Video Article

# Structure-function Studies in Mouse Embryonic Stem Cells Using Recombinase-mediated Cassette Exchange

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## Abstract

Gene engineering in mouse embryos or embryonic stem cells (mESCs) allows for the study of the function of a given protein. Proteins are the workhorses of the cell and often consist of multiple functional domains, which can be influenced by posttranslational modifications. The depletion of the entire protein in conditional or constitutive knock-out (KO) mice does not take into account this functional diversity and regulation. An mESC line and a derived mouse model, in which a docking site for FLPe recombination-mediated cassette exchange (RMCE) was inserted within the ROSA26 (R26) locus, was previously reported. Here, we report on a structure-function approach that allows for molecular dissection of the different functionalities of a multidomain protein. To this end, RMCE-compatible mice must be crossed with KO mice and then RMCE-compatible KO mESCs must be isolated. Next, a panel of putative rescue constructs can be introduced into the R26 locus via RMCE targeting. The candidate rescue cDNAs can be easily inserted between RMCE sites of the targeting vector using recombination cloning. Next, KO mESCs are transfected with the targeting vector in combination with an FLPe recombinase expression plasmid. RMCE reactivates the promoter-less neomycin-resistance gene in the ROSA26 docking sites and allows for the selection of the correct targeting event. In this way, high targeting efficiencies close to 100% are obtained, allowing for insertion of multiple putative rescue constructs in a semi-high throughput manner. Finally, a multitude of R26-driven rescue constructs can be tested for their ability to rescue the phenotype that was observed in parental KO mESCs. We present a proof-of-principle structure-function study in p120 catenin (p120ctn) KO mESCs using endoderm differentiation in embryoid bodies (EBs) as the phenotypic readout. This approach enables the identification of important domains, putative downstream pathways, and disease-relevant point mutations that underlie KO phenotypes for a given protein.

## Video Link

The video component of this article can be found at https://www.jove.com/video/55575/

## Introduction

It is estimated that mammalian genomes contain about 20,000 protein-coding genes. Alternative splicing and posttranslational modifications further increase the protein repertoire. Proteins have a modular structure[1] and often contain multiple interaction domains, which allow their recruitment into different protein complexes and their participation in multiple cellular processes[2]. One example is the multi-functional protein called p120ctn. p120ctn is encoded by the *Ctnnd1* gene and consists of a large central armadillo repeat domain flanked by an N-terminal and a C-terminal region. The armadillo domain of p120ctn binds to a highly conserved juxtamembrane domain of classical cadherins, which are involved in cell-cell adhesion, but it also binds to the transcriptional repressor Kaiso. The N-terminal domain of p120ctn interacts with different kinases, phosphatases, small RhoGTPases, and microtubule-associated proteins[3]. Interestingly, as a result of alternative splicing, p120ctn isoforms can be generated from four alternative start codons[4]. p120ctn isoform 1A is the longest, as it is translated from the most-5' start codon and contains the full-length N-terminal segment. In p120ctn isoforms 3 and 4, this N-terminal segment is deleted partially and completely, respectively. Understanding the precise role of proteins (or protein isoforms) and their domains in different cellular functions remains a challenge.

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Gene targeting in mESCs enables the study of the function of a protein through the genetic deletion of the corresponding gene and has widely contributed to the identification of developmentally important and disease-relevant genes and pathways. This breakthrough in reverse genetics was the result of advances in the fields of mESC isolation and gene targeting due to homologous recombination[5]. Homologous recombination is a process in which DNA fragments are exchanged between two similar or identical nucleic moieties after double-stranded (ds) DNA breaks. Normally, HR is inefficient because dsDNA breaks are infrequent. Recently, the efficiency of homology-directed gene targeting could be increased using site-specific nucleases[6,7], but unfortunately, these are prone to off-target effects[8]. A more reliable technique to enable gene targeting is RMCE, which is based on site-specific recombination systems such as Cre/loxP or FLPe/Frt. LoxP and Frt sequence are found in *bacteriophage P1* and *Saccharomyces cerevisiae*, respectively, and consist of 34 bp, including an asymmetric 8 bp sequence that determines the orientation of the site. On the other hand, the orientation of, for instance, two loxP sites within a DNA stretch will determine whether the floxed DNA becomes excised or inversed upon Cre-mediated recombination[9]. Moreover, Cre can also induce a translocation if two sites are located on different chromosomes. RMCE takes advantage of heterospecific recombination sites that do not cross-react and that are embedded in a genomic locus. In the presence of a donor plasmid that contains a DNA fragment flanked by the same heterospecific sites, the recombinase will insert this DNA fragment into the RMCE-compatible genomic locus because of double-simultaneous translocation (**Figure 1**). Here, only correctly RMCE-targeted clones can render drug resistance thanks to a promoter on the incoming vector that restores a "trapped," promoter-less Neomycin resistance gene ($Neo^R$) present in the R26 genome of the docking cells (**Figure 1**)[10,11]. This results in a very high targeting efficiency, often close to 100%[11,12]. In conclusion, RMCE-based targeting is highly efficient and can be used for structure-functions studies; however, it requires a pre-engineered genomic locus.

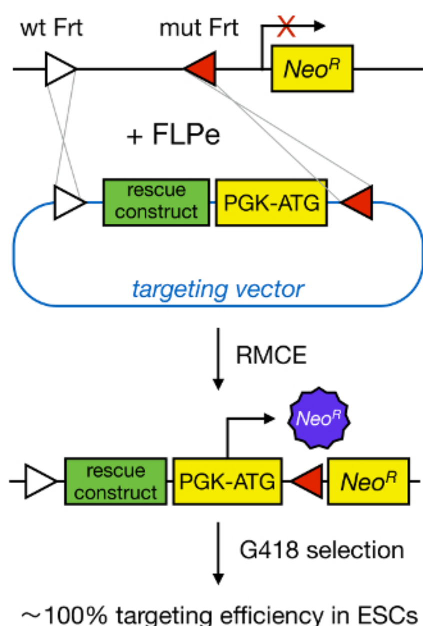


**Figure 1. Schematic Representation of RMCE-mediated Targeting.** RMCE allows for the exchange of DNA segments from an incoming targeting vector to a defined genomic locus if both harbor two heterospecific Frt sites (depicted by white and red triangles). In addition, the engineered genomic locus contains a promoterless and truncated neomycin-resistance ($Neo^R$) gene. By providing a promoter and start codon in the incoming DNA fragment, only correct recombination events restore neomycin resistance, resulting in high targeting efficiencies. Please click here to view a larger version of this figure.

Genome engineering in mESCs allows for the generation of RMCE-compatible mice. In 1981, two groups succeeded in capturing pluripotent cells from the inner cell mass (ICM) of blastocysts and in maintaining them in culture[13,14]. mESCs are capable of self-renewal and differentiation into all types of embryonic and adult cells, including the germ-cell lineage. Therefore, gene targeting in mESCs enables reverse-genetic studies through the development of constitutive or conditional (using the Cre/LoxP system) KO mice. However, the classical way to isolate mouse ES cells is very inefficient. Several major improvements have greatly increased the success rate for deriving mESC lines, including the use of a defined serum-replacement (SR) medium[15], alternating between mESC medium containing SR and fetal bovine serum (FBS)[16], and the use of pharmacological compounds such as pluripotin or 2i[17]. Pluripotin, a small synthetic molecule, allows for the propagation of mESCs in an undifferentiated state in the absence of leukemia inhibitory factor (LIF) and mouse embryonic fibroblasts (MEFs)[18]. Finally, it has been shown that mESCs can be isolated with a very high efficiency (close to 100%) when an SR/FBS medium alternation protocol is combined with LIF and pluripotin[19,20]. These protocols enable the efficient isolation of RMCE-compatible KO mESCs that can subsequently be used for structure-function studies.

This paper describes a method that enables one to identify the key domains or residues within a protein that are responsible for specific cellular processes. To this end, a pipeline of advanced technologies that enable efficient mESC isolation, targeting vector assembly, and mESC targeting was created. As such, large panels with protein isoforms, domain mutants, and downstream effectors can be introduced in KO mESCs and can be evaluated for their ability to rescue the *in vitro* KO phenotype.

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## Protocol

All experiments on mice were conducted according to institutional, national, and European animal regulations.

# 1. Isolation of RMCE-compatible KO mESCs

1. Breed heterozygous KO mice with RMCE-compatible mice, such as ROSALUC mice[10] or ROSA26-iPSC mice[21]. Both RMCE-compatible mice were maintained on a mixed 129/C57BL6/Swiss background.
   NOTE: Crossing with heterozygous KO mice is advised to overcome embryonic lethality in homozygous KO mice.
2. Use PCR to select heterozygous KO mice containing an RMCE cassette in the R26 locus[12].
3. Breed RMCE-compatible, heterozygous KO mice with heterozygous KO mice and isolate RMCE-compatible, homozygous KO blastocysts.
   1. Set up time matings in the evening and check for copulation plugs the next morning.
      NOTE: Plugs are made of coagulated secretions from the coagulating and vesicular glands of the male. These plugs fill the vagina of the female and persist for 8 - 24 h after breeding. Plugged females are considered to be carrying 0.5 dpc (days post coitum) embryos.
      1. To check for plugs, lift the female by the base of her tail and by examine her vaginal opening for a whitish mass. Spread the lips of the vulva slightly with an angled probe when the plug is difficult to see. Separate plugged females from their male.
   2. Collect blastocysts at 3.5 dpc.
      1. Euthanize pregnant females by the approved method (*e.g.,* cervical dislocation). Make a midventral incision and dissect the uterus and oviduct (still attached to each other) using fine scissor and forceps.
      2. Bend a 26-gauge needle into a 45° angle. Attach a 1-mL syringe filled with M2 medium to this bent needle and use it to flush the blastocysts from the uterus into the lid of a 10-cm dish.
         1. Insert the needle into the end of the uterus that is closest to the oviduct. Hold the needle in place with fine forceps while pushing the plunger; swelling of the uterus indicates a successful flushing.
      3. Use a mouth pipette (with a diameter of 100 - 200 µm) to collect all embryos and wash them twice in a drop of fresh M2 medium. Immediately after washing them, transfer the blastocysts to the culture plates (see below).
         NOTE: The dissection and handling of blastocysts of should be done in laminar air flow.
4. Isolate RMCE-compatible KO mESCs
   1. Prepare a 12-well plate with mitomycin-C-treated DR4 MEFs (see the **Table of Materials**) one day before the blastocyst isolation.
      NOTE: These MEFs were isolated from Tg(DR4)1Jae/J mice that contain four drug-selectable genes and confer resistance to neomycin, puromycin, hygromycin, and 6-thioguanine[22].
      1. Coat all culture plates with 0.1% gelatin. Add 0.1% gelatin to the culture plates, incubate for 5 min at 37 °C in 5% $CO_2$, and aspirate the gelatin solution. Seed one quarter of a vial of P2 MEFs in a 12-well plate and grow them in 2 mL of MEF medium (see **Table 1**, the **Table of Materials**) to a confluent monolayer[19].
      2. Inactivate them with mitomycin-C (10 µg/mL) for 3 h and wash them twice with phosphate-buffered saline (PBS)[19].
   2. Using a mouth pipette, plate the blastocysts onto gelatinized 12-well plates (1 well/embryo), with the mitomycin-C-treated MEFs in SR-ES cell medium (2 mL/well) supplemented with either 2 µM pluripotin or with 2i (1 µM Erk inhibitor PD0325901 and 3 µM Gsk3 inhibitor CHIR99021). Incubate at 37 °C in 5% $CO_2$.
   3. Refresh the SR-ES cell medium (supplemented with pluripotin or 2i) every 2 - 3 days.
   4. Examine each blastocyst under a stereomicroscope at 4.0X magnification and check for hatching and attachment to the MEF layer.
      NOTE: When blastocysts hatch, they lose the zona pellucida that encapsulates them. Wells with unattached blastocysts need to be refreshed using the mouth pipetting.
   5. Pick individual ICM outgrowths (using a stereomicroscope) after 10 - 12 days of culture using a P10 pipette with disposable tips. Transfer the outgrowth in approximately 10 µL of medium to a V-shaped, 96-well plate containing 30 µL/well of PBS (at room temperature).
   6. Add 50 µL of 0.25% trypsin to each well using a multichannel pipette and incubate for 3 min at 37 °C in 5% $CO_2$.
   7. Add 100 µL of FBS-containing mESC medium; dissociate the ICM outgrowths into single cells by pipetting 10-15 times; and transfer the dissociated cells to mitomycin-C-treated, 96-well MEF plates that were prepared one day before the ICM colonies are picked.
   8. From this step onwards, omit pluripotin or 2i from the mESC medium. On the next day, change the medium from FBS- to SR-containing mESC medium (100 µL/well).
   9. Expand the established mESC lines from 96- to 24-well format[19].
      1. Wash the cells with 200 µL of PBS, add 50 µL of trypsin, and incubate for 5 min at 37 °C in 5% CO. Add 100 µL of FBS-based mESC medium; dissociate by pipetting 10 - 15 times using a multichannel pipette; and transfer the dissociated cells to mitomycin-C-treated, 24-well MEF plates.
      2. Change to SR-based medium on the next day. Expand the mESCs in a similar fashion from 24- to 6-well format. Make 3 - 4 freezings from a confluent 6-well plate[19].
   10. Identify RMCE-compatible, homozygous KO mESCs using PCR primers for the R26 locus[23] and KO allele of choice (in this case, p120ctn; PCRs for p120ctn null and floxed alleles were described before[12]).

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| *MEF medium* |
| --- |
| Dulbecco's modified Eagle's medium (DMEM) |
| 10% fetal bovine serum (FBS) |
| L-glutamine (2 mM) |
| Sodium pyruvate (0.4 mM) |
| penicillin (100 U/mL) |
| streptomycin (100 µg/mL) |
| ***SR-based mESC medium*** |
| DMEM/F12 |
| 15% serum replacement (SR) |
| L-glutamine (2 mM) |
| non-essential amino acids (0.1 mM) |
| penicillin (100 U/mL) |
| streptomycin (100 µg/mL) |
| β-mercaptoethanol (0.1 mM) |
| recombinant mouse LIF (2,000 U/mL) |
| ***FBS-based mESC medium*** |
| KO DMEM |
| 15% FBS |
| L-glutamine (2 mM) |
| non-essential amino acids (0.1 mM) |
| penicillin (100 U/mL) |
| streptomycin (100 µg/mL) |
| β-mercaptoethanol (0.1 mM) |
| recombinant mouse LIF (2,000 U/mL) |
| ***Differention medium*** |
| Iscove's Modified Dulbecco's Medium (IMDM) |
| 15% FBS |
| 5% CD Hybridoma Medium (1x) liquid |
| L-glutamine (2 mM) |
| 1-thioglycerol (0.4 mM) |
| ascorbic acid (50 µg/mL) |
| penicillin (100 U/mL) |
| streptomycin (100 µg/mL) |

**Table 1. Culture Media.** All media were stored at 4 °C and warmed to 37 °C 30 min before use.

## 2. Generation of an RMCE-compatible Targeting Vector Using Recombination Cloning

1. Clone the rescue constructs into recombination-compatible vectors using restriction enzyme (RE)-based or PCR-based[24] cloning techniques. Make sure that the cDNAs contain a stop codon.
    1. Design AttB-tagged primers[24]. Ensure that the forward primer contains the following elements: a GGGG stretch, an AttB1 site, a linker, a Kozak sequence, and about 25 nucleotides of rescue cDNA (starting with its ATG). Make sure that the reverse primer has a similar composition: a GGGG stretch, an AttB2 site, a linker, and about 25 nucleotides of rescue cDNA (reverse complement).
    2. Amplify the rescue cDNA *via* PCR to obtain AttB-flanked cDNA.
    3. Perform a 10-µL BP reaction with 100 ng of AttB-flanked cDNA and 150 ng of recombination-compatible donor vector, which contains a kanamycin-resistance gene.
    4. Transform 5 µL of the BP mixtures in heat-shock-competent MC1061 *Escherichia coli* (*E. coli*) bacteria (similar to those described in step 2.3).
    5. Identify colonies containing the correct rescue cDNA-containing vectors (similar to those described in step 2.4)

2. Perform a 10-µL LR reaction using 100 ng of rescue cDNA-containing vector; 150 ng of Cre-excised pRMCE-DV1 vector[11] (LMBP 8195); and 2 µL of recombinase mix, which contains a phage-encoded integrase and excisionase and a bacterial integration host factor. Incubate for 2 h at 25 °C.
NOTE: An LR reaction is a recombination reaction in which an entry clone containing attL sites and a destination vector containing attR sites are recombined by the LR clonase enzyme mix. This results in an expression clone containing attB sites flanking the gene of interest.
3. Transform 5 µL of the LR mixtures in heat-shock-competent MC1061 *E. coli* bacteria.
    1. Add 5 µL of LR mixtures to a ribbed, skirted, 2-mL screw-cap tube with 40 µL of heat-shock-competent *E. coli* bacteria and incubate for 20 min on ice. Incubate for 5 min at 37 °C.
    2. Add 1 mL of Luria broth (LB) medium and incubate for 1 h at 37 °C. Plate 50 µL on ampicillin (Amp; 100 µg/mL)-containing agar plates and grow overnight at 37 °C.

4. Identify the colonies with the correct targeting vector.
    1. Pick 5 colonies randomly using a p200 tip. Transfer the tip to a glass test tube containing 2 - 5 mL of LB medium and grow overnight at 37 °C.
    2. Extract the plasmid DNA from the bacterial cultures using commercially available kits.
    3. Validate them using RE digests and sequencing. Cut 0.5 - 2 µg of plasmid using 0.2 µL of RE (20 U/µL) and 1 µL of the corresponding 10x buffer in a 10-µL reaction. Incubate for at least 1 h at 37 °C and separate on a 1% agarose gel. Select for colonies with the predicted pattern of DNA fragments.

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1. Analyze the confirmed vectors (50 ng/µL) with Tlox F (ATC ATG TCT GGA TCC CCA TC) and IRES R (GGG GCG GAA TTC GAT ATC AAG) primers (5 pmol/µL) using Sanger sequencing.

## 3. RMCE-mediated mESC Targeting of Rescue Constructs to the R26 Locus

1. Start a culture of RMCE-compatible KO mESCs and passage them at least twice on MEFs in FBS-based mESC medium. Split the mESCs on a gelatinized 6-well plate.
2. On the next day, refresh the cells, at about 50% confluency, with 1.5 mL of FBS-based mESC medium and transfect the mESCs with a Cre-excised pRMCE-DV1 targeting vector containing rescue cDNA.
    1. Make a DNA mix. Add 1 µg of targeting vector and 1 µg of FLPe-expression plasmid[25] to 250 µL of pure DMEM medium.
    2. Make a lipofection mix. Add 7 µL of lipofection-based transfection reagent (*e.g.,* Lipofectamine 2000) to 250 µL of pure DMEM medium and incubate for 5 min at room temperature (RT).
    NOTE: Similar RMCE targeting efficiencies were obtained using other lipofection-based reagents (*e.g.,* Lipofectamine LTX and Effectene).
    3. Mix the DNA mix with the lipofection mix and incubate for 20 min at RT. Pipette the transfection mixture onto the refreshed mESCs. Swirl gently and leave overnight.
3. One day after transfection, split all mESCs from the 6-well plate to a 10-cm culture dish with a confluent layer of DR4 MEFs and 10 mL of FBS-based mESC medium.
4. Two days after transfection, select mESC clones with the correct FLPe-mediated cassette exchange by adding 0.2 mg/mL G418 (100x) to the medium.
NOTE: Make a kill curve for each batch of G418 to identify the lowest concentration of G418 that kills all mESCs.
5. Refresh the mESCs daily with G418-containing mESC medium. Colonies should appear after 7 - 10 days, so pick and expand these as per step 1.4.
6. Confirm the correct clones *via* PCR[11].

## 4. Differentiation of mESCs in Embryoid Bodies (EBs)

1. Start a culture of KO mESCs with R26-driven rescue constructs and passage them at least twice on MEFs in FBS-based mESC medium. Passage the mESCs once on gelatinized 6-well plates to get rid of the MEFs.
2. Wash with PBS and trypsinize the nearly-confluent mESC cultures. Plate dissociated mESCs in different dilutions (1/20 and 1/40) onto non-adherent, bacterial-grade petri-dishes in differentiation medium.
3. Allow EBs to form in these dishes for 30 days. Refresh the medium every 2 - 3 days using the following procedure: transfer the EB suspension to a 50-mL tube, let the EBs settle by gravity, remove the supernatant, add fresh medium, and transfer the EB suspension back to a bacterial-grade dish.
4. Analyze the targeted mESCs and EBs by immunofluorescence and transmission electron microscopy using protocols that were described previously[12].

## Representative Results

The procedure to isolate RMCE-compatible KO mESC lines is depicted in **Figure 2**. Two consecutive breedings are required to obtain RMCE-compatible KO blastocysts. First, heterozygous KO mice are crossed with RMCE-compatible mice to obtain RMCE-compatible, heterozygous KO mice. These mice are then used for timed matings with other heterozygous KO mice to obtain 3.5-dpc, RMCE-compatible, homozygous KO blastocysts. The chance of obtaining such an embryo is one in eight, as predicted by Mendelian inheritance. Therefore, an efficient mESC isolation protocol is required. Using pluripotin-based or 2i-based protocols, we are able to isolate mESCs lines, including RMCE-compatible p120ctn KO mESCs, with an efficiency of about 94% (n = 114 blastocysts) and 64% (n = 22 blastocysts), respectively[12,19]. Typically, blastocysts hatch after between 3 and 6 days in *in vitro* (DIV) culture; this is followed by the attachment of ICM outgrowths to MEFs or to gelatin-coated dishes. On day 12, large ICM outgrowths are formed that faithfully give rise to mESC lines (**Figure 2**). Due to this highly efficient mESC isolation procedure, it is feasible to obtain RMCE-compatible KO mESCs from one or two plugged females.

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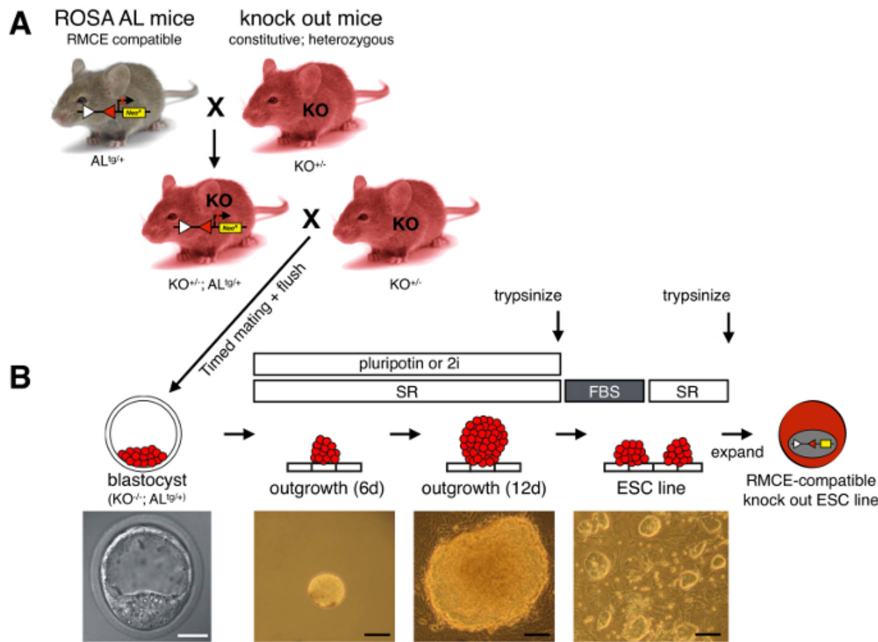


**Figure 2. Isolation of RMCE-compatible KO mESCs. (A)** Breeding strategy to obtain homozygous, RMCE-compatible KO mESCs. **(B)** Scheme of pluripotin- or 2i-based mESC isolation procedure. White scale bar: 25 µm. Black scale bars: 100 µm. Please click here to view a larger version of this figure.

To map the domains or amino acids within a protein that are important for specific cellular functions, one can now reintroduce, via RMCE, full-length or mutant constructs in KO mESCs and test them for their ability to revert the KO phenotype. Targeting vectors for many different domain mutants and point mutants can be efficiently generated by recombinational cloning (**Figure 3A**). Recombinational cloning is based on the recognition of specific attachment (Att) DNA sequences by the recombinase mix, which mediates an exchange of Att-flanked DNA fragments between different plasmids. The recombinational cloning system selects only correctly recombined vectors by switching between vectors that contain different antibiotic-resistance genes and by inserting a counter-selectable marker, the "control of cell death B" (ccdB) gene, into the destination vector (**Figure 3A**). We have generated over 25 Cre-excised pRMCE-DV1 targeting vectors with a near 100% efficiency (some examples are shown in **Table 2**).

| RMCE targeted mESC lines | cDNAs | Efficiency of targeting vector assembly (# of colonies picked) | # of mESC colonies picked | Targeting efficiency |
|---|---|---|---|---|
| R_p120_1A_CAAX | hp120ctn1A_CAAX | 100 % (3) | 3 | 100% |
| R_mSnail | mSnail | 100 % (5) | 12 | 100% |
| R_ZEB1 | hZEB1 | 100 % (5) | 18 | 94% |
| R_ZEB2 | hZEB2 | 100 % (5) | 18 | 94% |
| R_N-cadherin | N-cadherin | 100 % (3) | 8 | 100% |
| Total | | | 59 | 97% |

**Table 2. Overview of Targeting Vector Assembly and RMCE-mediated mESC Targeting.**

Different rescue constructs embedded in a cre-excised pRMCE-DV1 targeting vector can easily be targeted to KO mESCs using RMCE (**Figure 3B**). We previously reported on the generation of 133 targeted mESC clones for 19 different rescue constructs that were introduced into the R26 locus of p120ctn KO mESCs *via* RMCE with an average efficiency of 93%[12]. Here, we report on the targeting of additional constructs with similar high efficiencies, including a CAAX-tagged p120ctn 1A, epithelial-to-mesenchymal transition (EMT) inducers of the SNAI and ZEB family, and N-cadherin, in p120ctn KO mESCs (**Table 2**).

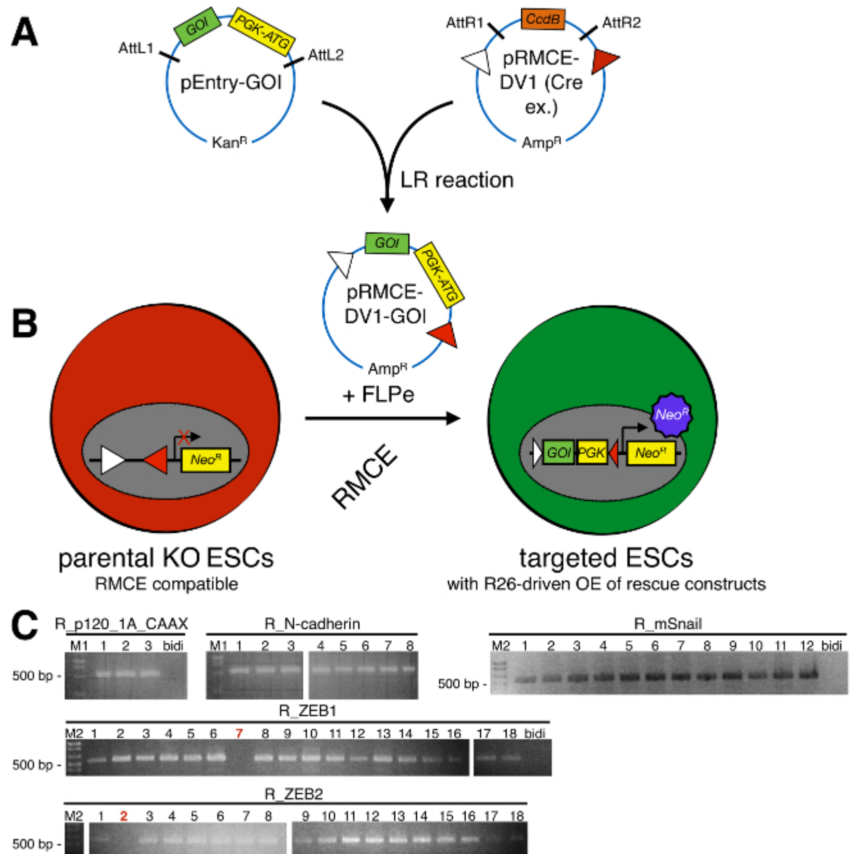


**Figure 3. RMCE-based Targeting in KO mESCs. (A)** Recombinase-mediated assembly of RMCE-compatible targeting vectors. **(B)** Targeting of putative rescue constructs to the R26 locus of homozygous KO mESCs via RMCE. **(C)** PCR-based validation of RMCE-targeted mESC clones. DNA was extracted from RMCE-targeted mESC clones and was amplified by PCR using a forward primer that is embedded in the endogenous R26 locus and a reverse primer that is located in the incoming pRMCE-DV1 construct. A 560-bp band is observed for correctly targeted clones. Unconfirmed clones are shown in red. M1: 1 kb DNA ladder. M2: 1 kb Plus DNA ladder. Please click here to view a larger version of this figure.

Once a panel of rescue mESCs is obtained, it needs to be screened for its ability to reverse the KO-induced phenotype. It was recently shown that p120ctn KO mESCs fail to differentiate into cystic EBs as a consequence of defective endoderm polarization[12]. Cystic EB morphology can be used as a phenotypic readout to screen different rescue constructs (**Figure 4A**). As proof of concept, we showed that R26-driven p120ctn isoform 1A (R_p120_1A) could rescue the p120ctn KO phenotype (**Figure 4B, 4C**)[12]. In addition, this screen-identified E-cadherin, but not RhoA, is a crucial partner of p120ctn during endoderm specification (**Figure 4B and C**)[12]. The ease of introducing rescue constructs to p120ctn KO mESCs allowed us to test additional hypotheses. First, would E-cadherin-independent membrane anchoring of p120ctn enable cystic EB formation? To test this, we fused the K-Ras membrane-targeting motif (CAAX) to the carboxy terminus of p120ctn, introduced it via RMCE to p120ctn KO mESCs, and made EBs from them. However, this construct serves a dominant negative that does not allow binding and stabilization of E-cadherin (**Figure 4D**) and, as a consequence, does not rescue the p120ctn KO phenotype (**Figure 4C**). Second, are inducers of EMT involved? EMT is an essential developmental process that also occurs during mESC differentiation and is orchestrated by the SNAI and ZEB family of transcription factors, which directly repress various epithelial marker genes like E-cadherin[26,27]. In line with these findings, the expression of EMT inducer ZEB2 was found in control EBs, but not in p120ctn-null EBs (**Figure 4E**). Nonetheless, p120ctn KO mESCs with the ROSA26-based expression of EMT inducers ZEB1, ZEB2, or Snail failed to restore cystic EB formation (**Figure 4C**). Third, can other classical cadherins, such as N-cadherin, functionally replace E-cadherin during endoderm specification? To address this, N-cadherin rescue lines were generated. Previously, it was shown that the ectopic expression of E-cadherin in p120ctn KO EBs partially rescued the formation of cystic EBs[12]. Interestingly, in a similar setup, forced N-cadherin expression failed to rescue (**Figure 4C and F**), indicating that E-cadherin is the major cadherin subtype required for EB adhesion and cannot be replaced by N-cadherin. These abovementioned examples highlight the ease with which biological questions can be addressed by the system.

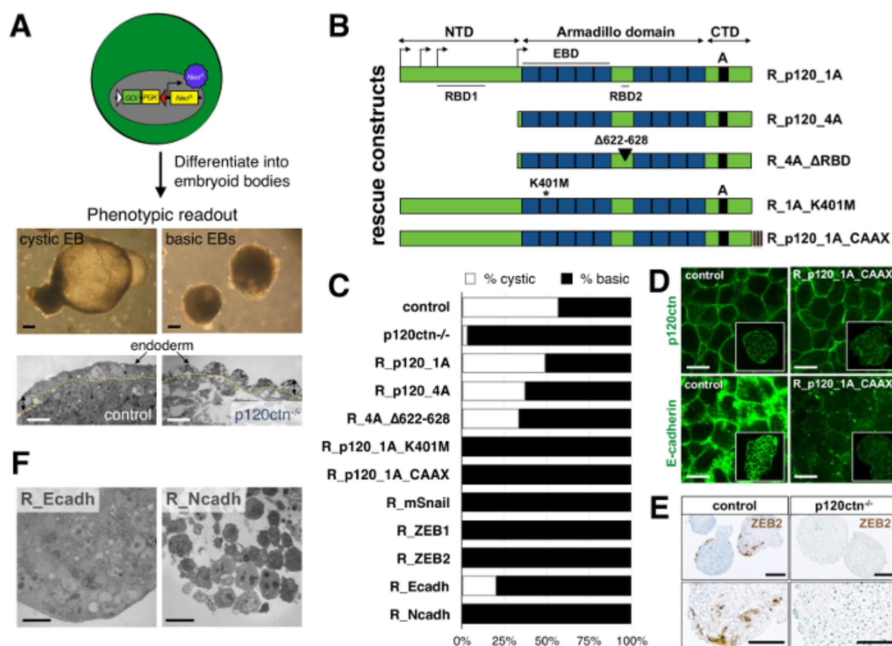


**Figure 4. Phenotypic Screening of RMCE-targeted Rescue mESCs. (A)** The formation of cystic EBs (captured by light microscopy; top panels) and proper endoderm polarization in EBs (captured by transmission electron microscopy; bottom panels) were used as the phenotypic readout to test the ability of targeted mESCs to rescue the p120ctn KO phenotype. White scale bar: 25 µm. Black scale bar: 200 µm. **(B)** Overview of p120ctn rescue constructs. p120ctn contains a central armadillo domain, consisting of nine armadillo repeats (blue boxes), that is flanked by a N-terminal domain (NTD) and C-terminal domain (CTD). p120ctn isoforms contain one out of four alternative start codons (arrows), possibly feature sequences encoded by alternatively used exons A and C (black boxes), and include or exclude two Rho GTPase binding domains (RBDs). p120ctn isoforms 1A and 4A use the first and fourth translation initiation site, respectively. The position of mutations of important AA and the artificial addition of a membrane-targeting motif (CAAX) are shown. EBD: E-cadherin binding domain. **(C)** Graph depicting the percentage of basic or cystic EB morphology in p120ctn KO cells that express different R26-driven rescue constructs. **(D)** Immunofluorescence staining for p120ctn (mouse monoclonal anti-p120ctn) and E-cadherin (mouse monoclonal anti-E-cadherin) on control G4 mESCs or on p120ctn KO mESCs with the R26-driven expression of CAAX-tagged p120ctn isoform 1A. The insets show the entire mESC colony. Scale bar: 10 µm. **(E)** Immunohistochemistry for ZEB2 (mouse monoclonal anti-ZEB2; 7F7; made in-house) on floxed (control) and p120ctn KO EBs. A 3.5-fold magnification is shown in the bottom panels. Scale bar: 200 µm (top), 100 µm (bottom). **(F)** Transmission electron microscopy of DIV12 EBs from p120ctn KO cells expressing E- or N-cadherin from the R26 promoter. Scale bar: 10 µm. Please click here to view a larger version of this figure.

To conclude, we report on a pipeline of robust technologies that combines a near-100% efficiency in mESC isolation with targeting vector assembly and mESC targeting. This pipeline enables us to unravel the versatility of proteins by means of structure-function studies in mESCs.

## Discussion

Our mESC isolation method is user-friendly and does not require advanced skills or equipment, such as microsurgery of blastocysts. Thus, this technology is accessible to a large proportion of the scientific community. Anyone with basic cell culture experience can propagate ICM outgrowths and establish mESCs lines. However, the flushing and handling of blastocysts requires some practice. A mouth pipette is used to transfer blastocysts and consists of a micropipette, a micropipette holder, tubing, and an aspirator mouthpiece[28]. Micropipettes can be custom-made by heating the finest part of a Pasteur pipette for a few seconds, removing the pipette from the flame, and pulling both ends. Select needles with a diameter slightly larger than a blastocyst (100 - 200 µm). Needles can be reused after being washed with distilled water (3x) and EtOH (3x). In our experience, the efficiency of pluripotin-based mESC isolation is higher compared to 2i-based protocols[12]. However, the use of pluripotin at high concentrations has been shown to induce genomic instability[19].

RMCE-based R26-targeting in mESCs typically yields 20 - 40 colonies after G418 selection. If more colonies are observed, this implies that the G418 concentration is not optimal and allows non-targeted mESCs to survive, affecting the overall targeting efficiency. Therefore, it is advised that, for each mESC line and for each new G418 batch, a kill curve is made to identify the lowest G418 concentration that kills all non-targeted mESCs. If no colonies are observed, this is indicative of an inefficient transfection. In that case, it is worthwhile to optimize the transfection procedure using reporter plasmids that allow the expression of EGFP or LacZ. If the transfection protocol works but still no colonies are observed, check the RMCE-compatible and cre-excised pRMCE-DV1 vector and the FLPe expression plasmid with RE digest and sequencing. It is advisable to make a big batch of parental mESCs and FLPe vector and to freeze several aliquots of them to reduce variation between experiments.

One drawback of this structure-function strategy is that the rescue constructs are not expressed from the endogenous locus, but rather from within the ROSA26 locus, with a ubiquitous, moderately active promoter. Compared to other technologies, where supraphysiological overexpression levels are often seen, this system enables physiological transgene expression, which is stable during *in vitro* differentiation

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experiments and across various cell lineages. It was observed that heterozygote, ROSA26-mediated transgene expression results in a p120ctn protein expression that was about half of the endogenous p120ctn levels of wildtype control mESCs but that was sufficient to rescue the observed p120ctn KO phenotypes[12]. Similarly, it was demonstrated previously that a heterozygote, ROSA26-mediated Zeb2 transgene expression level is sufficient to rescue the Zeb2 knockout phenotypes observed *in vitro* and *in vivo* in various cell lineages[29]. Another potential pitfall is the fact that genes that are indispensable for mESC self-renewal are not amenable for such structure-function studies. To exclude this, it is advised to characterize newly established KO mESCs for the following criteria: proliferation, colony morphology, and expression of stemness genes. It is also recommended to check the expression of the gene of interest, both in mESCs and in lineage-committed cells.

In addition to performing structure-function studies in constitutive KO mESCs, one could also take a conditional approach using the Cre/loxP system. The latter approach allows a lineage-specific KO without affecting the surrounding and supporting cells. In addition, conditional KO mESCs allow for the performance of structure-function studies if there is a phenotype in the constitutive KO mESCs. To generate RMCE-compatible, conditional-KO mESCs, homozygous floxed mice with a cell-type-specific Cre driver are bred with RMCE-compatible mice; mESC are isolated from their progeny. The advantage of this system is that the Cre recombinase is not expressed in mESCs and only becomes active when the mESCs are differentiated in the respective cell lineage. In RMCE-compatible, conditional-KO mESCs, Cre recombination will induce the simultaneous inactivation of the endogenous gene and the R26-driven expression of the rescue construct by using a conditional pRMCE-DV1 targeting vector (LMBP 08870)[11].

In addition, the structure-function system allows for the study of mutations that are found in human disease and enables us to test the impact of these mutations on the functions of the protein. As an example, we have identified ZEB2 mutations in patients with myeloproliferative neoplasm, made targeting vectors containing these ZEB2 mutants, shuttled them to the R26-locus of Zeb2 KO mESCs via RMCE, and examined the effect of the mutations on the stability of the protein itself[30]. This technology gives a greater understanding of the biochemistry of such mutants in mESCs, as well in any given lineage, since mESCs are pluripotent.

In conclusion, this mESC-based structure-function approach relies on efficient mESC isolation and targeting technology and allows for the identification of important domains and functions of a given protein within a defined biological context. By sharing mice (frozen sperm is available upon request) and plasmids (distributed from BCCM/LMBP and Addgene), we want to open this technology to the entire scientific community.

## Disclosures

The authors have nothing to disclose.

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