved in the control and proteasomal degradation of Geminin (targeted by APC^{Cdh1}) and Cdt1 (targeted by SCF^{Skp2})[26]. Cdt1 and geminin are critical regulators of the licensing of replication origins, with opposite functions and biological effects[26]. By fusing Cdt1 and Geminin with a variety of fluorescent proteins, a number of tools were engineered to accurately discriminate the cell cycle status of individual cells, either microscopically or by flow cytometry[22, 27–30], both *in vitro* and *in vivo*. Additional live-cell sensors, such as CDK2-activity fluorescent reporters[31], have also been used for *in silico* cell synchronization and study of protein biological functions by microscopy.

These novel approaches provide an efficient way to identify, visualize and select cells in specific phases of the cell cycle. However, some of them do not allow the isolation of large number of cells for downstream experiments, such as multi-omic or functional analyses. More importantly, they must be still combined with other technologies to perturb the levels of the protein of interest (POI) and assess its biological role throughout the cell cycle. Despite the advancements of genetic tools such as CRISPR/Cas9-based gene editing[32], gene silencing[33], or inducible gene expression approaches[34], none of these systems displays readiness of activity compatible with the kinetics of cell cycle progression. Conversely, an alternative to obtain rapid degradation of the POI is represented by targeted proteolysis using PROteolysis-Targeting Chimeras (PROTACs), or polypeptide tags (also known as degrons)[35,36]. In one of the most commonly used degron systems, the POI is fused with an Auxin-Inducible Degron (AID) sequence, such as the 7 kDa degron termed mini-AID (mAID), in cell lines expressing the *Oryza sativa* TIR1 (*Os*TIR1) F-box protein[37–39]. When the phytohormone auxin (indole-3-acetic acid IAA) is provided, OsTIR1 binds the mAID-POI and induces its quick proteasomal degradation[37,38]. However, despite their speed, reversibility, and fine-tuning, degron-based systems still lack cell cycle phase-specificity and require conventional cell synchronization[40].

Here, we report the engineering of the “Regulated OsTIR1 Levels of Expression based on the Cell-Cycle Status” (ROLECCS) technology, which combines the AID and the FUCCI systems. In this new tool, the *Os*TIR1 protein is fused to the fluorescent indicator mEmerald and the FUCCI tags Cdt1/Geminin, which are responsible for the restricted G_1 and S/G_2 expression, respectively. Upon auxin treatment, only the cells expressing the fusion-protein OsTIR1-mEmerald-Cdt1/Geminin, (i.e. in the desired cell cycle phase) degrade the mAID-POI. We further developed a second ROLECCS system (ROLECCSv2), which is triggered by the synthetic auxin analog 5-phenyl-indole-3-acetic acid (5-Ph-IAA), overcoming the major drawbacks

of auxin treatment, such as basal level protein degradation in the absence of the phytohormone[35]. We tested our ROLECCS systems for the cell cycle phase-specific control of both exogenous overexpressed targets and endogenous CRISPR/Cas9 gene-edited proteins. Finally, we assessed the capability of ROLECCS to discriminate between some of the G1 and S/G2 specific functions of the tumor suppressor TP53 in preserving genomic stability.

Results

Designing and engineering a cell cycle phase-specific OsTIR1

To engineer a cell cycle phase-specific degron system, we generated a variant of the mAID system where the expression of OsTIR1, necessary for the recognition and degradation of the mAID-tagged protein upon auxin exposure, was dependent on the G_0/G_1 or $S/G_2/M$ phase.

In our design, the OsTIR1 coding gene was fused in-frame with a mEmerald fluorescent reporter (a brightly fluorescent monomeric variant of GFP[41]) that allows the identification of cells expressing these constructs by fluorescence microscopy and flow cytometry. Then, we added the sequences corresponding to either human Cdt1 (aa 30–120) or Geminin (aa 1-110) to restrict OsTIR1 expression to different phases of the cell cycle, like in the FUCCI system. For convenience, the hCdt1 (30–120) and hGeminin (1-110) tags are indicated hereafter as Cdt1 and GEM, respectively. We also generated a construct where no additional tag was added, to allow OsTIR1-mEmerald expression independently on the phase of the cell cycle (Fig. 1).

In our design, engineered variants of OsTIR1-mEmerald, OsTIR1-mEmerald-Cdt1, and OsTIR1-mEmerald-GEM genes are actively transcribed throughout the cell cycle. However, the presence of the Cdt1 and the Geminin tags determine the Regulated OsTIR1 Levels of Expression based on the Cell Cycle Status (ROLECCS system). We predicted that the OsTIR1-mEmerald protein would be stably present throughout the cell cycle. Therefore, auxin treatment would trigger OsTIR1 enzymatic activity and degradation of the mAID-tagged protein of interest in any cell, independent of the cell cycle status (from now on: asynchronous ROLECCS, ROLECCS AS) (Fig. 1A).

On the other hand, the presence of OsTIR1-mEmerald-Cdt1 (from now on: ROLECCS G1) protein would be restricted the G_1/early S phase, because ubiquitylation by SCF^{Skp2} E3 ligase leads to its prompt degradation during S-phase transition. Thus, addition of auxin would lead to OsTIR1-mediated proteasomal degradation of the POI exclusively in those cells in G_1/S phase during the treatment (Fig. 1B).

Similarly, presence of OsTIR1-mEmerald-GEM (from now on: ROLECCS G2) protein would be restricted during the late S-G_2-M phase, peaking during the G_2, as APC^{Cdh1}-mediated ubiquitylation and degradation is rapidly triggered during M/G_1 transition. Consequently, auxin treatment would cause degradation of the

POI exclusively in cells going through the late S-G_2-M phase of the cell cycle during the treatment (Fig. 1C). To provide flexibility to the system and make it usable in different paradigms, the three CMV-driven ROLECCS constructs were subcloned in *ad hoc* vector[37] that allows either transient expression or CRISPR/Cas9-mediated integration into the *AAVS1* safe harbor site of the human genome (**Supplementary Fig. 1A-C**).

ROLECCS G1 and ROLECCS G2 expression during cell cycle.

To demonstrate that ROLECCS G1 and ROLECCS G2 expression is restricted to specific phases of the cell cycle, we first assessed their relative abundance in transiently transfected HEK-293 cells. As shown in Fig. 2A, each ROLECCS construct was abundantly expressed at 72h from transfection. Unlike the previously published FUCCI probes[22], all the ROLECCS proteins were present both in the nucleus and in the cytoplasm of transfected cells.

To obtain cell populations expressing a uniform level of the ROLECCS proteins, we generated stable HEK-293 cell lines. To this aim, the AAVS1 ROLECCS vectors were integrated into the *AAVS1* safe harbor locus by CRISPR/Cas9-mediated gene knock-in. Also in this case, sustained and ubiquitous expression of the ROLECCS proteins was observed by nuclear/cytoplasmic protein fractionation (Fig. 2B), and direct fluorescence imaging (Fig. 2C).

Next, we aimed to demonstrate that ROLECCS G1 and ROLECCS G2 protein levels oscillate reciprocally during cell cycle transition. Live cell imaging was performed on ROLECCS AS, G1 and G2 knock-in HEK-293 to monitor cell division and green fluorescence in real time. Figure 2D (**top**) and **Supplementary Video 1** show that ROLECCS AS expression did not change during a full cell cycle. Conversely ROLECCS G1 was not visible in actively dividing cells (Fig. 2D, **middle and Supplementary Video 2**), becoming detectable immediately upon completion of cell division, as assessed by contrast phase imaging. Finally, ROLECCS G2 was visible only in actively dividing cells, with the fluorescence intensity peaking at G_2/M transition (cells with round shape in contrast phase imaging, Fig. 2D, **bottom and Supplementary Video 3**).

To orthogonally validate ROLECCS G1 and ROLECCS G2 as cell cycle indicators, we sorted *AAVS1*-integrated ROLECCS HEK-293 based on their green fluorescence level and cellular complexity (Side Scatter, SSC) (**Supplementary Fig. 2A-D**), as described in the Methods section. DNA content analysis demonstrated that GFP^{high}-sorted ROLECCS G1 population mostly comprised cells in the G_1/early S phase (90.3 ± 6.5%), in comparison with GFP^{med} and GFP^{low} sorted populations (56.6 ± 10.6% and 17.9 ± 4.7% respectively) (Fig. 2E and 2F). Conversely, GFP^{high} ROLECCS G2 population showed a significant enrichment in late S/G_2 phase cells (94.1 ± 0.6%), compared to GFP^{med} and GFP^{low} sorted cells (50.2 ± 9.7% and 5.2 ± 2.4%, respectively) (Fig. 2G and 2H). Conversely, unsorted ROLECCS G1 and ROLECCS G2 populations displayed cell cycle distribution typical of unsynchronized HEK-293 cells.

Altogether, our findings indicate that engineered ROLECSS G1 and G2 protein levels are efficiently restricted to specific phases of the cell cycle, and their fluorescence intensity can be used as a good

surrogate marker of cell cycle distribution.

Biological activity of ROLECCS.

The addition of large tags to proteins might affect their biological activity[42]. Therefore, we wanted to assess that the enzymatic activity of OsTIR1-containing SCF complexes was not hampered by the mEmerald-Cdt1 and mEmerald-GEM tags of the ROLECCS G1 and G2, respectively. We transiently transfected *AAVS1*-integrated ROLECCS AS, ROLECCS G1, and ROLECCS G2 HEK-293 cells with a mAID-mCherry fluorescent reporter and measured its protein levels. As shown in **Supplementary Fig. 3A**, mAID-mCherry levels were appreciably reduced upon auxin treatment when performed at 8h after reporter vector transfection, indicating that the biological activity of OsTIR1 was preserved. However, transient transfection could lead to multiple sub-populations of ROLECCS-expressing cells with different levels of mAID-mCherry due to inconsistent transduction. Moreover, at later time points, auxin-dependent degradation of mAID-mCherry was negligible (not shown). Therefore, we hypothesized that an overexpressed target could be efficiently degraded only if the molar ratio between the POI and the ROLECCS was favorable to the latter, as in the very first hours (< 8h) after transfection. This is in line with the findings of other groups, which have generated All-in-One systems to achieve equimolar levels of OsTIR1 and its targets[43].

As shown in Fig. 3A-C, we created 3 all-in-one lentiviral vectors (pLentiROLECCS AS, G1, and G2) in which the ROLECCS proteins were fused with mAID-mCherry using an autoproteolytic P2A sequence[44]. This approach allows the simultaneous and equimolar expression of both OsTIR1 and its target which, upon translation, are released as independent molecules. Figure 3D shows that transient and stable transfection of pLentiROLECCS vectors led to sustained expression of both the ROLECCS proteins and of mAID-mCherry.

Next, we treated HEK-293 cells stably expressing LentiROLECCS G1 or LentiROLECCS G2 with auxin for 1h. Cells were sorted based on their GFP fluorescence intensity and SSC and analyzed by Western Blot. Figure 3E-F show that downregulation of the mAID-mCherry protein levels was specifically achieved in GFP^{med} and GFP^{high} populations upon auxin treatment. Western blot analysis also confirmed that these sorted cell populations expressed the highest levels of ROLECCS G1 and ROLECCS G2 (**Supplementary Fig. 3B-E**). Notably, highest levels of ROLECCS G1 corresponded to highest expression of Cdt1 (a G_1-specific marker, frequently identified as a doublet corresponding to Cdt1/phosphoCdt1[45]) and to the lowest levels of Cyclin B1 (a late-S/G_2 marker). Importantly, downregulation of the target mAID-mCherry was only observed in sorted GFP^{high} ROLECCS G1 cells upon auxin treatment, and not in the untreated or GFP^{low} auxin-treated controls (**Supplementary Fig. 3B-C)**. Similarly, ROLECCS G2 accumulation was observed in cell populations displaying highest levels of Cyclin B1 and lowest levels of Cdt1, but target downregulation was only observed upon auxin treatment (**Supplementary Fig. 3D-E**). These results were confirmed using 2 independent HEK-293 LentiROLECCS clones.

To confirm the cell cycle phase-specificity of ROLECCS G1 and G2 proteolytic activity, HEK-293 cells stably expressing LentiROLECCS G1 or LentiROLECCS G2 were treated with RO-3306, a potent cell cycle inhibitor, able to block the cell cycle in the G2/M phase of the cell cycle[46] (**Supplementary Fig. 4A and D**). For ROLECCS G1-transduced cells, we observed a significant inhibition of ROLECCS-mediated auxin-dependent proteolytic activity against mAID-mCherry, due to the reduction of the relative abundance of cells in the G1 phase upon RO-3306 treatment (**Supplementary Fig. 4B-C**). Conversely, RO-3306 enhanced the activity of ROLECCS G2, due to the increase of the relative abundance of the cells in the S/G2 phase.

Finally, we wanted to assess how promptly ROLECCS G1 and ROLECCS G2 could induce targeted proteolysis during cell cycle progression, in real time. To this aim, we performed live-cell confocal microscopy imaging on MCF 10a normal breast epithelial cells, transduced with LentiROLECCS G1 or LentiROLECCS G2. Figure 3G **and H** show that degradation of mAID-mCherry was noticeable even before the green-fluorescent signal from the ROLECCS G1 and G2 could be detected. Moreover, DNA imaging using Hoechst stain confirmed that ROLECCS G1 fluorescence was detected immediately after completion of cell division, when the cells enter the G1 phase of the cell cycle. Contrariwise, the ROLECCS G2 fluorescence increased during the progression through S and G2 phase, and promptly decreased upon cell division, in agreement with the data shown in Fig. 2.

Taken together, our data indicate that the ROLECCS system allows fast and temporally restricted degradation of mAID-tagged targets based on the cell cycle phase.

The ROLECCS and the ROLECCSv2 systems allow cell cycle phase-specific downregulation of endogenous proteins

It has been previously demonstrated that the mAID system is suitable for the downregulation of endogenous protein, when the gene of interest is modified by CRISPR/Cas9-mediated knock-in to include the mAID sequence[37]. We aimed to demonstrate that the ROLECCS system allows the same capability, but specifically in the phase of the cell cycle of interest. We decided to test whether the ROLECCS system could accomplish the cell cycle phase-specific downregulation of TP53, a well-known transcriptional factor playing a central role in the control of cell cycle progression, and genomic stability, especially in response to DNA-damaging agents[47–49]. Moreover, one of the main mechanisms of physiological negative regulation of TP53 is its MDM2-mediated ubiquitylation and proteasomal degradation[47]. Therefore, we postulated that ROLECCS-mediated synthetic degradation of TP53 could represent a valid alternative to its physiological mechanism of regulation.

First, we generated HCT116 cell lines where both wild-type *TP53* alleles were modified by CRISPR/Cas9-mediated knock-in (HCT116 TP53-mAID-mCherry). For gene editing purposes, the stop codon of the endogenous *TP53* gene was replaced by a mAID-mCherry fusion cassette (as described in Methods section) (Fig. 4A, **Supplementary Fig. 5A**). Appropriate editing by site-specific integration of the donor cassette was verified by PCR using integration-specific primer sets, as shown in **Supplementary Fig. 5B-C**.

Western blot analysis showed that gene-edited TP53 had a marked molecular size increase (final predicted molecular weight ~ 87KDa, compared to WT TP53, 53KDa), due to the presence of the mAID and mCherry tags (Fig. 4B). Of note, when probed with a TP53-specific antibody, edited clones displayed additional lower molecular weight bands, possibly due to an unstable linker sequence, previously described at the N-terminal domain of mCherry[50]. Importantly, gene-edited TP53 was still upregulated by DNA damaging agents such as cisplatin treatment, and its nuclear and cytoplasmic localization followed the expected distribution pattern[47,48] (Fig. 4B). The additional lower bands displayed a similar trend upon genotoxic stress. Notably, gene-edited TP53 preserved transcriptional activity on the p21 and BAX promoters, as shown by Real Time (Fig. 4C) and Western Blot (Fig. 4D) analysis, upon cisplatin treatment. Endogenous TP53 expression was also increased at the transcriptional level (Fig. 4C) upon treatment.

Next, we further edited HCT116 TP53-mAID-mCherry cells inserting the ROLECCS constructs in the *AAVS1* safe harbor site, generating HCT116 TP53-mAID-mCherry ROLECCS cell lines. As shown in Fig. 4E, sustained expression of ROLECCS AS, G1, and G2 with the expected molecular weight was achieved in at least 2 independent clones. Since this analysis was performed on asynchronously growing HCT116 TP53-mAID-mCherry ROLECCS cells, the three ROLECCS constructs apparently displayed different expression levels. However, these differences are likely due to the fact that ROLECCS G1 and ROLECCS G2 are expressed only in phase-specific cell subpopulations, while ROLECCS AS is equally expressed throughout the cell cycle. We also noticed a mild reduction in the levels of edited TP53 in comparison with parental HCT116 cells, compatible with the partial leakiness observed for the AID system[51,52]. For this reason, for all the functional studies, cells were pre-treated with auxinole (as described in the Methods section), a previously reported inhibitor of OsTIR1[51], to neutralize the activity of the ROLECCS system in the absence of auxin.

Finally, to validate that the ROLECCS system could allow cell-cycle specific target degradation of an endogenous target, we treated HCT116 TP53-mAID-mCherry ROLECCS G1 and G2 cells with auxin. At 1h after auxin treatment, cells were sorted (**Supplementary Fig. 6A-D**) based on their green fluorescence and SSC, as described in the Methods section. As shown in Fig. 4F-G, TP53 downregulation was noticeable in unsorted populations both in ROLECCS G1- and G2-expressing cells. However, upon sorting, we observed that TP53 downregulation upon auxin treatment was only achieved in GFP^{med} and GFP^{high} sorted populations, in comparison with GFP^{low} cells for both ROLECCS constructs. Importantly, Cdt1 and Cyclin B1 levels confirmed that ROLECCS G1 GFP^{med} and GFP^{high} represented a cell population enriched in G_1/early S phase of the cell cycle. On the other hand, ROLECCS G2 GFP^{med} and GFP^{high} cells were mostly representing cells in the late S/G_2 phase (see also **Supplementary Fig. 9**). Similar results were obtained using two independent clones for each ROLECCS protein (**Supplementary Fig. 7A-D**).

After these initial experiments, we noticed that the efficiency of the ROLECCS technology showed so far was only partially efficient, especially for ROLECCS G2. Nonetheless, these experiments required prior treatment with auxinole to prevent any leakiness shown by the *Os*TIR1 F-box protein, as described elsewhere[35,52]. These drawbacks have been shown to be dramatically reduced by using an engineered

variant of *Os*TIR1 containing a point mutation (F74G), which makes the system responsive to a synthetic variant of auxin, named 5-phenyl-indole-3-acetic acid (5-Ph-IAA)[35]. The *Os*TIR1(F74G) is able to induce efficient degradation of mAID-tagged proteins with no noticeable leakiness, more rapidly than the original AID system, in response to lower (more than 500 times) concentrations of the ligand, both *in vitro* and *in vivo*. Therefore, we decided to implement the same point mutant in our ROLECCS technology, generating the ROLECCS G1v2 and the ROLECCS G2v2 systems. HCT116 TP53-mAID-mCherry cells were edited inserting the ROLECCS v2 constructs in the *AAVS1* safe harbor site, generating HCT116 TP53-mAID-mCherry ROLECCS v2 cell lines, as described above (**Supplementary Fig. 8A**), where TP53 expression levels could be abrogated promptly and reversibly (**Supplementary Fig. 8B**). Figure 5A-B show that, similarly to the previous ROLECCS constructs, ROLECCS G1 v2 and G2 v2 were specifically expressed in the G1 or lateS/G2/M phase of the cell cycle, respectively. Sorting (**Supplementary Fig. 8C-D**) of GFP^{high} ROLECCS G1 v2 resulted in a significant enrichment of cells in the G1 phase of the cell cycle, while GFP^{low} cells were significantly enriched in cells progressing through S/G2/M, as demonstrated by propidium iodide staining after sorting (Fig. 5A). Conversely sorting of GFP^{high} ROLECCS G2 v2 resulted in a significant enrichment of cells in the S/G2/M phase of the cell cycle, while GFP^{low} cells were significantly enriched in cells progressing through G1 (Fig. 5B). We then performed WB analysis on protein extracts from samples sorted as described above, and assessed the levels of TP53-mAID-mCherry (Fig. 5C-F). Notably, both ROLECCS G1 v2 and ROLECCS G2 v2 displayed a noticeable and significant downregulation of their target, specifically when ROLECCS v2 levels were highest (GFP^{high}, corresponding to enriched G1 or S/G2/M, respectively). Importantly, GFP^{low} ROLECCS v2 systems did not display noticeable differences in the levels of their target, when comparing control vs 5-Ph-IAA treated samples.

Altogether, these data indicate that the ROLECCS and the ROLECCSv2 systems can be used to achieve the phase-specific downregulation of an endogenous target, appropriately gene edited to include a mAID tag.

Cell cycle phase-specific TP53 degradation has different effects on micronuclei formation

TP53 is one of the most frequently mutated genes in human cancer[47,49,53]. Its most well studied biological function is the regulation of expression of genes involved in cell cycle arrest, DNA repair, and apoptosis[49,53]. Loss of TP53 results in altered DNA damage response, reduced cell death even in response to anti-neoplastic treatments, and genomic instability[49,53]. For these reasons, TP53 is considered the main “guardian of the genome”[47,54]. Micronuclei are the result of missegregated chromosomes that, upon mitotic exit, can recruit a lamin B-positive nuclear envelope, creating subcellular structures that are frequently identified in genetically unstable human tumors[55]. One of the still not completely understood roles of TP53 in maintaining genome stability is its capability of preventing micronuclei formation or regulating the faith of micronucleated cells[56–58]. To gain insights into novel potential cell cycle-phase specific functions of TP53, we assessed the capability of HCT116 TP53-mAID-mCherry ROLECCS AS, G1, and G2 cells to spontaneously form micronuclei upon auxin treatment.

Asynchronously growing cells were treated for 1h with auxin, harvested, sorted as described above (**Supplementary Fig. 8C-D**) and plated on glass coverslips in complete media containing auxin for 24h. In parallel, cells were similarly collected and used for both WB analysis to confirm TP53-mAID-mCherry degradation upon auxin treatment (**Supplementary Fig. 9A**) and enrichment of cells in G1 or G2 (**Supplementary Figs. 9B-D**). ROLECCS AS cells were used as control for generalized (non-cell cycle phase-specific) TP53 degradation. At 24h from the plating, cells were fixed and stained for Lamin B and DNA to visualize nuclei and micronuclei, and the relative abundance of micronucleated cells was quantified by fluorescence microscopy (Fig. 6A **and C**). Interestingly, neither TP53 degradation in any phase of the cell cycle (ROLECCS AS, black) nor specifically in the G1 (ROLECCS G1, red) led to an altered formation of micronuclei that could reach statistical significance. However, when TP53 was specifically degraded in S/G2/M phase (ROLECCS G2, green), we observed a significant increase in the number of micronucleated cells. To demonstrate that cell sorting is not required to perform this assay, and it is not related to the observed phenotype, we repeated the same experiment on asynchronously growing HCT116 TP53-mAID-mCherry ROLECCS AS, G1, and G2 cells, continuously treated with auxin for 24h. As shown in Fig. 6B, ROLECCS G2, but not ROLECCS G1 and AS, displayed a significant increase in the number of micronucleated cells upon auxin treatment. These results indicate that TP53 plays different cell cycle phase-specific roles in preventing accumulation of micronucleated cells.

Discussion

The temporal discrimination of protein functions is critical to fully understand how the same factor might carry out different tasks during different phases of the cell cycle, ultimately leading to diverse biological outcomes. Therefore, “timing is everything”[59].

The development of mAID systems has allowed sharp and quick modulation of the levels of a protein of interest[36,60,61]. Considering the relatively short duration of cell division cycle, a rapid depletion of the protein of interest is of paramount importance. In this report, we introduced a novel tool to rapidly and reversibly regulate levels of virtually any protein in a cell cycle status-dependent manner, using the “Regulated *Os*TIR1 Levels of Expression based on the Cell-Cycle Status” (ROLECCS) system.

We generated two different ROLECCS proteins (ROLECCS G1 and ROLECCS G2), by fusing the *Oryza sativa* TIR1 (*Os*TIR1) F-box protein, the fluorescent indicator mEmerald and the FUCCI tags Cdt1 and Geminin, respectively. The ROLECCS system exerts its targeted proteolytic activity based on a Boolean-logic computational process (cell is actively dividing/not dividing). In fact, the presence of the phytohormone auxin and the appropriate cell cycle status are both simultaneously required to trigger the biological functions of ROLECCS proteins. As a result, the degradation of the mAID-tagged POI is temporally restricted to a specific cell cycle status, and only in the presence of auxin (**Figure 1 and Figure 6D**).

In our first design, the ROLECCS system is specifically integrated by CRISPR/Cas9 knock-in in the *AAVS1* safe harbor genomic locus (**Figure 2**). In these settings, we observed appropriate phase-specific

expression of the ROLECCS proteins. Western blot, flow cytometry and live cell imaging using fluorescence and contrast-phase microscopy confirmed that ROLECCS G1 and ROLECCS G2 expression had a maximum expression peak in G1 and late S/G2, respectively (**Figure 2** and **Supplementary Videos 1-3**). However, we noticed that exogenous targets (e.g. transiently transfected mAID-tagged POIs) were effectively down-regulated only at very short time points after the transfection. We hypothesized that this was due to molar excess of the transfected target POI in comparison with ROLECCS proteins, especially at longer time points.

For this reason, we designed All-in-One constructs, similar to others recently reported[43], allowing the simultaneous expression of the ROLECCS proteins and their mAID-tagged targets (pLentiROLECCS), using mAID-mCherry for our tests. Our results supported the conclusion that ROLECCS proteins require to be at least in equimolar ratio to their targets to achieve consistent target degradation upon auxin treatment. Therefore, the pLentiROLECCS system is a flexible and relatively simple way to generate cell lines in which an exogenous POI can be modulated on-demand (upon auxin treatment) in specific phases of the cell cycle (**Figure 3**).

Our ultimate goal was to generate a system to synthetically control endogenous protein levels based only on the cell cycle status, minimizing potential artificial factors such as the use of an exogenous promoter. Therefore, as proof-of-principle, we attempted to regulate the levels of a protein encoded by an endogenous gene, fused by CRISPR/Cas9 knock-in with the mAID tag. We chose the gene *TP53* because of its well-known role in the regulation of cell cycle progression[48]. Moreover, gene editing of this gene with the mAID tag was previously reported[37]. Here, we show that cell cycle specific TP53 degradation was effectively achieved with both the ROLECCS G1 and the ROLECCS G2 systems (**Figure 4**).

We also noticed that proteolysis of TP53, although significant, was not complete, especially with the ROLECCS G2 system. Therefore, the first iteration of ROLECCS G1 and G2 systems allowed us to demonstrate the appropriate restricted expression of the system in quiescent or dividing cells, respectively, but did not display sufficient biological activity for downstream uses.

Recently, a point mutant of OsTIR1 (F74G) was reported, establishing the mAID version 2 (mAID2) system, which does not respond to natural auxin but only to a synthetic ligand (5-Ph-IAA). Interestingly, this point mutant displayed no detectable leaky degradation of the target, was responsive to 670-times lower concentration of the ligand[35] and it was functional also *in vivo* using mouse models. For these reasons, we decided to implement this mutation in our first ROLECCS system, which resulted in the ROLECCS v2 technology. The ROLECCS v2 displayed a more potent and reliable cell cycle phase-specific targeted proteolysis of TP53.

Last, we wanted to assess whether our targeted proteolytic system could allow the identification of novel biological functions of TP53 related to cell cycle status, such as accumulation of micronucleated cells[56,57]. First, our results confirmed that the ROLECCS system is a valuable tool to study protein functions and identify biological outcomes, which would not be noticeable or reach statistical

significance when using conventional degradation or inhibition approaches. Second, our data indicate that the biological role of TP53 in preventing micronuclei accumulation is related to differential biological functions exerted in different phases of the cell cycle. In fact, our findings suggest that, at least in our model, TP53 abrogation during the S/G2, but not in the G1 phase of the cell cycle results in accumulation of micronucleated cells even in the absence of DNA-damaging agents (**Figure 6**). Interestingly, when TP53 was abrogated in any phase of the cell cycle (using ROLECCS AS), a small, but not statistically significant, increase in the relative abundance of micronuclei was observed, highlighting the importance of the use of a phase-specific targeted proteolytic system. Future studies taking advantage of the ROLECCS system could allow to understand the mechanism through which S/G2/M phase-specific TP53 downregulation leads to an increase of micronucleated cells. One potential explanation of the observed phenotype is that TP53 actively regulates phase-specific genes involved in the prevention of the formation of micronuclei, especially in G2/M phase. On the other end, absence of TP53 could alter micronuclei formation similarly in G1 or S/G2/M, but with different outcomes regarding cell proliferation or activation of apoptosis.

The ROLECCS systems described here are the initial prototypes of multiple potential cell cycle phase-specific degron technologies. For example, the choice of different FUCCI tags, as described previously, could lead to a more accurate phase-specific protein degradation. In fact, the FUCCI tags used for the ROLECCS proteins in the present study are partially co-expressed in late-G_1/early-S phase. However, it has been previously demonstrated the FUCCI systems (PIP-FUCCI, FUCCI(CA) and FUCCI(SCA)) could be used to achieve a sharp down-regulation of the ROLECCS system during cell cycle transitions[27,62].

Protein biological functions and cell cycle progression are intimately connected and reciprocally affected. Hence, the cell cycle status should be taken into account for the study of any biological phenomenon. Thanks to its phase specificity, rapidity, reversibility, and low overall perturbation of other biological processes, the ROLECCS technology represents a unique tool for the investigation of biological phenomena and their relationship with the cell cycle progression.

Methods

Plasmids

All the plasmids used in this study were generated by Gibson Assembly using NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs, E2621L) as per manufactory's instructions. To construct pAAVS1-ROLECCS AS, pAAVS1-ROLECCS G1, and pAAVS1-ROLECCS G2 plasmids, multiple fragments were PCR amplified from different donor plasmids and assembled as follow: pMK232 CMV-OsTIR1-PURO (Addgene #72834[37]) was used as donor plasmid for the expression of OsTIR1 from the *AAVS1* locus, the mEmerald tag was PCR amplified from mEmerald-PLK1-N-16 vector (Addgene #54234; http://n2t.net/addgene:54234 ; RRID:Addgene_54234), while pEN435 - pCAGGS-TagBFP-hGeminin-2A-mCherry-hCdt1-rbgpA-Frt-PGK-EM7-PuroR-bpA-Frt Tigre targeting (Addgene #92139[28]) was used as template for both hGeminin and hCdt1 tags. The vector for the mAID-mCherry expression was generated

using the pEGFP-C1 backbone (Clontech), replacing the GFP gene with the mAID-mCherry cassette derived from pMK292 mAID-mCherry2-NeoR (Addgene #72830[37]). The bicistronic lentiviral vectors for ROLECCS AS, ROLECCS G1, ROLECCS G2, and mAID-mCherry expression were similarly obtained, although linker sequences (P2A) were synthesized (IDT) and cloned by Gibson assembly. AID version2 ROLECCS system (ROLECCSv2), bearing the OSTIR1 (F74G) mutant (PMID 33177522), was obtained from pAAVS1-ROLECCS AS, pAAVS1-ROLECCS G1, and pAAVS1-ROLECCS G2 plasmids, via site directed mutagenesis PCR.

To generate the donor plasmids for *TP53* editing, the genomic region (~2000bp) encoding for the natural stop codon of *TP53* was first cloned into the pUC19 vector (New England Biolabs, N3041S) by Gibson assembly. More specifically, genomic DNA from H460 cell line (TP53 wild-type)[63] was used as template to amplify the *TP53* genomic region (Chromosome 17: 7,668,421-7,687,490, Transcript: TP53-201 ENST00000269305.9) of 1 kb upstream and 1 kb downstream the *TP53* translation stop codon. These regions were further used as homology arms for HDR-mediated CRISPR/Cas9-mediated knock-ins. Secondly, the homology arms containing plasmid was mutated using QuickChange II XL Site-Directed Mutagenesis Kit (Agilent, #200522) to delete the single-guide RNA (sgRNA) recognition sequence, to prevent Cas9 from re-cutting after homology-directed repair-mediated insertion at the desired genetic locus. Finally the mAID-mCherry cassette containing a selection marker was amplified from pMK292 mAID-mCherry2-NeoR (Addgene #72830[37]) or pMK293 mAID-mCherry2-Hygro (Addgene #72831[37]) and inserted between the homology arms (about 1000bp each), replacing the *TP53* stop codon, making sure that the tags sequences were cloned in frame with the gene of interest, in order to generate a fusion protein. A schematic overview of the donor vectors is presented in **Figure 4A**.

To construct the CRISPR/Cas9 *TP53* gene targeting vector, a single-guide RNA (sgRNA) (5'-ACTGACAGCCTCCCACCCCC-3') was designed (http://crispr.mit.edu) to specifically target TP53 translation stop site and it was cloned into pX330-U6-Chimeric_BB-CBh-hSpCas9-hGem (1/110) (Addgene #71707) according to the protocol of Ran et al.[64]. The same protocol was followed to clone the sgRNA used for the ROLECCS transgene insertion in the *AAVS1* locus[37] (PMID: 27052166), into the pX330-U6-Chimeric_BB-CBh-hSpCas9-hGem (1/110)[65].

All the plasmids will be deposited on Addgene or are available from the investigators upon kind request.

Cell Culture, Transfection, and Clones Isolation

The HCT116 and HEK-293T (HEK-293) cell lines were purchased from American Type Culture Collection (ATCC CCL-247; ATCC CRL-11268) and were cultured in RPMI-1640 medium (Millipore Sigma) supplemented with 10% FBS (Millipore Sigma). MCF 10a were also obtained from ATCC and cultured in DMEM/F12 (#21041025 ThermoFisher, Waltham, MA) containing 0.5 mg/ml hydrocortisone, 250 ng/ml insulin, 10 µg/ml transferrin (#H0888, I2643, T8158 Sigma-Aldrich, respectively), and 20 ng/ml epidermal

growth factor. Cells were grown in a 37 °C humid incubator with 5% CO^2. Identity of cell lines was validated upon and after the establishment of stable clones by STR profiling. To generate HEK-293 constitutively expressing pAAVS1-ROLECCS AS, pAAVS1-ROLECCS G1, and pAAVS1-ROLECCS G2, 2×10^5 cells were plated in a six-well plate, and 24h later CRISPR/Cas9 and donor plasmids were transfected using Lipofectamine™ 2000 Transfection Reagent (Thermo Fisher Scientific, #11668019) in Opti-MEM™ I Reduced Serum Medium (Thermo Fisher Scientific, #31985070). Cells were then grown up to a subconfluent T175 cm^2 flask in medium supplemented with 10% FBS and, about ten days after transfection, antibiotic selection was started using 2 µg/ml Puromycin Dihydrochloride (Thermo Fisher Scientific, A1113803) in medium. Resistant populations were expanded up to a subconfluent T175 cm^2 flask and cells were sorted and collected at FACSAria II (Becton Dickinson) for brightest mEmerald expression, using 488nm laser excitation. Parental HEK-293 cells were used as negative control of mEmerald expression to design the gates. Single cell clones were grown in 96-well plates and screened by WB for ROLECCS AS, G1 and G2 expression. Two different clones for each constructs were chosen among the ones with comparable ROLECCS expression. To obtain HEK-293 constitutively expressing pLENTIROLECCS AS, G1 or G2, 2×10^5 cells were plated in a six-well plate and the next day they were transfected with plasmids using Lipofectamine™ 2000 Transfection Reagent (in Opti-MEM™ I Reduced Serum Medium). After 48 hours from transfection, transfected cells selection was started using 5 µg/ml Blasticidin S HCl (Thermo Fisher Scientific, A1113903) in complete medium. Resistant population was expanded up to a subconfluent T175 cm^2 flask and double positive cells for mEmerald and mCherry expression were sorted and collected at FACSAria II, using 488nm and 561 nm laser excitations. Parental HEK-293 cells were used as negative control of mEmerald and mCherry expression to design the gates. Single cell clones were grown in 96 well plates and screened by WB for ROLECCS AS, G1 or G2 and mAID-mCherry expression. Two different clones for each construct were chosen among the ones with comparable proteins expression.

To generate *TP53*-edited HCT116 cells, 2×10^5 cells were plated in a six-well plate and CRISPR/Cas9 along with donor plasmids were transfected using Lipofectamine™ 3000 Transfection Reagent in Opti-MEM™ I Reduced Serum Medium. Cells were then grown up to a subconfluent T175 cm^2 flask in medium supplemented with 10% FBS and, about ten days after transfection, antibiotic selection was started using 700 µg/ml G418 Sulfate (Thermo Fisher Scientific, #10131035) and 100 µg/ml Hygromycin B (Thermo Fisher Scientific, 10687010) in medium. Double resistant population was expanded up to a full T175 cm^2 flask and cells were sorted and collected at FACSAria II for brightest mCherry expression, using 561 nm laser excitation. Parental HCT116 cells were used as negative control of mCherry expression to design the gates. Single cell clones were grown in 96-well plates and screened by WB for TP53-mAID-mCherry expression. Two different clones were chosen among the ones with homozygous expression of TP53 edited protein. After genotyping (see next section), the two clones were gene edited to constitutively express the pAAVS1-ROLECCS or pAAVS1-ROLECCSv2 variants, following the transfection and selection protocol aforementioned for HEK-293 cells.

To test biological functionality of ROLECCS constructs, HEK-293 *AAVS1*-integrated clones were transfected with 150 ng of mAID-mCherry expressing plasmid (see “Plasmid Generation” section) using Lipofectamine 2000. After 7 hours from transfection, cells were treated with 500 µM auxin for one hour. Cells were then collected and processed for WB analysis.

Lentivirus production and transduction

Lentiviruses were produced in Lenti-X 293T cells (# 632180, Takara Bio, Shiga, Japan) using Lenti-X Packaging Single Shots kit (# 631276, Takara Bio) according to the manufacturer instructions. At 48h from transfection, supernatant was cleared by cellular debris by centrifugation and transduction of MCF 10A cells was performed as suggested by the manufacturer.

Genomic PCR and genotyping

To obtain genomic DNA, cell pellets were resuspended in lysis solution (100 mM Tris-HCl [pH 8.0], 200 mM NaCl, 5 mM EDTA, 1% SDS, and 0.6 mg/ml proteinase K), and incubated at 55°C overnight. After ethanol and sodium acetate precipitation, DNA pellets were washed in 70% ethanol and resuspended in water. The DNA solution was incubated at 60 °C for 15 min and at least 1 hour at room temperature before proceeding. Genomic PCR was performed using Q5® High-Fidelity 2X Master Mix (New England Biolabs, M0492L) according to the manufacturer’s instruction. To genotype *TP53*-edited HCT116 clones, purified DNA (50ng) was analyzed by PCR to verify biallelic insertion of mAID-mCherry tag along with antibiotic resistance, in the right genetic locus, using the following primers: A (5’- GGAAAAGGGGCACAGACCCT-3’); B (5’- CATGGCCAGCCAACTTTTGCAT-3’); E (5’-GCACACCTATTGCAAGCAAG -3’): F (5’-TGCTCCTGCCGAGAAAGTAT-3’); C (5’-GGATGTTCCGAGAGCTGAAT-3’); D (5’-GAAGAACGTGATGGTTTC-3’) (**Supplementary Figure 5**). PCR products were loaded on 1 or 2% agarose TBE with ethidium bromide gel, along with 100bp or 1kb DNA ladders (Thermo Fischer Scientifc) to verify correct amplicons length and then purified using QIAquick PCR Purification Kit (Qiagen). Purified PCR DNA was submitted for sequencing to assess the frame and the integrity of the edited sequence, using the following primers: pSHALfwd (5’-ACTGAATACAGCCAGA-3’); pSHALrev (5’-ACTGAATACAGCCAGA-3’); A (5’-TTGGAACTCAAGGATGCCCAGG-3’); mAIDfwd (5’-GAAGAACGTGATGGTTTC-3); D (5’-GAAGAACGTGATGGTTTC-3’).

Treatments

To induce the degradation of mAID-fused proteins, cells were treated with 500 µM indole-3-acetic acid (auxin, IAA, dissolved 500 mM in water) (I5148, Millipore Sigma,) for ROLECCS experiments, or 1 µM 5-phenyl-indole-3-acetic acid (5-Ph-IAA) (Targetmol, T8885) for ROLECCS v2 experiments, for 1 hour, unless

otherwise stated. To suppress the partial degradation of TP53-mAID-mCherry in HCTT116 constitutively expressing pAAVS1-ROLECCS AS, pAAVS1-ROLECCS G1 and pAAVS1-ROLECCS G2 control cells were pretreated overnight with 200 µM auxinole (BioAcademia, Inc., Japan; #30–001, dissolved 200 mM in DMSO) and maintained in auxinole for the duration of the experiments where indicated.

To induce TP53 activation, cells were treated with 20 µM cis-Diamineplatinum(II) dichloride (cisplatin, stock diluted in 0.9% NaCl, Millipore Sigma, #479306) for the indicated time points.

Protein extractions, subcellular fractionations, and western blots

Total protein extractions were performed as follows: cells were collected and washed with PBS before adding adequate amount of lysis buffer (1% NP-40, 1mM EDTA pH 8.00, 50Mm Tris-HCl pH 7.5, 150 mM NaCl) containing a protease and phosphatase inhibitor cocktail (cOmplete™, EDTA-free Protease Inhibitor Cocktail; PhosSTOP™ inhibitor tablets, Millipore Sigma). Subcellular protein fractionations were performed using the NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, 78833), according to manufacturer's instructions. Protein concentration was checked by Bradford assay (Biorad). After denaturation at 100°C for 5 min, equal amounts of proteins (2.5-25 µg) were separated using SDS-PAGE, loading samples on TGX™ Precast Protein Gels (Bio-Rad). Proteins were transferred to 0.45 µm nitrocellulose membrane (Biorad) and blocked in 5% non-fat milk or BSA/TBST for 1 hour at room temperature. Membranes were probed with primary antibodies at 4°C overnight and subsequently incubated with a secondary antibody at room temperature for 1 hour. Antibodies used were anti-GFP (B-2) (Santa Cruz Biotechnology, sc-9996), anti-TP53 (DO-1) (Santa Cruz Biotechnology, sc-126), anti-GAPDH (14C10) Rabbit mAb (HRP Conjugate) (Cell Signaling Technology, #3683), anti-Cyclin B1 (D5C10) XP Rabbit mAb, (Cell Signaling Technology, #12231), anti-CDT1 (D10F11) Rabbit, (Cell Signaling Technology, #8064), anti-mCherry (Millipore Sigma, AB356482), anti-nucleophosmin (Cell Signaling Technology, #3542), anti-vinculin monoclonal Antibody (VLN01, Thermo Fisher # MA5-11690), anti-p21 Waf1/Cip1 (12D1) Rabbit mAb, (Cell Signaling Technology, #2947), anti-Bax (D2E11) Rabbit mAb, (Cell Signaling Technology, #5023).. Secondary antibodies were HRP-conjugated anti-mouse or rabbit IgG from from Millipore Sigma. Detection was performed using Immobilon Forte Western HRP substrate (Millipore Sigma) or the SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and X-ray blue films, in dark room. Blots in the same figure/panel were probed multiple times with the indicated antibodies and, when necessary, membranes were stripped using Restore™ PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific, 46428) and re-probed with the desired antibody. Images were acquired using standard protocols and Western Blot bands were quantified using ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA).

RNA isolation and quantitative real time PCR

Total RNA was isolated from cells using TRIzol Reagent (Thermo Fisher Scientific, 15596026) according to the manufacturer's instructions. The concentration and purity of RNA samples were determined using Nano-Drop spectrophotometer ND-1000 (Thermo Fischer Scientific). Total RNA (500 ng) was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, 4368813) according to manufacturer's instructions. Quantitative Real Time PCR experiments were performed using the TaqMan Fast Advance Master Mix (Thermo Fischer Scientific, 4444965) according to the manufacturer's instructions, using the following TaqMan gene Assays (Thermo Fischer Scientific): TP53 gene (assay ID: Hs01034249_m1, CDKN1A gene (assay ID: Hs00355782_m1), BAX gene (assay ID: Hs99999001_m1), GAPDH gene (assay ID: Hs02786624_g1). The experiments were performed twice on two independent clones. GAPDH mRNA was used as reference control.

Gating and sorting strategy

For FACS sorting, all samples were washed once in 1X PBS and resuspended in sorting buffer (1X PBS, 1mM EDTA, 24mM HEPES pH 7.0, 1% FETAL Bovine Serum (Heat-Inactivated), 0.2 µm filtered) before performing flow cytometric analysis. Instruments used were either FACSAria II (Becton Dickinson) or MA900 (Sony Biotechnology). For experiments requiring further cell cycle analysis, cells were collected using in a pre-cooled (4^{o} C) tube collector, and kept refrigerated for the entire time of the sorting and post-sorting, before further processing. After gating cells to exclude debris, dead cells, and doublets, cells were plotted for SSC-A and FITC-A (mEmerald expression). Three different gates, labelled GFP^{low}, GFP^{med}, and GFP^{high}, were designed to identify three distinct cell populations with different intensity of FITC (that is mEmerald expression). We also took into consideration the SSC as indicator of cells complexity to help recognize cells progressing into different phases of the cell cycle, especially to discriminate cells in G_2 phase. Therefore, in the instance of sorting ROLECCS G1 cells, the GFP^{high} gate include cells with high FITC intensity and low SSC, as these are cells expected to be in G_1 phase and so with relatively low cellular complexity and high expression of OsTIR1-mEmerald-Cdt1 protein. In the case of ROLECCS G2 cells, the GFP^{high} population is the one with high SSC and high FITC level as the OsTIR1-mEmerald-Geminin expression increases in S/G_2 as the cellular complexity does. GFP^{low} and GFP^{med} gates are here being indicated and analyzed as counter-proof that our reporters are not expressed in the not specific cell cycle phase, therefore, when cells are sorted for low or medium expression of mEmerald (FITC) they are not in the cell cycle phase under study. Sorting cells expressing ROLECCS G1 or ROLECCS G2 is intended as sorting for GFP^{high} cell populations, as the portion of the asynchronously growing cells, where the on demand degradation of the POI solely happens. Gating strategy was optimized for each cell line. Representative gating strategies are shown in **Supplementary Figures 2**, **6** and **8**. Cells were collected at 4 °C during sorting, when using MA900 Multi-Application Cell Sorter.

DFlow cytometry data were analyzed using FlowJo™ Software_v 10.6.1. analysis software (Ashland, OR: Becton, Dickinson and Company; 2019)

Cell cycle analysis using propidium iodide DNA staining

Cells were washed in 1X PBS (Millipore Sigma) before being fixed in 70% ethanol at -20 °C for at least 2 hours. After fixation, cells were pelleted and washed once in 1X PBS and then resuspended in staining solution (PBS containing 10 mg/mL propidium iodide (Thermo Fisher Scientific), 0.05% Triton X-100 (Millipore), 2.5 µg/mL RNAse A (Thermo Fisher Scientific)). After samples incubation at 37 °C for 30 min protected from light, flow cytometry analysis was performed using FACSCalibur Flow Cytometer (Becton Dickinson), modelling at least 5000 events per sample. Results were analyzed using ModFit software, v5.0 (Verity Software House).

Cell imaging

For Incucyte live cell imaging experiments presented in Figure 2D, HEK-293 ROLECCS *AAVS1*-integrated clones were plated in 96-well plates and time-lapse analyses were performed using IncuCyte® S3 Live-Cell Analysis System (Essen BioScience). Images were acquired every 15 minutes using bright-field and GFP channels. Data were exported and presented as unprocessed.

Images of fluorescent features of HEK-293 ROLECCS *AAVS*- integrated clones were obtained at Zeiss Axioskop 40 Microscope, using Zen Pro software (ZEISS). Cells were plated in cellview cell culture dish (glass bottom, Greiner Bio-One, 627870) and nuclear counterstaing was performed adding one drop of NucBlue™ Live ReadyProbes™ Reagent (Invitrogen, R37065) to the medium. DAPI (blue) and mEmerald (green) were acquired using EGFP and DAPI channels at 63x magnification.

Live-cell microscopy (Figure 3G-H) was conducted as previously described[66], with the following modifications. 96-well #1.5 imaging plates (#P96-1.5H-N CellVis, Mountain View, CA) were coated with 50 µg/ml rat tail collagen (#11179179001 Sigma-Aldrich, St Louis, MO) for 1 hour prior to cell seeding. MCF 10A ROLECCS cell lines were then seeded at a density of 5000 cells/well and allowed to adhere for 30-45 minutes prior to the addition of growth media containing auxinole and incubated overnight at 37°C. The imaging plate was then washed twice into imaging media (phenol free DMEM/F12; #21041025 ThermoFisher, Waltham, MA) containing 0.5 mg/ml hydrocortisone, 250 ng/ml insulin, 10 µg/ml transferrin (#H0888, I2643, T8158 Sigma-Aldrich, respectively), 20 ng/ml epidermal growth factor (#AF-100-15 Peprotech, Cranbury, NJ), and vehicle, auxinole, or auxin, based on conditions. Cells were immediately imaged using a Nikon Ti2E inverted microscope fitted with a Bold Line stage top environmental chamber (Okolab, Pozzuoli, Italy) and SOLA II solid-state light source (Lumencor, Beaverton, OR). A single stage position was chosen within each well of the plate and time lapse images were captured every 20 minutes, for 24-48hours, using a 20X 0.75 NA objective and Prime 95B camera (Teledyne Photometrics, Tucson, AZ). Automated imaging was performed using NIS-Elements AR software.

Imaging data analysis was performed as previously described using custom MATLAB software[66,67].

Micronuclei immunofluorescence

Micronuclei formation was evaluated by immunofluorescence as follows. Asynchronously growing HCT116 TP53-mAID-mCherry ROLECCS AS/G1/G2 cells were seeded on sterile glass coverslips (Gold Seal Cover Glass, thickness 1.5', Thermo Fisher) pre-coated with poly-L-lysine (Millipore Sigma #P4707) in 6 well plate in medium containing 200 µM auxinole, the day before. Cells were then treated with 500 µM auxin or left untreated for 24 hours. When cells were sorted for GFP^{high} only, asynchronously growing HCT116 TP53-mAID-mCherry ROLECCS AS/G1/G2 cells were cultivated the day before in the presence of 200 µM auxinole. Cells were then treated with 500 µM auxin or left untreated for 24 hours and processed for sorting as previously described. GFP^{high} population was collected directly on sterile glass coverslips, pre-coated with poly-L-lysine in 6 well plate containing medium supplemented with 200 µM auxinole or 500 µM auxin for 24 hours.

After medium removal, cells were fixed with cold 4% PFA/PBS (Paraformaldehyde Solution, 4% in PBS, Thermo Scientific™, J19943.K2) for 15 minutes at room temperature (RT). Fixative solution was aspirated, and cells were incubated with 0.3 M Glycine solution (Glycine USP, Gojiara Fine Chemicals, GC1004 dissolved in dH_2O and filtered) for 5 minutes at RT. Cells were rinsed once with 1X PBS for 5 min at RT. Coverslips were incubated in full medium, wrapped in parafilm and foil and stored at 4 °C overnight. Immunofluorescence staining of micronuclei was performed as follow. After cells fixation, coverslips were washed twice with 1X PBS, cells were then permeabilized with 0.2% Triton X-100 in PBS for 10 minutes at RT. Cells were washed once with 1X PBS and blocked in 5% BSA/PBS (filtered) for 1 hour at RT. Next, coverslips were incubated with rabbit anti-Lamin B1 primary antibody (ab16048, Abcam), diluted 1:1000 in 1% BSA filtered solution, for 1 hour at RT in a covered humidified chamber. Cells were then washed with 1X PBS three times for 5 minutes each, and incubated with anti-rabbit Alexa Fluor 488 (diluted 1:400, Thermo Fisher #A-11008) and Phalloidin-iFluor 647 (diluted 1:1000, ab176759, Abcam) in 1% BSA filtered solution for 1 hour at RT in a covered humidified chamber. Next, coverslips were washed with 1X PBS three times for 5 minutes each and mounted on Superfrost Plus Microscope Slides (Fisherbrand, 12-550-15), using VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories, H-1200-10). Slides were left under chemical hood for at least 15 minutes to let mounting medium to harden and stored in the dark at 4 °C at least overnight, before evaluation.

Micronucleated cells were counted manually from DAPI stained cells. Micronuclei were identified as separate (non-overlapping) extra-nuclear structures with rounded shape, that were positive for DAPI (blue) and encased by nuclear envelope positive to laminin B1 staining (green). For quantification purposes, at least 100 cells were counted for each condition and experiment were repeated at least 3 times.

Images were captured at 63x magnification using a Zeiss Axioskop 40 Microscope (Zen Pro Software, Zeiss) or EVOS™ M5000 Imaging System (EVOS™ M5000 Software).

Synchronization experiments

HEK-293 LentiROLECCS G1 or G2 were synchronized in G2/M as follow. The day before 150000 cells were plated in 6 well plates, cells were then treated with 10 μM RO-3306 (In Solution Cdk1 Inhibitor IV, dissolved 4 mg/ml in DMSO, Calbiochem, Millipore Sigma, 217721). After 23 hours from treatment, 500 μM auxin was added for 1 hour. Cells were then collected for cell cycle analysis and western blot, as described above.

Statistical analysis and data availability

All the experiments are representative of at least two independent experiments (technical and/or biological replicates). The number of replicates for each experiment is specified in the relative figure legend. For statistical analysis, two-tailed t-test was performed and data were considered statistically significant for $p<0.05$.

Original unprocessed data used for the preparation of the manuscript are available upon kind request. All data supporting the findings of this study are available within the paper and its Supplementary Information files (Source Data File).

Declarations

Supplementary Data

A detailed description of supplementary figures is reported in the Supplementary Information file.

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Author Contributions

M.C., D.P. and C.M.C. conceived and designed the project. M.C., D.P., A.T., W.O.M. conceived and planned the experiments. M.C., D.P., A.T., J.M., G.L.R.V., C.L., W.O.M. executed the experiments. A.E.D. and D.L. performed and analyzed live microscopy experiments. M.C., D.P., A.T., G.L.R.V., C.L., W.O.M. analyzed the data. B.M. provided technical support for flow cytometry experiments and cell sorting. M.C. and D.P., wrote the manuscript. A.T., J.M., G.L.R.V., C.L., W.O.M., V.C. and C.M.C. provided critical feedback and contributed to the final version of the manuscript.

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Figures

Figure 1

Schematic representation of the design for Regulated OsTIR1 Levels of Expression based on the Cell Cycle Status (ROLECCS) variants. (**A**) OsTIR1-mEmerald protein (asynchronous ROLECCS, ROLECCS AS, 92 KDa) is stably expressed throughout the cell cycle. Upon auxin treatment, OsTIR1 enzymatic activity elicits the degradation of the mAID-tagged protein of interest (POI) in any cell, independently of the cell cycle status. (**B**) The expression of the ROLECCS G1 variant (OsTIR1-mEmerald-Cdt1, 103 KDa) is restricted to the G_1/early S phase by the presence of the Cdt1 tag, when the SCF^{Skp2} E3 ligase activity is off. This, in turn, leads to auxin-dependent ubiquitylation and proteasome degradation of mAID-tagged POIs. In cells transitioning during S, G_2 and M phases, SCF^{Skp2} activity is naturally restored, leading to ROLECCS G1 degradation by ubiquitylation, and stabilization of the POI even in the presence of auxin. (**C**) The Geminin tag of the ROLECCS G2 variant (OsTIR1-mEmerald-GEM, 105 KDa) ensures its restricted expression during the late S-G_2-M phase, as APC^{Cdh1}-mediated ubiquitylation and degradation is rapidly triggered during M/G_1 transition. Therefore, auxin treatment induces degradation of the POI exclusively in cells going through the late S-G_2-M phase of the cell cycle.

Figure 2

Characterization of ROLECCS AS, G1, and G2 cellular distribution and during cell-cycle progression. (**A**-**B**) Representative WB analysis of nuclear/cytoplasmic distribution of ROLECCS proteins upon transient (72hrs) transfection (**A**) or *AAVS1* integration (**B**) in HEK-293 cells. ROLECCS AS, ROLECCS G1, ROLECCS G2 (see main text) were detected using anti-GPF antibody that recognizes the mEmerald tag of the proteins (see arrows). Nucleophosmin (NPM) and GAPDH antibodies were used as loading and purity control for nuclear and cytoplasmic soluble protein fractions, respectively. Not transfected (NT) or wild-type (WT) HEK-293 were used as negative control. WCL indicates Whole Cell Lysate. (**C**) Direct fluorescence images of HEK-293 *AAVS1*-integrated clones. DAPI staining (blue) was used to label nuclei, EFGP (green) signal was detected from ROLECCS variants (AS, G1, G2). (**D**) Time-frame pictures of duplicating HEK-293 *AAVS1*-integrated clones. Note the cell cycle-dependent changes in fluorescence of specific ROLECCS variants (AS, G1, G2) (green). Arrows indicate cells that are completing a cell cycle. (**E**, **G**) Cell-cycle distribution histograms of HEK-293 *AAVS1*-integrated clones expressing ROLECCS G1 and G2, obtained by propidium iodide staining and flow cytometry analysis. Red peaks indicate G1 and G2 phase, stripes indicate S phase. Cells were prior sorted based on GFP levels (GFP^{low}, GFP^{med}, GFP^{high}), as described in Supplementary Figure 2. Not sorted (unsorted) populations are reported for comparison. Data are representative of four independent experiments. (**F**, **H**) Quantification of experiments reported in E and G. GFP^{low}, GFP^{med}, GFP^{high} subpopulations were analyzed for cells composition as percentage of cells in G_1+earlyS and cells in mid-lateS/G_2/M, using ModFit software v5.0. Error bars indicate mean ± SD. *** $p < 0.001$, N.S. not significant. Statistics (two-tailed t-test) is calculated *versus* respective unsorted populations. Data are the average of four independent experiments.

Figure 3

Biological activity of ROLECCS proteins. (**A-C**) Schematic representation of lentiviral vectors (pLentiROLECCS AS, G1, and G2) and their corresponding translated proteins with respective molecular weight. (**D**) WB analysis of transient (24 hours) and stable transfection (bulk population) of pLentiROLECCS vectors in HEK-293 cells. Anti-GPF antibody was used to detect ROLECCS proteins (see arrows), anti-mCherry antibody was used to detect mAID-mCherry. Not transfected HEK-293 (NT) and GAPDH were used as negative and loading control respectively. (**E**) Densitometric quantification of mAID-mCherry normalized on GAPDH intensity of WB analyses of HEK-293 cells transfected with pLentiROLECCS G1, presented in Supplementary Figure 3 B (clone 1) and C (clone 2). Relative quantification *versus* GFP^{low} sorted population is reported. (**F**)Densitometric quantification of mAID-mCherry normalized on GAPDH intensity of WB analyses of HEK-293 cells transfected with pLentiROLECCS G2, presented in Supplementary Figure 3 D (clone 1) and E (clone 2). Relative quantification *versus* GFP^{low} sorted population is reported. Data are representative of four independent experiments. (**G-H**) Live-cell confocal microscopy imaging on MCF 10a normal breast epithelial cells, transduced with LentiROLECCS G1 (**G**) or LentiROLECCS G2 (**H**). Upon 5-Ph-IAA cells treatment, red fluorescent signal (mAID-mCherry) faded away before the green-fluorescent signal (ROLECCS G1, panel **G** and ROLECCS G2, panel **H**) could be detected. Hoechst staining (greyscale) of DNA content was performed to follow cell cycle division, confirming ROLECCS G1 expression increase after completion of cell division (**G**) and ROLECCS G2 detection during the progression through S and G2 phase (**H**). (**G**) Interval between still images is 100 minutes. Single cell traces (mAID-mCherry as red trace, ROLECCS G1 as green trace) of three different cells are representative and do not correspond with the images above. (**H**) Interval between still images is 120 minutes. Single cell traces (mAID-mCherry as red trace, ROLECCS G2 as green trace) are representative and do not correspond with the images above.

Figure 4

ROLECCS system downregulates endogenous proteins in a cell cycle-specific fashion. (**A**) Diagram of *TP53* gene editing strategy in HCT116 via CRISPR/Cas9-mediated knock-in. The stop codon was replaced by mAID-mCherry fusion cassette, cloned between 1-kb long Homology Arms. To achieve targeting of both *TP53* alleles, two donor plasmids (TP53-3'END Donor 1 and Donor 2) were used, bearing Neomycin (NeoR) or Hygromycin (HygroR) resistance genes, respectively. The antibiotic resistance genes are under the transcriptional control of independent promoters (SV40 and PGK, respectively). (**B**) WB analysis of nuclear/cytoplasmic distribution of TP53 protein (TP53-mAID-mCherry, 87 kDa) in HCT116 TP53-mAID-

mCherry clone 1 (cl 1) and clone 2 (cl 2). HCT116 wild type (WT) were loaded as control for TP53 activation upon cisplatin (20 μM) treatment for 48 hours. NPM and GAPDH were used as purity and loading controls for nuclear and cytoplasmic soluble protein fractions, respectively. WCL indicates Whole Cell Lysate. Images are representative of two independent experiments. (**C**) Messenger RNA fold change of TP53, p21 and BAX genes in HCT116 TP53-mAID-mCherry cells treated with cisplatin (20 μM for 24 hours) quantified by Real Time PCR. GAPDH gene was used as housekeeping control and data were normalized on not treated samples. Error bars indicate mean ± SD. *** $p < 0.001$, ** $p < 0.01$, N.S. not significant. Statistics (two-tailed t-test) is calculated *versus* not treated. Experiment was repeated twice on two independent clones. (**D**) WB analysis of cisplatin-induced TP53-mAID-mCherry (87 kDa), p21 (21 kDa) and BAX (21 kDa) proteins increase in HCT116 TP53-mAID-mCherry. Cells were treated with 20 μM cisplatin and collected for protein extraction at 48 hours. Lysates were loaded in duplicate to probe membranes with antibodies against proteins with same molecular weight, GAPDH was used as loading control. Two independent HCT116 TP53-mAID-mCherry clones (cl 1, cl 2) were analyzed. Images are representative of two independent experiments. (**E**) WB analysis of characterization of HCT116 TP53-mAID-mCherry with *AAVS1*-integrated ROLECCS variants (AS/G1/G2). ROLECCS AS, ROLECCS G1, and ROLECCS G2 were detected using anti-GPF antibody (see arrows), TP53 wild type (WT) and TP53-mAID-mCherry (TP53-mAID-mCh) were detected using anti-TP53 antibody. GAPDH was used as loading control. HCT116 wild type (WT) were loaded for comparison. Two independent HCT116 TP53-mAID-mCherry ROLECCS clones (cl 1, cl 2) were analyzed. Images are representative of two independent experiments. (**F**) WB analysis of HCT116 TP53-mAID-mCherry *AAVS1*-edited with ROLECCS G1, clone 1 (cl 1) after sorting. Cells were treated with auxin or left untreated for one hour and then sorted for GFP intensity (ascending grey gradient triangle). Membrane was probed with anti-GFP antibody for ROLECCS G1 detection and mCherry antibody for TP53-mAID-mCherry (TP53-mAID-mCh) detection. Cdt1 and CyclinB1 were used as G_1 phase and G_2 phase specific markers, respectively. GAPDH was used as loading control. Not sorted (unsorted) cells were loaded for comparison. (**G**) WB analysis of HCT116 TP53-mAID-mCherry *AAVS1*-edited with ROLECCS G2, clone 1 (cl 1) after sorting. Treatments, sortings, and antibodies are the same as shown in panel F. Blots are representative of two independent experiments.

Figure 5

Cell cycle phase-specific expression and functionality of ROLECCSv2 proteins.

(**A**, **B**) Quantification of cell-cycle distribution experiments of HCT116 TP53-mAID-mCherry *AAVS1*-integrated clones expressing ROLECCS v2 G1 (**A**) and G2 (**B**). Cells were prior sorted based on GFP levels (GFP^{low}, GFP^{med}, GFP^{high}), as described in Supplementary Figure 8, and then stained with propidium iodide as described in Methods. GFP^{low}, GFP^{med}, GFP^{high} subpopulations were analyzed for cells composition as percentage of cells in G_1+earlyS and cells in mid-lateS/G_2/M, using ModFit software

v5.0. Not sorted (unsorted) populations are reported for comparison. Data are the average of four independent experiments. Error bars indicate mean ± SD. **** $p < 0.0001$, N.S. not significant. Statistics (two-tailed t-test) is calculated *versus* respective unsorted populations. (**E**) Densitometric quantification of TP53-mAID-mCherry normalized on GAPDH intensity of WB analyses. Relative quantification *versus* GFP^{low} sorted population is reported. (**F**) Densitometric quantification performed as in (E). Densitometric analyses are the average of at least one experiment on 2 different clones (n=3). Error bars indicate mean ± SD. ** $p < 0.01$, * $p < 0.05$, N.S. not significant. Statistics (two-tailed t-test) is calculated *versus* respective GFP^{low} sorted population.

Figure 6

Micronuclei accumulation upon cell cycle phase-specific TP53 abrogation. (**A**) HCT116 TP53-mAID-mCherry ROLECCS AS, G1, and G2 were treated with auxin or left untreated for 1 hour and then GFP^{high} population was sorted and plated on glass coverslips in the presence of auxin or medium for 24 hours before fixation and IF staining for Lamin B. Dot plot graph represents percentage of micronucleated HCT116 TP53-mAID-mCherry ROLECCS cells with micronuclei per field. (**B**) Asynchronously growing HCT116 TP53-mAID-mCherry ROLECCS AS, G1, and G2 were seeded on glass coverslisps for 24h, then treated with auxin or left untreated for 24 hours before fixation and IF staining. Dot plot graph representing percentage of cells with micronuclei per field. Error bars in A and B indicate mean ± SD. **** $p < 0.0001$, *** $p < 0.001$, N.S. not significant. Statistics (two-tailed t-test) is calculated *versus* respective not treated. Data represent 4 independent experiments. (**C**) Representative images of micronuclei immunofluorescence staining in HCT116 TP53-mAID-mCherry ROLECCS cells. Micronuclei (MN, white arrow) are identified as separate extra-nuclear structures with rounded shape, positive for DAPI (blue) and encased by nuclear envelope positive to laminin B1 staining (green). Phalloidin-iFluor 647 staining (red) was used to stain actin, to facilitate single cell identification. (**D**) Schematic description of the ROLECCS system for cell cycle-specific targeted proteolysis. The ROLECCS system performs a Boolean logic computation. The contemporary presence of auxin and appropriate phase of the cell cycle are both simultaneously required to lead to targeted protein degradation. ROLECCS G1 and G2 are stable only through specific phases of the cell cycle (G_1/early S for ROLECCS G1, late S/G_2/M for ROLECCS G2), therefore their biological activity is restricted to those phases. However, auxin is required to trigger OsTIR1-mediated protein ubiquitylation, allowing proteasomal degradation of the POI only “on demand”, and only in the appropriate phase of the cell cycle.

Supplementary Files

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