Acute and rapid degradation of endogenous proteins by Trim-Away

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EDITORIAL SUMMARY This Protocol describes 'Trim-away', an approach for rapid protein depletion in different cell-types. TRIM21-mediated proteasomal degradation is induced by microinjection or electroporation of an antibody against the protein of interest.

TWEET A new Protocol describing the detailed procedures for 'Trim-away', an approach for rapid protein depletion in oocytes, primary cells, and cultured cells @SchuhLab @mpi_bpc @MRC_LMB @CellBiol_MRCLMB

COVER TEASER Rapid protein depletion in cells using 'Trim-away'

Please indicate up to four primary research articles where the protocol has been used and/or developed.

1. Clift, D. et al. A method for the acute and rapid degradation of endogenous proteins. *Cell*. 171, 1692-1706 (2017).

Abstract

Protein depletion is a key approach to understanding functions of a protein in a biological system. We recently developed the Trim-Away approach in order to rapidly degrade endogenous proteins without prior modification. Trim-Away is based on the ubiquitin ligase and Fc receptor TRIM21, which recognizes antibody-bound proteins and targets them for degradation by the proteasome. In a typical Trim-Away experiment, protein degradation is achieved in three steps: first, introduction of an antibody against the target protein; second, recruitment of endogenous or exogenous / overexpressed TRIM21 to the antibody-bound target protein; and third, proteasome-mediated degradation of the target protein, antibody and TRIM21 complex. Protein degradation by Trim-Away is acute and rapid, with half-lives of around 10-20 minutes. The major advantages of Trim-Away over other protein degradation methods are that it can be applied to any endogenous protein without prior modification; that it uses conventional antibodies which are widely available; and that it can be applied to a wide range of cell types including non-dividing primary human cells, where other loss-of-function assays are challenging. In this Protocol, we describe the detailed procedures for antibody preparation and delivery in mouse oocytes and cultured cells via microinjection and electroporation. In addition, we provide recommendations for antibody selection and validation and for generating TRIM21 over-expressing cell lines for cases where endogenous TRIM21 is limited. A typical Trim-Away experiment takes just a few hours.

Introduction

Protein depletion is one of the key tools for studying protein functions in cells and tissues and can be achieved by either interfering with protein synthesis or by inducing protein degradation. Protein synthesis can be blocked at various levels. At the genomic level, protein-coding genes can be disrupted using genome-editing technologies such as zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas system. These technologies are all based on nucleases that can be targeted to a specific DNA sequence and introduce a frame-shift mutation, a premature stop codon, or delete the coding sequence[1]. At the post-transcriptional level, messenger RNAs (mRNAs) can be targeted for degradation using RNA interference (RNAi). To this end, sequence-specific small interfering RNAs (siRNA) or short hairpin RNAs (shRNA) are introduced into cells. These become incorporated into RNA-induced silencing complexes (RISC), which bind to and degrade the target mRNA[2]. At the translational level, morpholino oligomers can be used to block the translation of mRNAs. These antisense oligonucleotides bind to the target mRNA and block the progression of the translation initiation complex from the 5' cap to the start codon[3].

While these techniques have been proven to be highly useful for studying various genes in different model systems, a common major limitation is that they are not suitable to deplete already synthesized proteins. In both metabolically inactive and long-lived metabolically active cells, certain proteins – particularly those in essential cellular structures – can persist for years after translation[4]. These long-lived proteins cannot be depleted by blocking protein synthesis at the gene or mRNA level. Another limitation of methods that act at the gene or mRNA level is the long delay between the time of their application and actual protein depletion, which is typically in the range of days. These methods are therefore not well suitable to investigate short-lived biological processes. For example, many regulatory proteins have multiple functions during different stages of mitosis, which takes only about 1 hour in HeLa cells[5]. If protein degradation is delayed, it is difficult to determine if the phenotypes are a direct consequence of protein depletion or a secondary consequence of earlier

defects[6]. Delays may also allow cells to activate compensatory mechanisms, which may modify or even mask the phenotypes[7].

To overcome these challenges, several methods have been developed that act directly on the protein level, targeting the protein itself for degradation. Some of these methods are based on controlling protein stability, for instance by fusing the target protein to destabilizing domains that are controlled by ligands[8,9]. Others are based on recruiting SKP1-CUL1-F-box (SCF) E3 ubiquitin ligases to the target protein, such as binding of an auxin-inducible degron to the F-box protein TIR1, and binding of GFP-tagged proteins to a GFP nanobody fused to the F-box domain of Slmb[10, 11]. Proteins can also be perturbed acutely by knock-sideways approaches, in which proteins are targeted away from their principle site of action, for instance by tethering them to mitochondria[12]. However, all of these assays require the endogenous protein to be first replaced by a modified variant. They are hence not suitable for studying protein functions in all cell types. For instance, application of these methods in non-dividing primary cells would often require the generation of transgenic animals. This is time-consuming and not feasible for many species. For tag-free degradation, ligand- and peptide-based techniques have been developed, but the number of proteins that can be targeted with these methods is very limited[13,14].

To achieve acute depletion of any endogenous protein without prior modification, we recently developed a post-translational protein depletion method, which we called 'Trim-Away'[15]. Trim-Away relies on an E3 ubiquitin ligase called TRIM21[16]. TRIM21 is involved in the intracellular immune response: it binds to antibody-bound pathogens and proteopathic agents and targets them for degradation[17-19]. The precise mode of action of TRIM21 is not yet fully understood, but it involves binding of TRIM21 to the Fc-region of an antibody and subsequent autoubiquitination of TRIM21. In a Trim-Away experiment, the high affinity of TRIM21 to the Fc-region of an antibody is exploited to target endogenous proteins for degradation. An antibody against the target protein is introduced, TRIM21 binds to the antibody-bound target protein and triggers the proteasome-mediated degradation of the antibody-antigen complex together with TRIM21[20,21]. Trim-Away has enabled us to degrade a wide variety of proteins within minutes of application in different cell types[15]. For instance, we could acutely deplete Rec8 in unmodified mouse oocytes[15], an experiment that required complex genetics in the past[22]. We have also been able to deplete the intracellular signalling molecule NLRP3 in human primary macrophages, which has not been possible with nucleic acid-based depletion techniques in the past[15]. Here, we describe (1) the design of Trim-Away experiments; (2) the selection and preparation of reagents for Trim-Away experiments; (3) the procedures for Trim-Away in mouse oocytes and pre-implantation embryos; (4) the procedures for Trim-Away in primary cells and cell lines; and propose (5) quality controls for Trim-Away experiments. We also highlight and give advice on critical steps in the procedure.

Overview of the Procedure

For a successful Trim-Away experiment, a specific antibody that targets an intracellular protein of interest must be delivered into cells that contain TRIM21. If the endogenous levels of TRIM21 are not sufficient for protein degradation, TRIM21 must also be introduced together with or before antibody delivery. In this Protocol, we outline different methods for introducing antibody and TRIM21 into cells and give advice on how to preselect specific antibodies for a successful Trim-Away experiment. A routine Trim-Away experiment involves the following three events (**Fig. 1**):

1. *Ensuring sufficient TRIM21 levels in target cells of interest*: TRIM21 is widely expressed in different cell types[23]. Depending on the expression level of the target protein, endogenous

TRIM21 levels may be sufficient for Trim-Away[15]. But as TRIM21 is continuously degraded during a Trim-Away experiment and typically not expressed at high levels, the endogenous level may be insufficient for complete target protein degradation. Additional TRIM21 can be introduced in the form of a transgene (i.e. DNA), mRNA or protein. Using TRIM21-encoding DNA, we have transiently transfected cell lines or created stable lines with a TRIM21 expression cassette integrated into their genome[15] (see also Box 3). Stable lines simplify the workflow in a Trim-Away experiment because only the delivery of the antibody is required for acute protein degradation. In certain cases, cells with low expression levels of TRIM21 have to be pre-selected by fluorescence activated cell sorting (FACS), because high expression of TRIM21 can sometimes lead to the formation of protein aggregates, which are non-functional[15]. We have also used *in vitro* transcribed mRNAs to transiently express TRIM21 in mouse oocytes[15]. Using mRNAs accelerates the expression of TRIM21 and allows fine-tuning of the expression level, but translation rates may vary for cells at different cell cycle stages. TRIM21 can also be introduced in the form of a recombinant protein, for instance by electroporation[15]. Recombinant TRIM21 (see also Box 4) eliminates the incubation time that is required for protein expression from DNA or mRNA before a Trim-Away experiment. However, recombinant TRIM21 can be exhausted if not replenished.

2. *Delivery of antibody*: Depending on the experimental setup, the antibody can be delivered by either microinjection (step 16) or electroporation (step 17 – 32). We have microinjected antibody into mouse oocytes and single adherent cells[15]. Microinjection allows tight control over the amount of antibody delivered into each cell, and is the method of choice when individual cells need to be analysed rapidly upon triggering protein degradation. For bulk cell populations, we recommend delivering the antibody by electroporation using the Neon Transfection System[15]. Unlike protein transfection reagents, electroporation can be used to simultaneously deliver the antibody into the cytoplasm of many cells without antibody accumulation in the endosome and/or lysosome. In addition to conventional antibodies, we have also used a fusion between a nanobody and the Fc-region of human IgG1 for protein degradation by Trim-Away[15]. We have introduced this nanobody-Fc fusion into mouse oocytes as mRNA, but delivery as DNA or protein into other cell types should work as well[15].
3. *Target protein degradation:* When the antibody has bound to the target protein, TRIM21 targets the antibody-antigen complex for degradation via the ubiquitin-proteasome pathway[20,21]. Degradation typically starts within 5 – 10 minutes after application of the antibody[15]. Complete depletion can be achieved within ~3 hours, depending on the abundance and accessibility of the target protein, as well as the amount of antibody that was introduced into the cell[15].

Applications of the method

Trim-Away utilizes TRIM21 to degrade endogenous proteins that are bound to an antibody. Our results suggest that any protein within a cell that is accessible by antibody can be degraded by Trim-Away. As TRIM21 proteins and antibodies are highly conserved amongst different mammalian species, Trim-Away works with a wide range of cells and antibodies[24]. Trim-Away acts very rapidly and does not require prior modification of the target protein. It also works in various cell types, including non-dividing primary cells. Trim-Away also allows loss-of-function studies in primary immune cells, which could not be studied efficiently with nucleic acid-based depletion methods in the past.

Some specific examples of what can be studied are (1) the function of a protein within a defined cell cycle stage without affecting its potential other functions during the other cell cycle stages; (2) the roles of proteins for the maintenance of cellular structures, even if these proteins are essential for the formation of these structures; (3) the functions of the cytoplasmic pool of certain proteins; (4)

the functions of certain proteins with a particular post-translational modification; (5) the functions of specific isoforms of certain proteins; and (6) structure-function relationships in certain proteins by depletion followed by rescue with different mutants.

Advantages and limitations of the method

In comparison to other currently available protein depletion techniques, Trim-Away has several unique advantages. Firstly, Trim-Away acts directly at the protein level. It is therefore effective against long-lived proteins, and the development of phenotypes does not rely on inherent turnover of the target protein. Secondly, Trim-Away does not require modification of the genome. It therefore greatly facilitates loss-of-function studies in models where DNA- and RNA-based depletion methods are often ineffective, including non-dividing primary cells. Thirdly, Trim-Away is a protein-based depletion method, and can be used in primary immune cells which have active nucleic acid-sensing machineries. Fourth, TRIM21 is widely-expressed in most tissues. This means that it may often be possible to take advantage of endogenously produced protein and not rely on ectopic overexpression or introduction of recombinant protein. Fifth, TRIM21 does not have a cellular housekeeping function, in contrast to E3s like von Hippel–Landau (VHL) that are typically used in PROTAC applications[31]. This means there is no competition for endogenous substrates and less likelihood that normal metabolism will be impacted. Lastly, Trim-Away utilizes off-the-shelf reagents for protein-targeting. Antibodies have been produced for almost all proteins and are widely commercially available.

As with any technique, there are limitations for Trim-Away. One limitation is that Trim-Away depends on the intrinsic proteasome activity. Hence, the efficiency of Trim-Away may be compromised in certain cell types and/or at certain cell cycle stages accordingly. As Trim-Away is a protein-targeting method, another limitation is that it is more challenging to evaluate off-targets when compared to gene- or mRNA-targeting methods. Thus, antibodies used for protein-targeting have to be carefully selected and validated before use, as discussed in the Experimental Design section (Antibody selection). In addition, antigen buried within the native protein or target stably localized in a particular cellular compartment such as the nucleus will not be accessible for degradation by the antibody. But the latter case may be circumvented by the use of the smaller nanobody-Fc fusion[15]. Finally, the usefulness of Trim-Away may be limited in certain models such as intact tissues, where it is challenging to deliver antibody by microinjection or electroporation. However, as there are intensive efforts to develop cell delivery reagents, it may become possible in the future to utilize Trim-Away even in intact tissue applications. An important potential limitation in Trim-Away is that TRIM21 both targets proteins for degradation and can activate innate immune signal transduction pathways like NF-κB[32]. This is because TRIM21 synthesises K63-ubiquitin chains, which when released by the proteasomal DUB RPN11 acts as an immune second messenger[21]. Fortunately, there is a high activation barrier for TRIM21-signalling, and NF-κB activation is highly sensitive to changes in antibody affinity, concentration and viral dose[33,34]. For instance, in our Trim-Away experiment targeting IκBα for degradation, NF-κB was only weakly activated in control cells where mTOR was depleted[15]. If activation of NF-κB is a concern during Trim-Away, this can be assayed for either through the use of an NF-κB-luciferase reporter or by qPCR for cytokine transcripts.

Experimental design

Antibody selection Similar to other antibody-based techniques, the specificity of the antibody is crucial for a successful Trim-Away experiment. A good antibody should degrade all antigens bearing the targeted epitope (i.e. on-target), but not irrelevant antigens (i.e. off-target). When selecting an antibody for Trim-Away, several parameters should be considered:

- *Antibody species*: The TRIM21-antibody interaction is highly conserved and maintained both within and between murine, canine, primate and human species[24]. Our preliminary results with anti-GFP antibodies raised in mouse, rabbit and goat suggest no significance difference in their Trim-Away efficiency at the same concentration. We have also successfully used antibodies raised in rat[15] . However, antibodies from less common hosts such as guinea pig and sheep should be tested before actual application. TRIM21 does not bind to chicken antibodies.
- *Isotype of antibody*: TRIM21 has the highest affinity to IgG, followed by IgM and IgA[25]. Binding of TRIM21 to IgD or IgE remains unknown. TRIM21 does not bind IgY.
- *Form of antibody*: Commercial antibodies are offered in different forms such as full length Ig, Fab and Fab2. As TRIM21 binds to the Fc-region of antibodies, Fab and Fab2 should be avoided. Considering the growing supply of nanobodies, we have also successfully used a fusion between a nanobody and the Fc-region of human IgG1 for Trim-Away experiments[15].
- *Clonality of antibody*: We have had success with both polyclonal and monoclonal antibodies. Because of lot variation between polyclonal antibodies, we recommend the use of monoclonal antibodies as they may yield better reproducible results.
- *Antigen of antibody*: The epitope should be accessible for the antibody under native conditions.
- *Antibodies against post-translationally modified proteins:* Our preliminary results suggest that Trim-Away can also be used to selectively degrade proteins with post-translational modifications, as long as specific antibodies are available. However, these antibodies need to be evaluated particularly carefully before usage. For instance, we observed non-specific binding of some polyclonal antibodies against phosphorylated kinetochore proteins to acentricolar microtubule organizing centres, which are also enriched in phosphorylated proteins in mouse oocytes (and the other way round).

Antibody validation Before proceeding to Trim-Away, we generally validate the antibody by at least two of the experiments listed below:

- *Co-localisation with the fluorescently tagged protein*: Most proteins localize correctly when they are tagged with a fluorescent protein. To evaluate if an antibody is specific, we compare the localization of the fluorescent protein with the pattern obtained by immunofluorescence using the antibody of interest. They should be identical. Exceptions to this are some long-lived proteins such as Rec8 and CenpA in mouse oocytes[22,26]. These proteins are only loaded correctly during particular stages of development, which do not allow further incorporation or displacement by their fluorescent reporter afterward. Also, comparison of the staining pattern with published data from other cell types is a useful way to test for specificity.
- *Antibody delivery followed by immunofluorescence*: To determine the ability of antibody to recognize the target protein in live cells, we deliver the antibody by either microinjection or electroporation, fix and stain with a secondary antibody. The delivered antibody should show the same localization pattern as immunofluorescence of the target protein using a different antibody. It is also possible to fluorescently label the antibody in order to observe correct binding to target protein in live cells[27]. However, in some cases antibody delivery may cause target protein degradation via endogenous TRIM21 and/or cause target protein mislocalisation due to inhibitory effects of antibody binding. We therefore recommend traditional immunofluorescence as the preferred method to validate antibodies.
- *Immunoprecipitation followed by Western blotting*: To determine the ability of antibody to recognize the target protein in native conformation in cells, we perform immunoprecipitation

on whole cell lysate in non-denaturing conditions. Specific antibody should enrich a single band corresponding to the target protein over background band(s) (if any) in Western blot. We do not recommend assessing the antibody by performing Western blotting alone, as that only provides information on the specific binding of antibody to the denatured epitope.

- *Cross-validation with other approaches*: To test for specific binding of an antibody, when possible we first deplete the target protein with other approaches such as genome-editing or RNAi. This is followed by immunofluorescence using the antibody of interest. Specific binding of the antibody is confirmed by a reduction in the signal relative to the control. Such validation may also be provided by some antibody suppliers.
- *Peptide pre-incubation followed by immunofluorescence*: To test for non-specific binding of an antibody, we pre-incubate the antibody with the immunizing peptide before immunofluorescence (**Box 1**). The degree of background binding can be estimated by the level of signal retained after blocking specific binding of the antibody. We highly recommend this assay for post translational modification-specific antibodies.

Source of TRIM21 TRIM21 is widely-expressed in most tissues[23]. This means that most cell types will contain endogenous TRIM21 protein, and this may be sufficient for Trim-Away to work simply by delivering antibody to unmodified cells. Indeed, we have found that RPE-1 cells and primary macrophages contain sufficient endogenous TRIM21 for Trim-Away of several different target proteins[15]. Cell types can be screened for TRIM21 expression levels by RT-PCR or by western blotting using anti-TRIM21 antibodies (see Reagents section). Relative expression levels of TRIM21 can be compared to low expressing cells (HEK293T) or high expressing cells (RPE-1). As an initial Trim-Away experiment, we suggest delivering antibody to the cells of interest and screening for target protein depletion. Depending on the cell type and relative expression levels of cellular TRIM21 and target protein, antibody delivery alone may be sufficient for target protein depletion. If target protein depletion does not occur, or is incomplete, then methods for increasing cellular TRIM21 levels can be considered from the following options:

- *TRIM21 overexpression*: In oocytes and early embryos we found it necessary to overexpress TRIM21 for successful Trim-Away. In these cell types microinjection of *Trim21* mRNA is the recommended method. In bulk cell populations it is important to ensure that all cells in the population overexpress TRIM21. To achieve this we recommend generating stable cell lines as described in box 3.
- *Recombinant TRIM21 protein*: For some cell types, particularly primary cells, it is not possible to generate stable cell lines overexpressing TRIM21. In this case it may be preferable to co-deliver recombinant TRIM21 protein together with antibody. Recombinant TRIM21 protein can be readily produced from bacteria as described in box 4. Bacterially-produced proteins may contain bacterial contaminants that could perturb certain cell types such as immune cells. In these cases it may be preferable to purify TRIM21 from insect or mammalian cells.

Trim-Away by microinjection of oocytes and early embryos In our initial study, we microinjected *Trim21* mRNA into arrested germinal vesicle (GV) oocytes, incubated the oocyte for expression and recovery for 3 hours, followed by microinjection of the antibody[15]. Depending on the biological questions to be addressed, antibodies can be microinjected immediately before meiotic release or during meiosis. While sequential injections can be mastered by scientists with extensive experience in micromanipulation, a significant reduction in oocyte survival and developmental rates is frequently observed for less experienced operators.

We have therefore developed a simplified protocol that involves single microinjection, which minimizes harm to the cells. We here present protocols for both hydraulic- and pressure-based

microinjection systems. Hydraulic-based systems allow injection of a defined volume, but require the use of mercury-filled needles[28]. While it is challenging to consistently inject a defined volume using pressure-based systems, these systems are more widely available, do not involve mercury and allow more rapid microinjections. The new protocols offer several benefits. Firstly, single microinjection improves survival and developmental rates to levels comparable to routine live imaging experiments. Secondly, depletion starts immediately after microinjection as *Trim21* mRNA is translated, which can be advantageous for early embryos that cannot be arrested at a particular stage of development. Lastly, target proteins that are expressed at high endogenous levels (which can take up to 3 – 4 hours for full depletion) are more completely degraded before meiotic release, and phenotypes during early meiosis are more consistent between individual oocytes.

In addition, we optimized our protocol for preparing mRNAs encoding *Trim21* and reporters for live-cell imaging with several modifications. Firstly, the use of anti-reverse cap analog (ARCA) yields transcripts that are all capped in the correct orientation at the 5' end and hence, a higher translation efficiency than mRNAs that have been synthesised with standard cap analog. Secondly, the increased ratio of cap analog and GTP relative to other NTPs enables the synthesis of transcripts of up to 12 kb at high yield. Lastly, the incorporation of a column clean-up step removes all free nucleotides, allowing higher accuracy in determination of mRNA concentration and reproducibility in expression level.

Trim-away by electroporation of primary cells and cell lines A successful Trim-Away experiment in bulk cell populations depends on efficient delivery of antibody all the cells in a population. Antibodies are large molecules of ~150 kDa that cannot cross cell membranes. We initially tested antibody delivery by conventional chemical- and lipid-based transfection reagents in addition to several different commercially available protein and antibody transfection reagents. However, this most often led to antibodies being trapped inside intracellular vesicles with very little antibody actually reaching the cytosol. We also tested classical electroporation using conventional cuvettes. However, this caused high levels of cell death, and surviving cells tended to have morphologically changes and exhibited antibodies stuck to the cell surface, trapped inside intracellular vesicles and aggregated inside the cells. We and others have found that a new type of electroporation device with a capillary electrode[15,27,29,30], available commercially as the Neon Transfection System, can be used to deliver antibodies directly to the cytoplasm of almost 100% of cells without cell death. Cells are morphologically normal following electroporation with the Neon system, and re-adhere and proliferate at a similar rate as non-electroporated cells[15]. Thus, Trim-Away experiments can be performed by electroporation of antibodies and phenotypes can be analysed as soon as the target protein is depleted, which is usually within 3 hours. In addition, we found efficient antibody delivery can also be achieved in primary cells such as normal human lung fibroblasts, mouse bone-marrow-derived macrophages and human monocyte-derived macrophages[15]. Here we include an optimized antibody electroporation protocol that we found to be suitable for antibody delivery in all cell types tested so far.

Trim-Away controls To properly interpret a Trim-Away experiment certain controls can be included. To control for antibody delivery, we recommend using isotype control antibodies of the same clonality as the antibodies used for targeted protein degradation. Control antibodies are often raised against proteins that are not normally expressed in mammalian cells, such as GFP or yeast proteins, and are readily available from most antibody suppliers. Additional controls such as delivery of

antibody buffer alone and non-electroporated/microinjected cells can also be included. If a Trim-Away experiment requires TRIM21 overexpression, we also recommend including non-overexpressing cells as an additional control. Importantly, we found that both antibody delivery and TRIM21 overexpression did not perturb cell proliferation or induce major changes in global transcript levels[15].

Confirmation of protein depletion by Trim-Away Confirmation of protein depletion by Trim-Away can be achieved by standard methods used to detect cellular levels of endogenous proteins. For example immunofluorescence, flow-cytometry or western blotting methods are suitable. When using indirect antibody-based detection methods to detect cellular levels of target proteins following Trim-Away (and also other non-targeted cellular proteins), it is important to take into account that secondary antibodies will also detect residual electroporated/microinjected antibody present in the cell if the secondary antibodies are raised against the same species of antibody as the delivered antibody. This problem will arise if the antibody delivered to cells for Trim-Away is also used as a primary antibody for protein detection. When western blotting, this will result in detection of IgG heavy chain and light chain on the membrane (at ~50 kDa and ~25 kDa respectively), which may interfere with detection of endogenous proteins. When performing immunofluorescence or flow cytometry, this will result in higher levels of background signal. In all detection methods, this problem can be avoided by using primary antibodies of a different species to the antibodies delivered to the cells for Trim-Away. If there is no option but to use the same antibody (or antibodies from the same species) for Trim-Away *and* for detection, HRP-conjugated secondary antibodies that only bind to the native non-denatured IgG can be used for western blotting, and primary antibodies can be directly labelled with fluorophores for immunofluorescence and flow cytometry.

Confirmation of specificity in a Trim-Away experiment The specificity of depletion in a Trim-Away experiment is determined by the specificity of the antibody used. Thus antibodies should be selected carefully as described above (see Antibody selection). If multiple antibodies raised against different regions of the same target protein are available, these can be used to confirm the specificity of a Trim-Away phenotype. Rescue experiments can also be performed by overexpressing exogenous target protein. When performing rescue experiments, it should be taken into account that the antibody delivered during Trim-Away will likely also target the exogenous protein for degradation. In this case it may be necessary to increase expression levels of the exogenous protein in order to saturate cellular antibody and TRIM21 to allow for a rescue. Similarly, the amount of delivered antibody should be used at a minimum to aid rescue experiments. It may also be possible to rescue a phenotype by overexpressing exogenous protein from a different species that is not recognised by the antibody used for Trim-Away. Another way to confirm the specificity of depletion is to express a fluorescently tagged target protein. Upon acute delivery of the antibody against the target protein, the fluorescent signal should be reduced as a result of co-degradation of the fluorescent tag.

Materials

Reagents

Antibody preparation

- NAb protein A/G spin kit (0.2 ml; Thermo Scientific, cat. no. 89950)
- Water (for embryo transfer; Sigma-Aldrich, cat. no. W1503)
- Phosphate-buffered saline (PBS) tablets (Gibco, cat. no. 18912)

Isolation and culture of mouse oocytes and pre-implantation embryos

- Culture medium such as M2 medium (homemade or Sigma-Aldrich, cat. no. MR-015P)
- NaCl (Sigma-Aldrich, cat. no. S5886)
- KCl (Sigma-Aldrich, cat. no. P5405)
- $CaCl_2 \bullet 2H_2O$ (Sigma-Aldrich, cat. no. C7902)
- KH_2PO_4 (Sigma-Aldrich, cat. no. P5655)
- $MgSO_4 \bullet 7H_2O$ (Sigma-Aldrich, cat. no. 63138)
- $NaHCO_3$ (Sigma-Aldrich, cat. no. S3817)
- HEPES (Gibco, cat. no. 15630)
- Na lactate (Sigma-Aldrich, cat. no. L7900)
- Na pyruvate (Sigma-Aldrich, cat. no. 11360)
- Glucose monohydrate (Sigma-Aldrich, cat. no. 49158)
- Penicillin G (Sigma-Aldrich, cat. no. P3032)
- Streptomycin (Sigma-Aldrich, cat. no. S9137)
- Bovine serum albumin (BSA) (Sigma-Aldrich, cat. no. A3311)
- Phenol red (Sigma-Aldrich, cat. no. P3532)
- Oil (mineral; Sigma-Aldrich, cat. no. M5310 or paraffin; Merck Millipore, cat. no. 107174)
- Mice **! CAUTION** Animal experiments must be carried out according to institutional and national regulations. All experiments in this Procedure involving live mice were approved by the MRC Cambridge Ethical Review Committee and the UK Home Office under project license numbers PPL 70/8087 and PPL PCF3F9520.

Constitutive Trim-Away in mouse oocytes and pre-implantation embryos

- Hydrochloric acid (36.5 – 38.0% (vol/vol); Sigma-Aldrich, cat. no. H1758)
- Silicon grease Bayer (medium vacuum; Sigma-Aldrich, cat. no. 85402)
- Sigmacote (Sigma-Aldrich, cat. no. SL2)
- Mercury (Sigma-Aldrich, cat. no. 261017)
- NP-40 Surfact-Amps detergent solution (Thermo Scientific, cat. no. 28324)
- Dimethylpolysiloxane (20 cSt; Sigma-Aldrich, cat. no. DMPS2X or 50 cSt; Sigma-Aldrich, cat. no. DMPS5X)

Trim-Away by electroporation in cell lines and primary cells

- DMEM (high glucose; GlutaMAX Supplement; pyruvate; Gibco, cat. no. 31966)
- Fetal bovine serum (Gibco, cat. no. 10270)
- DPBS (no calcium; no magnesium; Gibco, cat. no. 14190)
- Trypsin-EDTA (0.05%; phenol red; Gibco, cat. no. 25300)
- Cell lines of interest: We have used 293 [HEK-293] (ATCC, cat. no. CRL-1573), NIH/3T3 (ATCC, cat. no. CRL-1658), hTERT RPE-1 (ATCC, cat. no. CRL-4000), U-2 OS (ATCC, cat. no. HTB-96), HCT 116 (ATCC, cat. no. CCL-247) and HeLa (ATCC, cat. no. CCL-2) cells, although any mammalian cell line may be used. Cell lines should be grown in the appropriate growth media and maintained by standard cell culture methods. **! CAUTION** Cell lines should be regularly checked to ensure they are authentic and not infected with mycoplasma.
- Primary cells of interest: We have used NHLF (Lonza, cat. no. CC2512), human monocyte-derived macrophages and mouse bone-marrow-derived macrophages, although any mammalian primary cells may be used.

- Mouse anti-TRIM21 (D-12; Santa Cruz Biotechnology, cat. no. sc-25351)
- Rabbit anti-TRIM21 (D1O1D; Cell Signalling Technology, cat. no. 92043S)

Equipment

Antibody preparation

- Amicon Ultra-0.5 centrifugal filter unit with Ultracel-100 membrane (Merck Millipore, cat. no. UFC100)

Isolation and culture of mouse oocytes and pre-implantation embryos

- TC-treated easy-grip style cell culture dish (35 mm; Corning, cat. no. 353001)
- Microcapillary tube (100 µl; Sigma-Aldrich, cat. no. P1174)
- Aspirator tube assemblies for calibrated microcapillary pipettes (Sigma-Aldrich, cat. no. A5177)
- Stereomicroscope (Zeiss, cat. no. SteREO Discovery.V8)
- Galaxy 48 R (Eppendorf, cat. no. CO482)

Constitutive Trim-Away in mouse oocytes and pre-implantation embryos

- Inverted microscope (Zeiss, cat. no. Axio Vert.A1)
- Crossline micrometer (Zeiss, cat. no. 474066-9901-000)
- Vibration-free table (Gutmann, cat. no 14.860)
- Stage micrometer for transmitted light (Zeiss, cat. no. 474026-0000-000)
- Coarse manipulator (Narishige, cat. no. MN-4)
- Three-axis joystick oil hydraulic micromanipulator (Narishige, cat. no. MO-202U)
- Adapter Zeiss 2 (for Eppendorf micromanipulation systems; Eppendorf, cat. no. 5192312002)
- TransferMan 4r (Eppendorf, cat. no. 5193000012)
- 22×22 mm #0 glass coverslips (Sigma-Aldrich, cat. no. C9802)
- Diamond pencil (CellPath, cat. no. MEA-0301-00A)
- Microinjection chamber (design adapted from ref. 28.)
- Capillary that fits into the groove of the microinjection chamber (e.g. Drummond Scientific Company, cat. no. 1-00-0300)
- Microcaps (Drummond Scientific Company, cat. no. 1-000-0500)
- Flaming/Brown micropipette puller (Sutter Instrument, cat. no. P-97)
- Calibrated syringe (10 µl; Hamilton, cat. no. 80300)
- CellTram Oil (Eppendorf, cat. no. 5176000076)
- Microloader (Eppendorf, cat. no. 5242956003)
- Femtotip II (Eppendorf, cat. no. 5242957000)
- FemtoJet 4i (Eppendorf, cat. no. 5252000013)
- Holding capillary (VacuTip; Eppendorf, cat. no. 5175 108.000 or VacuTip FCH; Eppendorf, cat no. 5175 240.006)
- CellTram Air (Eppendorf, cat. no. 5176000068)
- TC-treated easy-grip style cell culture dish (60 mm; Corning, cat. no. 353004)

Trim-Away by electroporation in cell lines and primary cells

- Corning tissue-culture treated culture dishes (100 mm × 20 mm; Sigma-Aldrich, cat. no. CLS430167)
- Tube (15 ml; PP; screw cap; Greiner Bio-One, cat. no. 188285)

- Eppendorf Safe-Lock tubes (1.5 ml; Eppendorf, cat. no. 0030120086)
- Neon Transfection System starter pack (Invitrogen, cat. no. MPK5000S)

Reagent setup

***Trim21* and reporter mRNA** mRNAs encoding *Trim21* and reporters for live-cell imaging such as meGFP-MAP4 and H2B-mCherry are prepared by *in vitro* transcription as described in Box 2 and can be stored at -80°C as long as no signs of mRNA degradation can be seen .

TRIM21-overexpressing stable cell lines TRIM21-overexpressing cell lines are prepared as described in Box 3 and can be cultivated at 37°C/5% CO_2 as long as no signs of senescence can be seen.

Recombinant TRIM21 protein.TRIM21 protein is prepared by purification from bacteria as described in Box 4 and can be stored at -80°C as long as no signs of protein degradation can be seen.

PBS for antibody preparation. Dissolve 5 g of PBS tablet in 500 ml of embryo-tested or nuclease-free water. Store at room temperature as long as no signs of contamination can be seen.

Homemade culture medium for isolating mouse oocytes and pre-implantation embryos. We routinely use modified M2 medium as described in ref. 35 for culture of mouse oocytes and pre-implantation embryos. The modified M2 medium contains 5.53 g/l NaCl, 0.36 g/l, KCl, 0.25 g/l $CaCl_2 \bullet 2H_2O$, 0.16 g/l KH_2PO_4, 0.29 g/l $MgSO_4 \bullet 7H_2O$, 0.35 g/l $NaHCO_3$, 4.97 g/l HEPES, 2.6 g/l Na lactate, 0.04 g/l Na pyruvate, 1.1 g/l glucose monohydrate, 4 g/l BSA, 0.06 g/l penicillin G, 0.05 g/l streptomycin and 0.002 g/l phenol red. We reduce the concentration of phenol red from 0.01 g/l to 0.002 g/l as phenol red is toxic for mouse oocytes and early embryos. Store at 4°C for up to up to two weeks.

Δ CRITICAL We obtain higher developmental rates with homemade M2 medium than with commercial M2 medium.

Δ CRITICAL Drugs such as 250 µM dibutyryl cyclic adenosine monophosphate should be added to the culture medium to maintain meiotic arrest for GV oocytes.

DMEM supplemented with 1X GlutaMAX and 10% (vol/vol) fetal bovine serum Add 55 ml of fetal bovine serum to 500 ml of DMEM. Store at 4°C until use.

Equipment setup

Mouth pipette for isolation and culture of mouse oocytes and pre-implantation embryos Pull microcapillary tubes above a gas burner. Break the tip by striking the pair of pipettes against each other to obtain a smooth opening of ~80 – 100 µm. Attach a pipette to the aspirator tube assembly. More details on mouth pipettes can be found in ref. 36.

Microinjection setup Install the inverted microscope on a vibration-free table. For the use of hydraulic-based systems, mount the coarse manipulator and the three-axis joystick oil hydraulic micromanipulator on one side of the arm. For the use of pressure-based systems, install the Adapter Zeiss 2 and mount a TransferMan 4r to both sides of the arm.

Coverslips Boil 0.5 L of deionized water in a microwave oven, add a few drops of dishwashing detergent and add 22×22 mm #0 glass coverslips one-by-one. Incubate for 20 min at room temperature and discard the detergent solution. Rinse with deionized water thrice, boil and rinse with deionized water thrice again. Transfer the coverslips one-by-one to 0.5 L of 5 M hydrochloric acid and incubate overnight at room temperature. Rinse five times with deionized water and three times with double distilled water. Transfer the coverslips one-by-one to 70% (vol/vol) ethanol for storage. Blot-dry excess ethanol solution and air-dry before use.

Δ CRITICAL Using unwashed coverslips for microinjection may compromise the survival rate of oocytes and early embryos during microinjection.

Microinjection chamber Cut a washed coverslip along the edges into 6 equal rectangular pieces (shelves) and a centre square piece with a diamond pencil. Attach a piece of double-sided tape of approximately the same dimension as a shelf (as a spacer of ~100 µm) close to the center of another washed coverslip at ~5 mm from one edge. Attach a shelf to the double-sided tape to form a ~0.5 mm loading space at the side closest to the edge. Apply a thin layer of silicon grease along the U-shape cut on both sides of the microinjection chamber. Attach the coverslip with the loading space facing away from the U-shape cut to the side of the microinjection chamber with a groove for placing the capillary. Attach another washed coverslip to the other side of the microinjection chamber. Gently press on the coverslips to spread the silicon grease. Fill the U-shaped reservoir with culture medium and keep the microinjection chamber in a humidified container at 37^{o}C before use. After loading and before microinjection, apply a thin layer of silicon grease along the groove and insert the capillary with mRNA and/or antibody.

Microinjection needle Siliconize microcaps by dipping one end in a beaker containing ~0.5 cm of Sigmacote. A column of Sigmacote will form due to capillary action. Invert microcaps end-to-end several times so that the entire length is fully covered with Sigmacote. Blot-dry excess Sigmacote at one end and air-dry for at least two weeks. Pull siliconized microcaps in a Flaming/Brown micropipette puller to obtain pairs of pipettes with an opening of ~0.5 – 1 µm after breaking the tip. Back-load ~1 µl of mercury as close to the tip as possible with a 10 µl calibrated syringe.

Δ CRITICAL Siliconizing microcaps largely facilitate the flow of mercury within the microinjection needle.

Δ CRITICAL Insufficient drying of Sigmacote increases the chance of breaking the mercury column during microinjection.

CellTram Oil Set up the CellTram Oil according to the manufacturer's instructions. Fill it with mineral oil.

FemtoJet 4i Set up the FemtoJet 4i according to the manufacturer's instructions.

CellTram Air Set up the CellTram Air according to the manufacturer's instructions.

Neon Transfection System for electroporation in primary cells and cell lines The Neon Transfection System is setup according to manufacturer's instructions.

Procedure

Antibody Preparation for Trim-Away (TIMING 1 – 2 hours)

Δ CRITICAL This section describes how to prepare the antibody of interest for Trim-Away. Ultrafiltration is necessary for removing contaminants, which are cytotoxic (e.g. sodium azide) or interfere with microinjection (e.g. glycerol).

1. If the antibody is from an unpurified source such as serum, ascites fluid and cell culture supernatant, or contains BSA or gelatin, pre-purify the antibody using the 0.2 ml Nab protein A/G spin kit.

Δ CRITICAL STEP This step is necessary for removing contaminating proteins, which lead to an overestimation of the IgG concentration. Gelatin is not compatible with the Amicon Ultra-0.5 100K devices used in Step 3.

2. Mix the components tabulated below in a 1.5 ml nuclease-free tube on ice.

Component	Amount per reaction (µl)	Final concentration
PBS (in embryo-tested or nuclease-free water)	400 – 450	
1 mg/ml Antibody	50 – 100	0.1 – 0.2 µg/µl

Δ CRITICAL STEP For co-injection of Trim21 mRNA and antibody using FemtoJet, PBS should be prepared in nuclease-free water instead to reduce the chance of RNase contamination.

3. Insert an Amicon Ultra-0.5 100 K device into a provided collection tube.
4. Apply 500 µl of diluted antibody from step 2 to the device.
5. Align the cap strap of the collection tube and the membrane of the device towards the center of the rotor.
6. Centrifuge at 14,000 ×g for 10 min at 4°C.

Δ CRITICAL STEP Higher centrifugal force may damage the membrane and cause sample loss.

7. Discard the flow-through.
8. Add 480 µl of PBS to the Amicon Ultra-0.5 100 K device and carefully mix well.

Δ CRITICAL STEP Mixing is necessary for avoiding high local concentration during ultrafiltration, which can cause protein aggregation and loss. Avoid touching the membrane with the pipette tip as this may damage the membrane and cause sample loss.

9. Repeat step 5-7 twice.

? TROUBLESHOOTING

10. Insert the inverted Amicon Ultra-0.5 100 K device into another provided collection tube.
11. Align the open cap towards the centre of the rotor.
12. Centrifuge at 1,000 ×g for 2 min at 4°C.
13. Use 2 µl to measure the IgG concentration with a NanoDrop spectrophotometer.

Δ CRITICAL STEP 1.0 OD_{280} is equivalent to 0.714 mg/ml IgG.

Δ CRITICAL STEP We typically retrieve ~20 µl of purified antibody at ~1.25 – 2.5 mg/ml from 50 µg of input.

? TROUBLESHOOTING

14. Snap-freeze in liquid nitrogen.

Δ CRITICAL STEP Purified antibody should be divided into 1 – 2 µl single-use aliquots to minimize freeze-thaw cycles in the absence of cryoprotectant.

■ **PAUSE POINT** Antibody can be stored at -80°C as long as no loss of activity can be seen.

Isolation and culture of mouse oocytes and pre-implantation embryos (TIMING >1 hour)

15. Isolate and culture mouse GV, meiosis II (MII) oocytes or early embryos under oil as previously described in ref. 37 – 42.

Trim-Away by microinjection of mouse oocytes and pre-implantation embryos (TIMING 4 – 5 hours)

16. To perform constitutive depletion (**Fig. 3a**) using hydraulic systems, follow option A. To perform constitutive depletion using pressure-based systems, follow option B. To perform acute depletion (**Fig. 3b**), follow option C.

(A) Constitutive depletion using hydraulic-based systems such as CellTram Oil (TIMING 4 – 5 hours)

(i) Mix the components tabulated below in a 1.5 ml nuclease-free tube on ice.

Component	Amount per reaction (µl)	Final concentration
2 µg/µl *Trim21* mRNA	0.5	400 ng/µl
Reporter mRNA(s)	Variable	Usually 30 – 300 ng/µl each
Nuclease-free water	To 2.5	

■ **PAUSE POINT** mRNA mix can be stored at -20°C or -80°C until use. We usually freeze-thaw the mRNA mix up to 4 times without noticeable RNA degradation.

(ii) Mix the components tabulated below in another 1.5 ml nuclease-free tube on ice.

Component	Amount per reaction (µl)	Final concentration
2 mg/ml Antibody/ antibodies from Step 14	1.25	1 mg/ml each
0.5% (vol/vol) NP-40 (in embryo-tested water)	0.25 – 0.5	0.05 – 0.1% (vol/vol)
PBS (in embryo-tested water)	To 2.5	

Δ CRITICAL STEP Antibody at a final concentration of 1 mg/ml is a good starting point for complete depletion of most target proteins. However, we suggest titrating individual antibodies to the minimal effective concentration, as some can have secondary inhibitory effects.

Δ CRITICAL STEP For antibody at a final concentration of <0.1 mg/ml, storage in a low protein-binding tube or preparation of fresh antibody mix is recommended.

Δ CRITICAL STEP NP-40 largely facilitates the flow of the viscous antibody mix within the microinjection needle and reduces the chance of clogging.

■ **PAUSE POINT** For short-term storage (up to 1 week), antibody mix can be stored at 4°C in a 1.5 ml tube with the opening sealed with Parafilm. However, changes in ionic strength due to repeated evaporation and condensation at the cap can denature the antibody/antibodies. For long-term storage (up to at least 1 month), the antibody mix can be stored at -20°C or -80°C until use. We usually freeze-thaw the antibody mix up to 4 times without noticeable loss of activity.

(iii) Centrifuge the mRNA mix and antibody mix at 20,000 ×g for 5 min at 4°C before capillary loading.

Δ CRITICAL STEP This step is necessary for reducing the amount of precipitated materials loaded into the capillary.

(iv) Load 0.5 µl of dimethylpolysiloxane into a capillary.

Δ CRITICAL STEP Avoid introducing air bubbles from step 16(A)(iv) to (viii).

Δ CRITICAL STEP Follow the order of loading (**Fig. 3c**) from step 16(A)(iv) to (viii).

(v) Load 0.5 µl of pre-cleared antibody mix.

Δ CRITICAL STEP Down to 0.25 µl can be used.

(vi) Load 0.5 µl of dimethylpolysiloxane.

(vii) Load 0.5 µl of pre-cleared mRNA mix.

Δ CRITICAL STEP Down to 0.25 µl can be used.

(viii) Load 0.5 µl of dimethylpolysiloxane.

? TROUBLESHOOTING

Δ CRITICAL STEP Loaded capillary should be kept on ice until use.

(ix) Transfer oocytes or early embryos into the loading space of the microinjection chamber using a mouth pipette under the stereomicroscope.

(x) Mount a microinjection needle onto the capillary holder attached to the CellTram Oil.

(xi) Attach the capillary holder to the micromanipulator.

(xii) Carefully tap the tip of the microinjection needle against the wall of the capillary to obtain an opening of ~0.5 – 1 µm and slowly push the mercury column to the front.

Δ CRITICAL STEP An opening of >1 µm will increase the risk of lysis after microinjection, but an opening of <0.5 µm will increase the chance of clogging.

(xiii) Determine the injection volume by loading different amount of polydimethylsiloxane into the microinjection needle and carefully expelling into the mRNA mix. A polydimethylsiloxane droplet will form. The corresponding volume can be calculated using the formula $4/3 \times \pi \times (d/2)^3$, where d is the diameter of the droplet.

(xiv) Load ~1 pl of polydimethylsiloxane into the microinjection needle.

(xv) Load 3.5 pl of mRNA mix.
(xvi) Load ~1 pl of polydimethylsiloxane.
(xvii) Load 3.5 pl of antibody mix.
(xviii) Load ~1 pl of polydimethylsiloxane.

Δ CRITICAL STEP mRNA mix and antibody mix should not be mixed within the microinjection needle to avoid RNA degradation before microinjection.

Δ CRITICAL STEP The antibody mix can be injected at a volume of up to 8.5 pl without harming the oocytes, which may be necessary for antibody/antibodies that cannot be concentrated further to the desired concentration.

(xix) Adjust the level of the microinjection needle until it is in focus with the plasma membrane of the oocyte or early embryo.
(xx) Carefully press the tip of the microinjection needle against the oocyte or early embryo until it penetrates the plasma membrane.
(xxi) Expel the column of polydimethylsiloxane-mRNA mix-polydimethylsiloxane-antibody mix into the cytoplasm.
(xxii) Quickly withdraw the microinjection needle to release the oocyte or early embryo.
(xxiii) Repeat step (xiv)-(xxii).

Δ CRITICAL STEP A successful microinjection is indicated by a cytoplasmic movement and the presence of two dimethylpolysiloxane droplets.

Δ CRITICAL STEP A video and an illustration describing hydraulic-based microinjection step-by-step can be found at https://health.uconn.edu/cell-biology/wp-content/uploads/sites/115/2017/07/follicleinjection.mp4?_=2 and in ref. 42, respectively.

? TROUBLESHOOTING

(xxiv) Transfer microinjected oocytes or early embryos from the microinjection chamber to a 50 µl drop of culture medium covered with oil in a 35 mm dish.
(xxv) Incubate at 37°C for 3 hr before performing an assay such as live imaging or immunofluorescence.

Δ CRITICAL STEP Full depletion of some target proteins can take up to 4 hr.

? TROUBLESHOOTING

(B) Constitutive depletion using pressure-based systems such as FemtoJet 4i (TIMING 4 – 5 hours)

(i) Mix the components tabulated below in a 1.5 ml nuclease-free tube on ice.

Component	Amount per reaction (µl)	Final concentration
2 µg/µl *Trim21* mRNA	0.25	200 ng/µl
Reporter mRNA(s)	Variable	Usually 15 – 150 ng/µl each
2 mg/ml Antibody/ antibodies from Step 14	0.625	0.5 mg/ml each
0.5% (vol/vol) NP-40 (in nuclease-free water)	0.25 – 0.5	0.05 – 0.1% (vol/vol)
PBS (in nuclease-free water)	To 2.5	

Δ CRITICAL STEP Antibody at a final concentration of 0.5 mg/ml is a good starting point for complete depletion of most target proteins. However, we suggest titrating individual antibodies to the minimal effective concentration, as some can have secondary inhibitory effects.

Δ CRITICAL STEP For antibody at a final concentration of <0.1 mg/ml, storage in a low protein-binding tube or preparation of fresh mRNA & antibody mix is recommended.

Δ CRITICAL STEP NP-40 largely facilitates the flow of viscous mRNA & antibody mix within the Femtotip II and reduces the chance of clogging.

■ **PAUSE POINT** The mRNA and antibody mix can be stored at -20°C or -80°C until use. We usually freeze-thaw the mRNA & antibody mix up to 4 times without noticeable denaturation and/or RNA degradation.

(ii) Centrifuge the mRNA and antibody mix at 20,000 ×g for 5 min at 4°C before loading.

Δ CRITICAL STEP This step is necessary for reducing the amount of precipitated materials loaded into the capillary.

(iii) Load 0.5 µl of mRNA and antibody mix to the tip of a Femtotip II using Microloader.

Δ CRITICAL STEP Avoid introducing air bubbles.

(iv) Mount a Femtotip II onto the capillary holder of the FemtoJet 4i.

(v) Mount a holding capillary onto the capillary holder of the CellTram Air.

Δ CRITICAL STEP We have also successfully used the FemtoJet 4i with a microinjection chamber instead of a holding capillary.

(vi) Attach the capillary holders to TransferMan 4r.

(vii) Transfer oocytes or early embryos to a drop of culture medium covered with oil on the lid of a 60 mm dish.

(viii) Determine the appropriate settings for the microinjection program to inject a volume of ~7 pl by first microinjecting different amount of a fluorescent dye into oocytes or early embryos and measuring the intracellular fluorescence intensity and the fluorescence intensity of a series of dye solutions of known concentration. The injection volume can then be calculated using the formula $F_{dye\ injected} \times V_{dye\ injected} = F_{cell} \times V_{cell}$, where $F_{dye\ injected}$ and $V_{dye\ injected}$ is the fluorescence intensity and volume of the dye solution microinjected, and F_{cell} and V_{cell} is the fluorescence intensity and volume of the oocyte or early embryo microinjected. More details can be found in ref. 43 and 44.

Δ CRITICAL STEP This step depends on the experience of the scientist and may be optional.

Δ CRITICAL STEP The mRNA and antibody mix can be injected at a volume of up to 12 pl without harming the oocytes, which may be necessary for antibody/antibodies that cannot be concentrated further to the desired concentration.

(ix) Adjust the level of the holding capillary until it is in focus with the oocyte or early embryo.

(x) Secure an oocyte or early embryo in place by applying a negative pressure at the holding capillary.

(xi) Adjust the level of the Femtotip II until it is in focus with the plasma membrane of the oocyte or early embryo.

(xii) Push the tip of the Femtotip II against the oocyte or early embryo until it penetrates the plasma membrane.
(xiii) Press the 'Inject' button on the FemtoJet 4i to expel ~7 pl of mRNA and antibody mix into the cytoplasm.
(xiv) Quickly withdraw the microinjection needle.
(xv) Release the oocyte or early embryo by applying a positive pressure at the holding capillary.
(xvi) Repeat step (ix) to (xv).

Δ CRITICAL STEP A successful microinjection is indicated by a cytoplasmic movement.

Δ CRITICAL STEP A video describing pressure-based microinjection step-by-step can be found in ref. 45.

? TROUBLESHOOTING

(xvii) Transfer microinjected oocytes or early embryos from the stage to a 50 µl drop of culture medium covered with oil in a 35 mm dish.
(xviii) Incubate at 37°C for 3 hr before performing an assay such as live imaging or immunofluorescence.

Δ CRITICAL STEP Full depletion of some target proteins can take up to 4 hr.

? TROUBLESHOOTING

(C) Acute depletion (TIMING >4 hours)

(i) Prepare the mRNA mix as in step 16(A)(i).
(ii) Microinject 3.5 pl using a hydraulic-based system as in step 16(A)(iii)-(xix) or a pressure-based system as in step 16(B)(ii)-(ix)
(iii) Transfer microinjected oocytes or early embryos to a drop of culture medium covered with oil.
(iv) Incubate at 37°C until the desired developmental stage.
(v) Prepare the antibody mix as in step 16(A)(ii).
(vi) Repeat step 16(C)(ii).
(vii) Immediately proceed to assay such as live imaging or immunofluorescence.

Trim-Away by electroporation in primary cells and cell lines (TIMING <1 hour)

17. Trypsinise the cells using standard cell culture procedure and transfer to a 15 ml tube.
18. Pellet the cells at 300 xg for 5 minutes at room temperature and discard the supernatant.
19. Resuspend the cell pellet in 10 ml of PBS and count the cells.
    Δ CRITICAL STEP The cells should be thoroughly resuspended in PBS to avoid cell clumping that can give inaccurate results in cell counting
20. Transfer n x (8×10^5) cells to a new 15 ml tube (where n = number of electroporation reactions)
    Δ CRITICAL STEP We routinely use 8×10^5 cells per electroporation reaction using a 10 µl Neon tip. However, it is possible to use any number of cells between 1×10^5 and 1.5×10^6 depending on the type and size of cells, without any detrimental effects on antibody delivery or cell survival.
21. Pellet the cells at 300 xg for 5 min at room temperature and discard all but ~1 ml of supernatant.
22. Using residual supernatant, transfer the cells to a new 1.5 ml tube.
23. Pellet the cells at 300 xg for 5 min at room temperature. In the meantime, proceed with Step 24.
24. During step 23, insert a Neon Tube into the Neon Pipette Station and fill the Neon Tube with 3 ml of Neon Buffer E.

25. Carefully remove the supernatant.
    Δ CRITICAL STEP Supernatant should be removed carefully using a Gilson pipette to avoid disturbing the cell pellet and subsequent cell loss.

26. Resuspend the cell pellet in n x 10 µl of Neon Buffer R (where n = number of electroporation reactions).
    Δ CRITICAL STEP Once the cells are resuspended in Neon Buffer R, it is important to proceed with electroporation immediately. Prolonged incubation (>30 min) in Neon Buffer R can reduce cell viability.

27. Aliquot 10.5 µl (~8 x 10^5) of cells per electroporation reaction into 1.5 ml tubes.
    Δ CRITICAL STEP It is important to avoid introducing air bubbles that can cause arcing during electroporation

28. Add 2 µl of antibody from Step 14 to the cells and mix gently.
    Δ CRITICAL STEP Antibody at a concentration of 1 mg/ml is a good starting point for complete depletion of most target proteins. However, we suggest titrating individual antibodies to the minimal effective concentration, as some can have secondary inhibitory effects.
    Δ CRITICAL STEP It is important to avoid introducing air bubbles that can cause arcing during electroporation. Gently tapping the tube can help remove any air bubbles. A total volume of 12.5 µl per electroporation reaction should be used as this helps prevent that any air bubbles are taken up into the Neon 10 µl Tip in step 28.

29. Take up the cell/antibody mix into the Neon 10 µl Tip.
    Δ CRITICAL STEP It is essential to avoid taking up air bubbles into the Neon 10 µl Tip that can cause arcing during electroporation. Use a single smooth pipetting action combined with gentle swirling is recommended. Avoid pipetting up and down. For detailed instructions on how to insert the Neon 10 µl Tip into the Neon Pipette refer to the Neon Transfection System manual.

    ? TROUBLESHOOTING

30. Insert the Neon Pipette with the sample in the tip into the Neon Tube containing 3 ml of Neon Buffer E assembled in the Neon Pipette Station. Push down vertically until a 'click' is heard.

31. Electroporate using the following parameters: 1400 V, 2 pulses, 20 ms per pulse.
    Δ CRITICAL STEP If there is a visible spark, discard the sample and return to step 28.

32. Transfer the sample to a 1.5 ml tube containing 1 ml of pre-warmed cell growth media without antibiotics. Electroporated cells can now be plated as determined by the scientist. An incubation time of ~3 hours is usually sufficient for target protein degradation.

    ? TROUBLESHOOTING

Timing

Step 1-14, Antibody preparation: 1 – 2 hours

Step 15, Isolation and culture of mouse oocytes and pre-implantation embryos: >1 hour

Step 16, Trim-Away by microinjection of mouse oocytes and pre-implantation embryos: 4 – 5 hours

Step 17-32, Trim-Away by electroporation in primary cells and cell lines: <1 hour

Troubleshooting

Step	Problem	Possible reason	Solution
Antibody preparation			
9	Antibody cannot be concentrated further to ~20 µl.	The membrane is clogged due to precipitated materials.	Pre-clear the antibody at 20,000 ×g for 5 min at 4°C and use the supernatant for step 2.
			Rotate the device by 180° to make use of the other membrane.
			Use Vivaspin ultrafiltration device instead as it can concentrate antibody down to 5 µl.
13	Antibody is lost after ultrafiltration.	The membrane is damaged.	Check the centrifugal force and do not touch the membrane with pipette tip.
		The antibody is binding to the regenerated cellulose membrane.	Use Vivaspin ultrafiltration device instead as it utilizes polyethersulfone rather than regenerated cellulose membrane.
13	Antibody concentration is much higher than expected.	The antibody solution contains stabilizing proteins such as BSA.	Pre-purify the antibody using 0.2 ml Nab protein A/G spin kit
Trim-Away by microinjection of mouse oocytes and pre-implantation embryos			
16(A)(iv)-(viii)	mRNA mix mixes with the antibody mix within the capillary.	The mRNA mix is loaded before the antibody mix.	Load antibody mix before the mRNA mix.
16(A)(xix)/ 16(B)(ix)/ 16(C)(ii)/ 16(C)(vi)	Microinjection needle or Femtotip II is clogged every few injections.	Precipitated materials are loaded into the capillary.	Pre-clear longer and/or break the tip more.
		Precipitated materials are loaded into the Femtotip II.	Pre-clear longer, break the tip more and/or use the 'Clean' function.
		The antibody concentration is too	Use 0.1% NP-40 and/or inject a larger

		high.	volume of diluted antibody mix.
	Oocytes and early embryos do not survive the microinjection.	The GV is damaged.	Inject close to the GV but do not penetrate the membrane of GV.
		The opening of the microinjection needle is too wide.	Adjust the program of the needle puller to obtain thinner microinjection needle.
			Break the tip of the microinjection needle just enough for the mercury to flow to the tip.
		The injection volume is too large.	Do not inject more than 12 pl (~6%) for mouse oocytes.
		The intracellular NP-40 concentration is too high.	Use 0.05% NP-40 and/or inject less antibody mix.
16(A)(xxi)/ 16(B)(xi)/ 16(C)(vii)	Target protein is not degraded.	The antibody does not recognize the antigen.	Use another antibody.
		TRIM21 does not recognize the antibody.	Confirm that the antibody contains an exposed Fc-region or use another antibody.
		The microinjection is not successful.	Confirm that the microinjection needle or Femtotip II penetrates the plasma membrane and there is a cytoplasmic movement due to liquid outflow.
		Oocytes and early embryos are treated with proteasome inhibitor such as MG132.	Do not use proteasome inhibitor during incubation or apply it after incubation.
	Depletion is not efficient or complete.	The endogenous level of the target protein is too high.	Incubate longer to allow degradation before the start of assay, use higher concentration of antibody and/or inject more antibody mix.
		The expression level of Trim21 mRNA is	Incubate longer to allow expression

		too low.	before the start of assay, use higher concentration of *Trim21* mRNA and/or poly(A)-tail *Trim21* mRNA.
		Trim21 mRNA is degraded.	Make sure that all materials are handled in an RNase-free manner.
	Oocytes and early embryos are not normally developing.	Microinjected oocytes and early embryos are left outside the incubator for too long.	Inject fewer oocytes and early embryos at a time and replace the medium in the microinjection chamber after each round of microinjection.
		The injection volume is too large.	Do not inject more than 12 pl (~6% of the oocyte volume) for mouse oocytes.
		The intracellular NP-40 concentration is too high.	Use 0.05% NP-40 and/or inject less antibody mix.
		The mRNA mix and/or antibody mix is/are contaminated by embryo-toxic substance(s).	Test the nuclease-free water and/or repeat the mRNA and/or antibody preparation.
		The antibody has secondary inhibitory effects.	Reduce the injection volume, antibody concentration and/or use another antibody.
		Reporter mRNA(s) is/are overexpressed.	Reduce the injection volume and/or mRNA concentration.
29	Air bubbles are present in the Neon 10 µl Tip.	Air bubbles are present in the sample/ inaccurate pipetting/ insufficient sample volume.	Gently tap the sample tube to remove air bubbles prior to taking up sample into Neon 10 µl Tip/ ensure the electroporation reaction sample has a volume of 12.5 µl/ take up the sample into the Neon 10 µl Tip slower.
32	Target protein is not degraded.	The antibody does not recognize the	Use another antibody.

		antigen.	
		The antibody does not recognise the native protein inside cells.	Use an antibody that has been tested to work by immunofluorescence
		The electroporated antibody cannot gain access to the target protein.	Allow longer incubation time/ incubation of cells for at least one cell division allows degradation of retained nuclear target proteins.
		TRIM21 does not recognize the antibody.	Use whole IgG molecules from mammalian hosts.
		Electroporation of antibody was not successful.	Check the antibody concentration prior to electroporation/ check for arcing (sparks) during electroporation likely caused by air bubbles in the Neon Tip.
		Cells are treated with proteasome inhibitor such as MG132.	Do not use proteasome inhibitor during Trim-Away.
	Depletion is not efficient or complete.	The endogenous level of the target protein is too high.	Incubate longer to allow degradation before the start of assay/ use higher a concentration of the antibody.
		TRIM21 levels are too low.	Use TRIM21-overexpressing stable cell lines/ co-electroporate recombinant TRIM21 protein together with the antibody.
	Cells have low survival rates.	Arcing (sparks) occurs during electroporation.	Ensure that there are no air bubbles in the Neon 10 µl Tip.
		Cells were incubated for too long in Neon Buffer R	Proceed to antibody electroporation within 10 min of resuspending cells in Neon Buffer R.

Anticipated results

Generation of TRIM21 stable cell lines as described in Box 3 should result in all cells overexpressing TRIM21 diffusely in the cytoplasm (Fig. 2a,b and 2d,e). Representative results for Trim-Away assays in cell lines overexpressing TRIM21 are shown in Figure 2c and 2f. The expression and purification protocol described in box 4 should give TRIM21 protein yields of at least 1 mg/ml (Fig. 2g). Figure 2h shows Trim-Away of ERK1 in five different unmodified human cells lines by either electroporating anti-ERK1 antibody alone or together with recombinant TRIM21 protein.

Representative results for a Trim-Away assay in mouse oocytes are shown in **Figure 4**. In the example shown, it can be observed that depletion of motor protein kinesin-5 (Eg5) by Trim-Away resulted in a monopolar spindle during meiosis I (MI) (**Fig. 4a**). This is consistent with inhibiting the motor activity of Eg5 with monastrol[46]. Subsequent expression of Eg5-meGFP from mRNA reverted the phenotype (**Fig. 3d** and **4b**), confirming the specificity of Eg5 depletion by Trim-Away.

Author contributions

D.C. and M.S. conceived and designed the study. D.C. carried out all experiments, with the following exceptions: C.S. simplified and optimized methods for conducting Trim-Away experiments in mouse oocytes and early embryos, and optimized the peptide binding essay. W.M. and D.C. optimized antibody electroporation. W.M. generated and characterised stable cell lines. D.C. and L.J. purified recombinant TRIM21 protein. C.S. and D.C. prepared figures. L.J. wrote the section on antibody purification; W.M. wrote the section on generating stable cell lines; C.S., D.C. and M.S. wrote all other sections of the manuscript. All authors edited the manuscript. M.S. supervised the study.

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Competing financial interests

The authors declare that they have no competing financial interests.

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Figures

Figure 1 | Schematic of the principle of Trim-Away.

Figure 2 | Trim-Away in cell lines. (**a** and **d**) NIH3T3, NIH3T3-mCherry-mTRIM21, HEK293T and HEK293T-mCherry-hTRIM21 cell lines were analysed by flow cytometry. At least 2000 cells were counted for each condition. Percentages correspond to mCherry-positive cells falling within the gate drawn. (**b** and **e**) NIH3T3-mCherry-mTRIM21 and HEK293T-mCherry-hTRIM21 cells were imaged by confocal microscopy, detecting mCherry fluorescence. Scale bar, 50µm. (**c**) NIH3T3 and NIH3T3-mCherry-mTRIM21 cell lines were electroporated with control IgG or anti-Pericentrin antibodies and analysed 16 hours later by immunoblotting. (**f**) HEK293T and HEK293T-mCherry-hTRIM21 cell lines were electroporated with PBS or anti-IKKα antibody and analysed 3 hours later by immunoblotting. (**g**) Coomassie-stained gel showing His-Lipoyl-hTRIM21 protein. Stars indicate bacterial contaminants

(*) and degradation products (**). (**h**) The indicated cell lines were electroporated with PBS, anti-ERK1 antibody, His-Lipoyl-TRIM21, or anti-ERK1 + His-Lipoyl-TRIM21 and analysed 3 hours later by immunoblotting. Data in panels a-f and panel h were originally published in ref. 15.

Figure 3 | Schematic of Trim-Away experiments in mouse oocytes. (**a**) For constitutive depletion, mouse oocytes are co-injected with *Trim21* mRNA and antibody, and are allowed for expression of Trim21 and subsequent target protein degradation for 3 – 4 hours before release. (**b**) For acute depletion, mouse oocytes are first injected with *Trim21* mRNA, and are allowed for expression of Trim21 for 3 – 4 hours before release. At the desired time point, the antibody is acutely injected into the oocytes. (**c**) For hydraulic-based microinjection system, the capillary is loaded with the first oil, antibody mix, the second oil, mRNA mix followed by the third oil. The liquids are taken up into the microinjection needle in the reverse order. (**d**) For rescue, mRNA encoding the degraded target protein is acutely injected into the oocytes.

Figure 4 | Trim-Away in mouse oocytes. (**a** and **b**) 3D time-lapse imaging of mouse oocytes expressing meGFP-MAP4 (green, microtubules) and H2B-mCherry (magenta, chromosomes), injected with *Trim21* mRNA and control IgG or anti-Eg5 (Sigma-Aldrich, cat. no. HPA010568). Z-projection, 5 sections, every 6 µm. Scale bar, 10 µm. Time, hour:min from nuclear envelope breakdown (NEBD). (**c** and **d**) 3D time-lapse imaging of Eg5-depleted mouse oocytes expressing mCherry-MAP4 (green, microtubules), injected with solvent or EG5-meGFP mRNA. Z-projection, 5 sections, every 6 µm. Scale bar, 10 µm. Time, hour:min from NEBD.

Box 1 Peptide pre-incubation assay (TIMING 2 – 4 days)

This Box describes the procedures for pre-incubating a primary antibody with its immunizing peptide prior to immunofluorescence. This assay allows the evaluation of non-specific binding of the antibody under native conditions.

Reagents

- Paraformaldehyde (4% (wt/vol) in PBS; VWR, cat. no. J61899)
- Immunizing peptide (lyophilized; from solid-phase peptide synthesis by company such as GenScript)
- Bovine serum albumin (BSA) solution (30% (wt/vol) in DPBS; Sigma-Aldrich, cat. no. A9576)
- PBS tablets (Gibco, cat. no. 18912)
- Triton X-100 solution (10% (vol/vol) in H_2O; Sigma-Aldrich, cat. no. 93443)

Reagent Setup

- **PBT** Add 1 g of PBS tablet and 5 ml of 10% (vol/vol) triton X-100 solution to 95 ml of water. Store at 4^{o}C until use.
- **PBT-BSA** Add 1 g of PBS tablet, 5 ml of 10% (vol/vol) triton X-100 solution and 10 ml of 30% (wt/vol) BSA solution to 85 ml of water. Store at 4^{o}C until use

Procedure

1. Add appropriate volume of solvent to dissolve the lyophilized peptide at a final concentration of 133 µM.

Δ CRITICAL STEP Recombinant protein has been used with success as well, albeit at a higher cost.

2. Fix the identical samples with 4% (wt/vol) paraformaldehyde in PBS for 15 – 30 min at 37°C.
3. Permeabilize the samples in PBT overnight at 4°C.
4. Block the samples in PBT-BSA overnight at 4 °C
5. Mix the components tabulated below and incubate at 4°C overnight.

Component	Amount per reaction (µl)	Final concentration
1 mg/ml (Primary) Antibody	0.2	10 µg/ml (66.7 nM)
133 µM Peptide	0 (for no-peptide control) or 2 (for peptide pre-incubated sample)	0 or 13.3 µM (200X of 66.7 nM)
PBT-BSA	to 20	

6. Pre-clear the mixes from step 5 by centrifuging at 20,000 ×g for 5 min at 4°C
7. Incubate one sample with the pre-cleared mix without peptide from step 4 (i.e. no-peptide control) and the other sample with the pre-cleared mix with peptide from step 4 (i.e. peptide pre-incubated sample) overnight at 4°C.
8. Wash the samples in PBT-BSA for 5 min at room temperature three times.
9. Incubate the samples with secondary antibodies diluted in PBT-BSA for 1 hour at room temperature.
10. Wash the samples in PBT for 5 min at room temperature three times.
11. Image the samples in PBS.
12. Assess non-specific bindings from the signal present in both the no-peptide control and peptide pre-incubated sample.

Box 2 *Trim21* and reporter mRNA preparation (TIMING 8 hours)

This Box describes the procedures for preparing mRNAs encoding *Trim21* and reporters for live-cell imaging. The resulting mRNAs are suitable for microinjection into mouse oocytes or early embryos for expression.

Reagents

- pGEMHE-mCherry-mTrim21 (Addgene plasmid #105522)
- pGEMHE-mEGFP-mTrim21 △ RING-Box (Addgene plasmid #105520)
- pGEMHE-mCherry-mTrim21 △ RING-Box (Addgene plasmid #105523)
- pGEMHE-mEGFP-mTrim21 △ PRYSPRY (Addgene plasmid #105521)
- pGEMHE-mCherry-mTrim21 △ PRYSPRY (Addgene plasmid #105524)
- Nuclease-free water (not DEPC-treated; Invitrogen, cat. no. AM9930)
- CutSmart Buffer (NEB, cat. no. B7204S)
- *Asc*I (NEB, cat. no. R0558S or R0558 L)
- Glycogen (RNA grade; Thermo Scientific, cat. no. R0551)
- Phenol-chloroform-isoamyl alcohol (IAA) mixture (25:24:1; Sigma-Aldrich, cat. no. 77617)
- Sodium acetate buffer solution (3 M; pH 7.0; Sigma-Aldrich, cat. no. S2404)
- Ethanol (Sigma-Aldrich, cat. no. 51976)
- Liquid nitrogen
- HiScribe T7 ARCA mRNA kit (with tailing; NEB, cat. no. E2060S)
- TURBO DNase (2 U/µl; Invitrogen, cat. no. AM2238)

- NucleoSpin RNA Clean-up XS (Macherey-Nagel, cat. no. 740903)
- Phenol-chloroform-IAA mixture (125:24:1; Sigma-Aldrich, cat. no. 77619)
- Ethylenediaminetetraacetic acid solution (0.5 M; pH 8.0; Sigma-Aldrich, cat. no. 03690)
- Sodium acetate buffer solution (3 M; pH 5.2; Sigma-Aldrich, cat. no. S7899)
- Sodium dodecyl sulfate (SDS) solution (10% (wt/vol) in H_2O; Sigma-Aldrich, cat. no. 71736)

Equipment

- Snaplock microcentrifuge tube (1.5 ml; RNase-/DNase-free; nonpyrogenic; Corning, cat. no. MCT-150-C)
- Centrifuge 5424 R (refrigerated; Eppendorf, cat. no. 5404000413)
- NanoDrop 2000 spectrophotometer (Thermo Scientific, cat. no. ND-2000)

Reagent Setup

- **0.25 M sodium acetate-5 mM EDTA-0.5% (wt/vol) SDS** Add 0.1 ml of 0.5 M EDTA solution, 0.5 ml of 10% (wt/vol) SDS solution and 0.833 ml of 3 M sodium acetate buffer solution (pH 5.2) to 8.567 ml of nuclease-free water. Divide into 1 ml aliquots and store at -20°C until use.
- **70% (vol/vol) ethanol** Add 35 ml of ethanol to 15 ml of nuclease-free water. Store at -20°C until use.

Equipment Setup

- **NanoDrop 2000 spectrophotometer** Set up the NanoDrop 2000 spectrophotometer according to the manufacturer's instructions.

Procedure

1. **Linearization and purification of DNA template (Steps 1-12):** Mix the components tabulated below in a 1.5 ml nuclease-free tube and incubate at 37°C for 1 hr.

Component	Amount per reaction (µl)	Final concentration
Nuclease-free water	20	
0.5 µg/µl Trim21 or reporter construct (in pGEMHE)	20	0.2 µg/µl
10X CutSmart Buffer	5	1X
10 U/µl *Asc*I	5	1 U/µl

Δ CRITICAL STEP We generally use pGEMHE as the vector because it contains a T7 promoter, multiple cloning sites flanked by 5'- and 3'-untranslated region (UTRs) of *Xenopus* β-globin gene, a short stretch of ~35 bp poly(A) sequence and linearization sites. Constructs in other vectors such as pCS2 can also be used, but the translation efficiency may be lower due to the lack of the 5' and 3' UTRs, and poly(A) tailing may be required.

2. Add 0.5 µl glycogen, 150 µl nuclease-free water and 200 µl phenol-chloroform-IAA (pH 8.0) to terminate the linearization reaction.

Δ CRITICAL STEP Addition of glycogen as co-precipitant is optional, but the yield of precipitated DNA is higher.

Δ CRITICAL STEP Phenol-chloroform-IAA is saturated and stored with an equilibration buffer. Do not take-in the upper aqueous layer to avoid dilution.

3. Invert end-to-end to mix well and centrifuge at 20,000 ×g for 5 min at room temperature.
4. Transfer ~180 µl of the upper phase to a new 1.5 ml nuclease-free tube, add 20 µl of 3 M sodium acetate (pH 7.0) and 400 µl of ice-cold 100% ethanol.

Δ CRITICAL STEP Do not use 100% isopropanol as more salts will be precipitated.

5. Invert end-to-end to mix well and snap-freeze in liquid nitrogen.
6. Centrifuge at 20,000 ×g for 30 min at 4°C.

Δ CRITICAL STEP Start centrifugation before the mixture is thawed.

7. Discard the supernatant and add 1 ml ice-cold 70% (vol/vol) ethanol in nuclease-free water.
8. Invert end-to-end to mix well and centrifuge at 20,000 ×g for 1 min at 4°C.

Δ CRITICAL STEP Do not vortex to avoid fragmentation of the pellet.

9. Discard ~900 µl of the supernatant and centrifuge at 20,000 ×g for 30 sec at 4°C.

Δ CRITICAL STEP This step is necessary for avoiding ethanol carryover.

10. Discard the rest of the supernatant and air-dry for 3 min at room temperature.

Δ CRITICAL STEP After drying, the centre of the pellet should remain white with the edge being transparent. Over-drying, as indicated by a completely transparent pellet, should be avoided.

11. Add 6 µl nuclease-free water and pipette up-and-down to fully dissolve the pellet.
12. Use 1 µl to measure the DNA concentration with a NanoDrop spectrophotometer. We typically recover ~7 – 8 µg linearized DNA.

■ **PAUSE POINT** Linearized DNA can be stored at -20°C until use.

13. ***In vitro* transcription of capped mRNA (Step 13):** Mix the components tabulated below in a 1.5 ml nuclease-free tube and incubate at 40°C for 4 hr.

Component	Amount per reaction (µl)	Final concentration
Nuclease-free water	19 or 21.5	
~1.2 µg/µl Linearized DNA	2.5 (for mRNA of <6 kb) or 5 (for mRNA of ≥6 kb)	0.05 or 0.1 µg/µl
2X ARCA/NTP mix	30	1X
T7 RNA polymerase mix	6	

Δ CRITICAL STEP We use the HiScribe T7 ARCA mRNA kit (with tailing) for successful transcription of full-length mRNAs of up to 12 kb at high yield. Other *in vitro* transcription kits can also be used, but the ratio of ARCA:GTP:CTP:ATP:UTP should be adjusted from 0.8:0.2:1:1:1 to 4:1:1:1:1.

Δ CRITICAL STEP For constructs with other promoters such as T3 or SP6, corresponding RNA polymerase should be used.

14. **Removal of DNA template (Step 14):** Add 3 µl of 2 U/µl TURBO DNase and incubate at 37°C for 15 min.

Δ CRITICAL STEP Other DNase I can be used as well.

15. **Optional: Poly(A) tailing of mRNA (Steps 15-16):** Mix components tabulated below and incubate at 37°C for 3 min.

Component	Amount per reaction (µl)	Final concentration
Nuclease-free water	192	
Mix from step 13	63	
10X Poly(A) polymerase reaction buffer	30	1X

Δ CRITICAL STEP We generally omit step 15 and 16 when we synthesize mRNAs from pGEMHE-based plasmids, add 237 µl nuclease-free water and directly proceed to step 17. However, poly(A) tailing can be advantageous for assays with a longer time frame (i.e. >24 hr) or for mRNAs transcribed from constructs without a short stretch of poly(A) tail.

16. Add 15 µl of Poly(A) polymerase and incubate at 37°C for 30 min.
17. **Removal of free nucleotides (Step 17):** Purify the mRNA using NucleoSpin RNA Clean-up XS and elute with 160 µl nuclease-free water.

Δ CRITICAL STEP This step can be omitted, but then the final RNA concentration cannot be directly determined with a NanoDrop spectrophotometer due to free nucleotide contamination.

18. **Purification of mRNA (Steps 18-23):** Add 0.5 µl glycogen, 40 µl of 0.25 M sodium acetate-5 mM EDTA-0.5% (wt/vol) SDS and 200 µl phenol-chloroform-IAA (pH 4.0).

Δ CRITICAL STEP Addition of glycogen as co-precipitant is optional, but the yield of precipitated mRNA is higher.

Δ CRITICAL STEP Phenol-chloroform-IAA is saturated and stored with an equilibration buffer. Do not take-in the upper aqueous layer to avoid dilution.

19. Invert end-to-end to mix well and centrifuge at 20,000 ×g for 5 min at room temperature.
20. Transfer ~180 µl of the upper phase to a new 1.5 ml nuclease-free tube and add 360 µl of ice-cold 100% ethanol.

Δ CRITICAL STEP Do not use 100% isopropanol as more salts will be precipitated.

21. Repeat step 5 to 10.
22. Add 11 µl of nuclease-free water and pipette up-and-down to fully dissolve the pellet.
23. Use 1 µl to measure the RNA concentration with a NanoDrop spectrophotometer.

Δ CRITICAL STEP RNA concentration can also be determined with other techniques such as agarose gel electrophoresis and comparison with RNA markers of known concentration or fluorescence-based Qubit RNA assays.

Δ CRITICAL STEP We typically obtain mRNA at ~1 – 3 µg/µl.

■ **PAUSE POINT** mRNA should be divided into 0.6 µl single-use aliquots and can be stored at -80°C as long as no signs of mRNA degradation can be seen.

Box 3 Preparation of TRIM21 stable cell lines (TIMING 2 – 3 weeks)

Though TRIM21 is near-universally expressed, its expression level varies between cell types. As TRIM21 is degraded alongside the antibody and target antigen during Trim-Away, levels of endogenous TRIM21 may be insufficient to elicit complete degradation of target antigen, especially where the target antigen is abundant. This can be remedied by the constitutive expression of TRIM21 stably integrated into the target cell genome. Here we describe a protocol for stable cell line generation by transduction with pseudotyped lentiviral particles. However, alternative approaches for stable cell line generation may also be used.

Reagents

- DMEM (high glucose; GlutaMAX; Gibco, cat. no. 31966)
- Fetal bovine serum (Gibco, cat. no. 10270)
- DPBS (no calcium; no magnesium; Gibco, cat. no. 14190)
- Trypsin-EDTA (0.05% (wt/vol), phenol red; Gibco, cat. no. 25300)
- Opti-MEM I reduced serum medium (Gibco, cat. no. 31985)
- psPAX2 (Addgene plasmid #12260)
- pSMPP-mCherry-hTRIM21 (Addgene plasmid #104972)
- pSMPP-mCherry-mTrim21 (Addgene plasmd #104971)
- pMD2.G (Adddgene plasmid #12259)
- FuGENE 6 transfection reagent (Promega, cat. no. E2691)

- Polybrene (Santa Cruz Biotechnology, cat. no. sc-134220)
- Puromycin dihydrochloride (Gibco, cat. no. A1113803)

Equipment

- Corning tissue-culture treated culture dishes (100 mm × 20 mm; Sigma-Aldrich, cat. no. CLS430167)
- 10 ml syringe (BD Plastipak, cat. no. 302188)
- 33 mm Ezee syringe filters (0.45 µm; PVDF; sterile; Elkay, cat. no. E25-PV45-50S)
- Corning Costar TC-treated multiple well plates (6 wells; clear; polystyrene plate; flat bottom; Sigma-Aldrich, cat. no. CLS3516-50E)
- Corning Costar TC-treated multiple well plates (24 wells; flat bottom; Sigma-Aldrich, cat. no. CLS3527)

Reagent Setup

- **DMEM supplemented with 1X GlutaMAX and 10% (vol/vol) fetal bovine serum** Add 55 ml of fetal bovine serum to 500 ml of DMEM. Store at 4°C until use.

Procedure

! CAUTION Ensure that virus work takes place at an appropriate level of biosafety according to institutional and national regulations.

1. Seed 2.5 x10^6 HEK293T cells in a 10 cm dish containing 10 ml of DMEM supplemented with 1X GlutaMAX and 10% (vol/vol) fetal bovine serum.
2. Incubate the cells for 16 – 24 h at 37°C in a 5% CO_2 atmosphere.
3. In a sterile 1.5 ml reaction tube, prepare 200 µL of Opti-MEM I, 2 µg of HIV GagPol expression plasmid (e.g. psPAX2), 2 µg of TRIM21 construct in lentiviral transfer vector (e.g. pSMPP-mCherry-hTRIM21) and 1 µg of VSV-G glycoprotein expression plasmid (e.g. pMD2.G).
4. Mix the plasmids well by gentle vortexing and bring to the bottom of the tube with a brief spin in a microcentrifuge.
5. Add 12 µl of FuGENE 6 transfection reagent to the plasmids and mix immediately by flicking. Bring liquid to the bottom of the tube with a brief spin in a microcentrifuge.
6. Incubate the transfection mix for 20 min at room temperature.
7. Add the transfection mix dropwise to the centre of the dish of HEK293T cells with gentle swirling.
   Δ CRITICAL STEP Mixing by pipetting or vigorous agitation should be avoided as it may dislodge HEK293T cells from tissue culture dishes.
8. Incubate the cells for 16 – 24 h at 37°C in a 5% CO_2 atmosphere.
9. Gently remove the media and replace with fresh, pre-warmed DMEM supplemented with 1X GlutaMAX and 10% (vol/vol) fetal bovine serum, and return cells to the incubator for a further 48 h.
10. (Optional) Examine the cells under a fluorescence microscope to ensure high levels of mCherry-hTRIM21 expression in virus producing cells resulting from transfection and single cycle re-infection.

11. Harvest the supernatant directly into a 10 ml syringe and filter at 0.45 µm, separate into 1 ml aliquots and store the virus particles at -80°C.

    ■ **PAUSE POINT** Viral particles can be stored indefinitely at -80°C
12. Seed adherent target cells at 1 x10^5 per well in 6 well plates in appropriate medium. Suspension cells can be seeded in 24 well plates in their normal media with polybrene at 10 µg/ml and transduced immediately (step 15).
13. Incubate the cells 16 – 24 h at 37°C in a 5% CO2 atmosphere.
14. Replace the media with fresh media containing 10 µg/ml polybrene (2X of final concentration).
15. Thaw the virus supernatant at room temperature.
16. On the first transduction of a new cell type, or after production of a new batch of virus supernatant, add virus at a range of concentrations to determine the optimal level for low multiplicity transduction (less than 0.1 transducing particles per cell). Prepare a five-fold serial dilution of virus in media such that each well will receive 1, 5, 25, 125, 625 µL of virus. Add virus to cells in a volume equal to the plating media such that final polybrene concentration is 5 µg/ml. Include a control well that does not receive virus.
17. Incubate the cells for 48 h at 37°C in a 5% CO2 atmosphere.
18. Analyse for transgene expression by fluorescence microscopy or flow cytometry. Select conditions where <10% of cells are expressing mCherry-TRIM21 to minimise the number of multiply-transduced cells.
19. Add puromycin to the cells at an appropriate concentration for the cell type, typically 0.5 – 5 µg/ml.
20. After 1 week of selection, all untransduced cells and the untreated control well should be killed. Expand the cell population for freezing and for use in Trim-Away experiments.

    Δ CRITICAL STEP Regularly ensure that the distribution of mCherry-TRIM21 is diffuse and cytoplasmic by fluorescence microscopy (Fig. 4b and 4e). Large crescent-shaped aggregates of mCherry-TRIM21 that are non-functional can form if levels of expression are too high or cells become over-confluent. This seems to be a cell type-specific phenomenon, and occurs in cell types where expression from viral promoters is particularly high, for instance in HEK293 cells. In this case, consider FACS sorting low mCherry-positive cells or deriving clonal lines and selecting low expressing colonies. Alternatively, consider using a tetracycline-inducible promoter to drive TRIM21 expression.

Box 4 Preparation of recombinant TRIM21 protein (3 days)

This Box describes the procedure for expression and purification of full-length human TRIM21 protein His-Lipoyl-hTRIM21 (Fig. 2g). His-Lipoyl-hTRIM21 is expressed from a T7-promoter driven bacterial expression plasmid HLTV-hTRIM21. HLTV-hTRIM21 is available from Addgene (see Reagents) and is a low copy number ampicillin-resistant plasmid with an N-terminal His tag that can be optionally cleaved via TEV protease.

Reagents

- OverExpress C41(DE3) chemically competent cells (Sigma-Aldrich, cat. no. CMC0017)
- HLTV-hTRIM21 (Addgene plasmid #104973)
- LB-Agar Miller (Formedium, cat. no. LMM02)
- 2XTY media (Formedium, cat. no. YDB)
- D-(+)-Glucose (Sigma-Aldrich, cat. no. G8270)
- Ampicillin sodium salt (MELFORD, cat. no. A0104)
- IPTG (MELFORD, cat. no. MB1008)
- Bugbuster plus Benzonase (Merck Millipore, cat. no. 70750-3)
- cOmplete, EDTA-free protease inhibitor cocktail (Sigma-Aldrich, cat. no. 04693132001)
- Trizma base (Sigma-Aldrich, cat. no. T1503)
- Sodium chloride (Sigma-Aldrich, cat. no. S3014)
- Ni-NTA agarose (Qiagen, cat. no. 30210)
- Imidazole (Sigma-Aldrich, cat. no. I5513)

Equipment

- Poly-Prep Chromatography Column (Bio-Rad, cat. no 7311550)
- ÄKTA pure (GE Healthcare Life Sciences)
- HiLoad 26/600 Superdex 200 pg (GE Healthcare Life Sciences, cat. no. 28989336)

Reagent Setup

- **100 mg/ml ampicillin** Add 10 g of ampicillin sodium salt to 100 ml of sterile water. Filter at 0.45 µm. Divide into 10 ml aliquots and store at -20^{o}C until use.
- **LB agar plates supplemented with 100 µg/ml ampicillin** Add 40 g of LB-Agar Miller to 1 L of sterile water. Autoclave at 120 ^{o}C for 15 min. Cool down briefly and add 1 ml of 100 mg/ml ampicillin before pouring plates.
- **2xTY medium** Add 31 g of 2XTY media to 1 L of sterile water. Autoclave at 120°C for 15 min.
- **0.1 M IPTG** Add 2.38 g of IPTG to 100 ml of sterile water. Filter at 0.45 µm. Divide into 10 ml aliquots and store at -20^{o}C until use.
- **10% (wt/vol) glucose** Add 10 g of glucose to 100 ml of sterile water. Autoclave at 120^{o}C for 15 min.
- **1 M Tris (pH 8.0)** Add 12.1 g of trizma base to 90 ml of sterile water. Adjust the pH to 8.0 and top up to 100 ml with sterile water. Store at room temperature until use.
- **5 M NaCl** Add 29.2 g of sodium chloride to 90 ml of sterile water. Top up to 100 ml with sterile water. Store at room temperature until use.
- **PBS (pH 8.0)** Add 5 g of PBS tablet to 450 ml of sterile water. Adjust the pH to 8.0 and top up to 100 ml with sterile water. Store at room temperature until use.
- **3 M Imidazole** Add 102.1 g of imidazole to 450 ml of sterile water. Adjust the pH to 8.0 and top up to 500 ml with sterile water. Store at room temperature until use.
- **Wash buffer** (20 mM Tris (pH 8.0)-200 mM NaCl-10 mM imidazole) Add 1.7 ml of 3 M imidazole, 10 ml of 1 M Tris (pH 8.0) and 20 ml of 5 M NaCl to 468.3 ml of sterile water. Store at room temperature until use.
- **20 mM Tris (pH 8.0)-200 mM NaCl** Add 10 ml of 1 M Tris (pH 8.0) and 20 ml of 5 M NaCl to 470 ml of sterile water. Store at room temperature until use.

Equipment Setup

- **ÄKTA pure** The HiLoad 26/600 Superdex 200 pg column is setup according to manufacturer's instructions

Procedure

1. Freshly transform C41(DE3) cells with HLTV-hTRIM21, plate the transformed cells onto a LB-agar plate supplemented with 100 µg/ml ampicillin overnight at 37°C.
2. Pick colonies and inoculate overnight cultures in 25 mL 2XTY supplemented with 2% (wt/vol) glucose and 100 µg/ml ampicillin. Shake at 220 rpm at 37°C.
3. Inoculate 1 L of 2XTY media with 10 ml of overnight culture, supplement with 0.1% (wt/vol) glucose and 100 µg/ml ampicillin. Shake at 220 rpm at 37°C.
4. At OD_{600} 0.8 – 1.2 (after ~3 h), induce with 0.1 mM IPTG and reduce the temperature to 22°C.
   Δ CRITICAL STEP Induction at ODs greater than 1.2 or overnight growth at higher temperatures will dramatically reduce yields.
5. Next day, harvest the cells in 1 L centrifuge bottles by centrifuging at 5.000 xg for 20 min at 4°C (e.g. using RC3B rotor). Discard the media.
   ■ **PAUSE POINT** Bacterial pellets can be frozen at -20°C, and protein preparation can be continued later.
6. Re-suspend the cells in 20 ml of Bugbuster + ½ crushed complete, EDTA-free protease inhibitor cocktail tablet per centrifuge tube. Add 1 M Tris (pH 8.0) to a final concentration of 20 mM and 5 M NaCl to 200 mM.
7. Keep the cells in 50 ml centrifuge tubes (40 ml per tube) on ice-water. Sonicate a minimum of 2 min with 10 s pulse followed by 10 s rest.
   Δ CRITICAL STEP Sonicated solution should be carefully monitored so that it does not become warm. Successful sonication is indicated by a noticeable reduction in the viscosity of the solution.
8. Spin down in SS34 rotor at 20.000 xg for 40 min at 4°C. Carefully decant the supernatant. Proceed to Step 9 during centrifugation.
9. In the meantime, wash 10 ml of Ni-NTA agarose in 50 ml PBS (pH 8.0). Spin at 2.000 xg for 3 min at room temperature and discard the supernatant. Repeat once.
   Δ CRITICAL STEP Centrifugation of Ni-NTA beads at higher revolutions for longer time periods will compress the resin and reduce its binding capacity.
10. Add Ni-NTA agarose to cleared supernatant from step 8 in centrifuge tubes, supplement with 10 mM imidazole and rotate to allow mixing at 4°C for ~1 h.
11. Spin down the resin at 2.000 xg for 3 min at 4°C and discard the supernatant.
12. Resuspend the resin in 40 ml wash buffer, re-spin and discard the supernatant. Repeat this step once.
13. Decant the washed resin to an empty gravity flow column. Wash the resin in place with 2 bed volumes of wash buffer.
14. Elute the protein from resin with wash buffer supplemented with 300 mM imidazole.
15. Analyse the eluted protein by SDS-PAGE to ensure that the purification has been successful. This should be visible as a single band running at ~66 kDa following Coomassie staining (Figure 2g). If so, proceed to step 16.
16. Apply eluted protein to an S200 gel filtration column equilibrated with 20 mM Tris (pH 8.0)-200mM NaCl. Select the size of the S200 column depending on the volume of eluate and total amount of protein to be purified. Follow the manufacturer's guidelines for column capacity and flow rate.

Δ CRITICAL STEP Eluted protein will not remain stable under high concentration of imidazole for prolonged periods, so it must be immediately buffer exchanged, desalted or gel-filtrated into 20 mM Tris (pH 8.0)-200mM NaCl.

Δ CRITICAL STEP Soluble aggregated materials will elute in the void volume of the column and should be discarded.

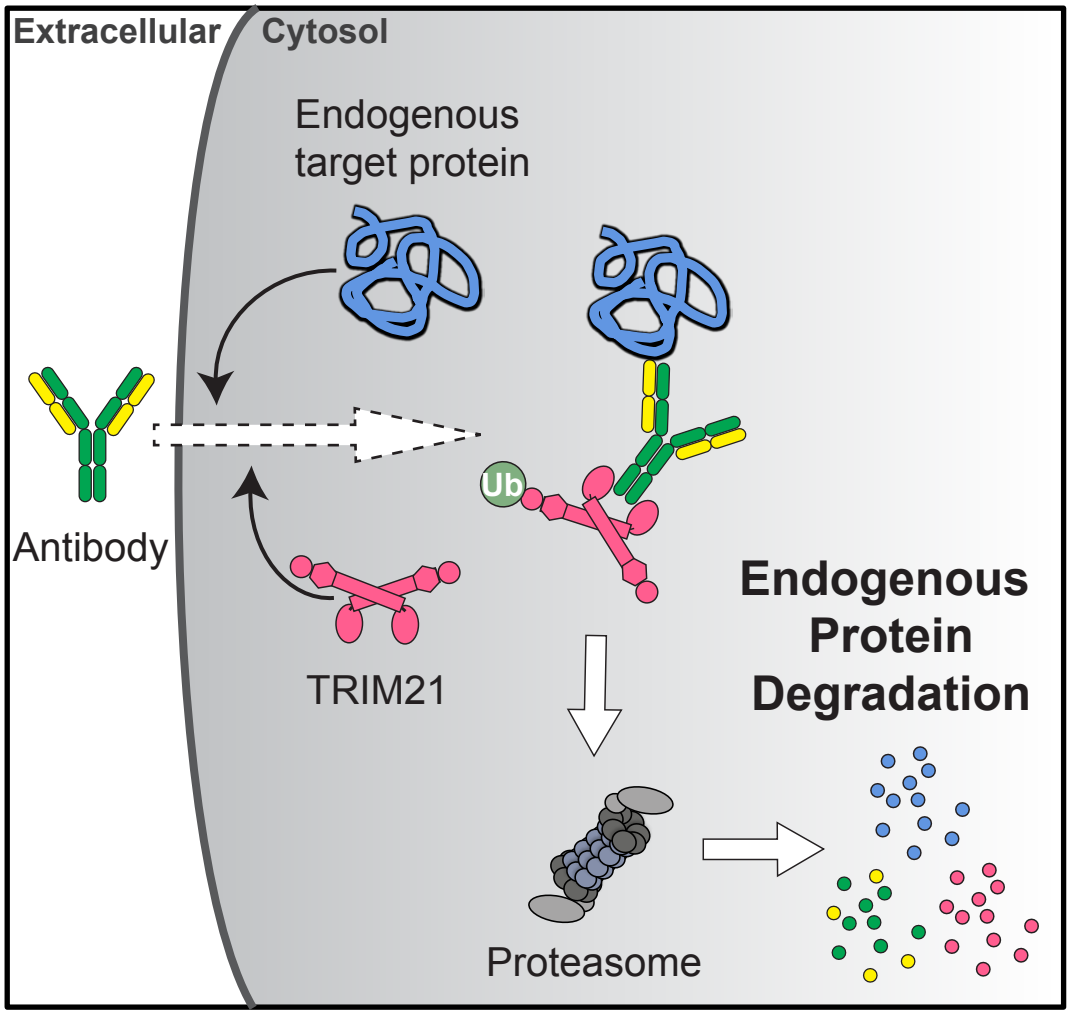
17. Analyse the peak fractions by SDS-PAGE to ensure that the correct peak is collected and to determine which fractions should be combined.

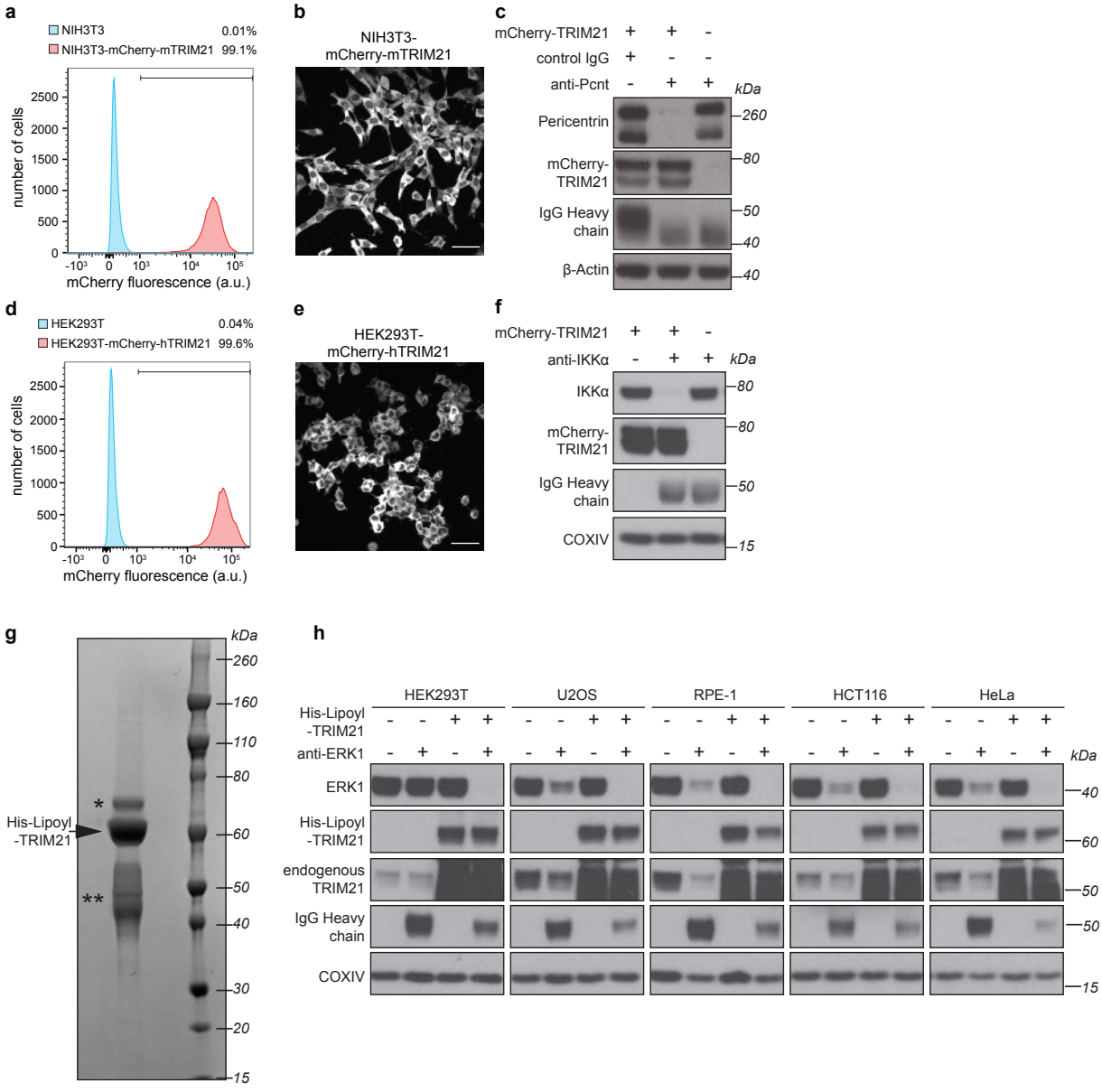
    Δ CRITICAL STEP TRIM21 is an elongated molecule with a large Stokes radius; if molecular weight standards are used to guide fraction collection, then note that TRIM21 will behave as if it is a trimer (i.e. 3X of its expected molecular weight (~165 kDa)) in an S200 gel filtration column.

    Concentrate the combined fractions of TRIM21 using 50 kDa cut-off centrifugal filter units, aliquot them and snap freeze them in liquid nitrogen.

    Δ CRITICAL STEP Protein solution should be carefully monitored during centrifugation and regularly mixed to prevent super-concentration at the cellulose membrane.

    PAUSE POINT The purified protein can be stored at -80^{o}C as long as no signs of protein degradation can be seen.


Extracellular
Cytosol
Endogenous
target protein
Antibody
Ub
TRIM21
Endogenous
Protein
Degradation
Proteasome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b
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mCherry-mTRIM21
c
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control IgG + - -
anti-Pcnt - + +
kDa
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d
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anti-IKKα - + +
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TRIM21
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IgG Heavy
chain
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40
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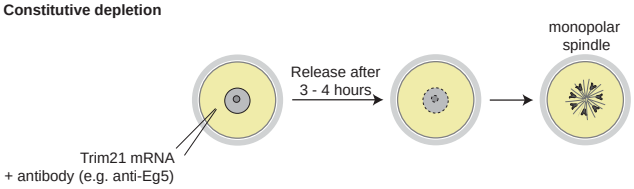
15

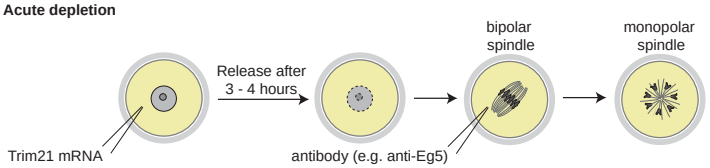
a

Constitutive depletion



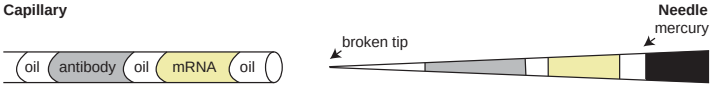
b

Acute depletion



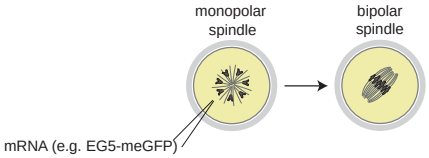
c

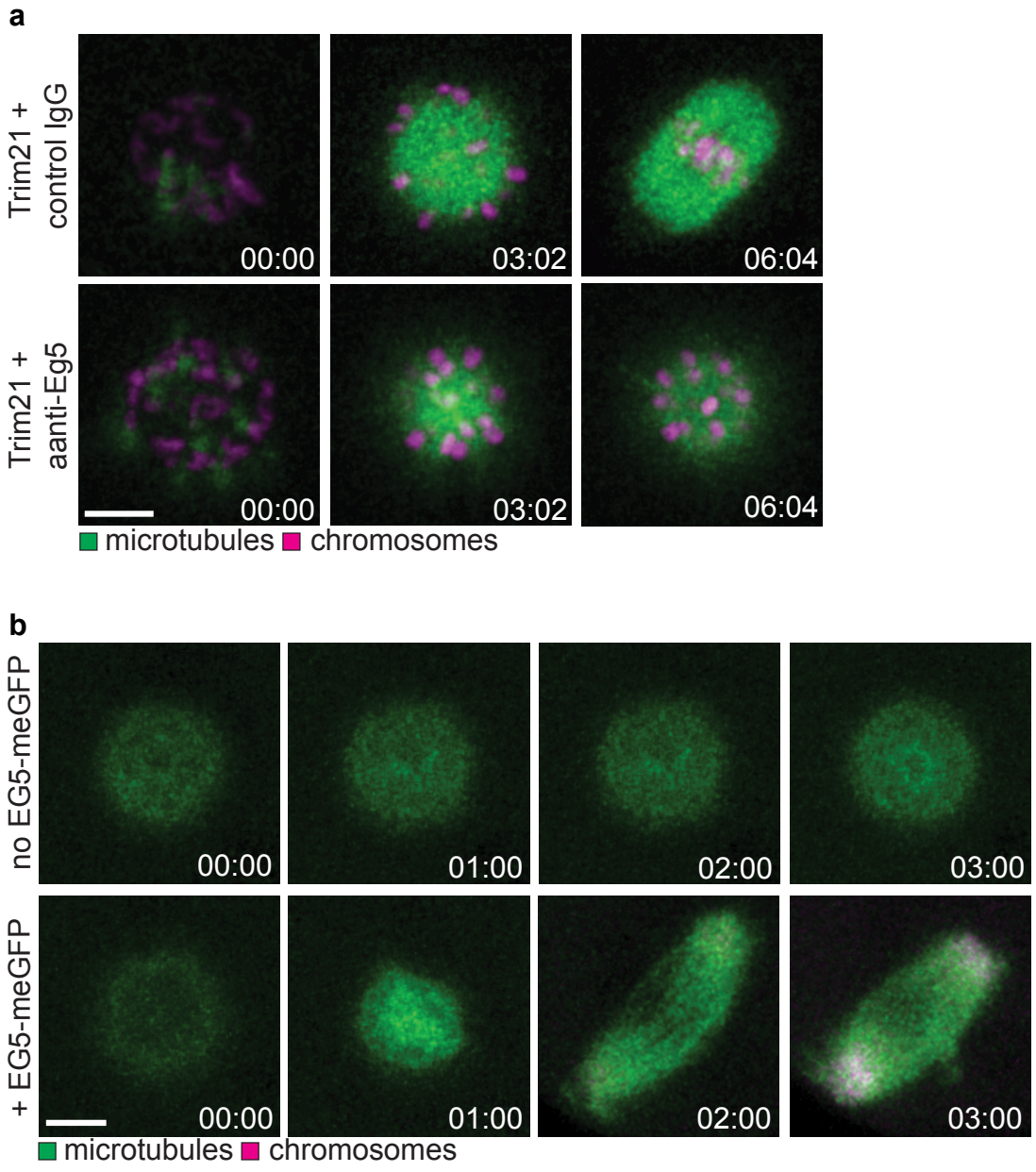
Capillary



d

Rescue

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+ EG5-meGFP
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microtubules chromosomes