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Genome Wide Conditional Mouse Knockout Resources

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Abstract

The International Knockout Mouse Consortium (IKMC) developed high throughput gene trapping and gene targeting pipelines that produced mostly conditional mutations of more than 18,500 genes in C57BL/6N mouse embryonic stem (ES) cells which have been archived and are freely available to the research community as a frozen resource. From this unprecedented resource more than 6,000 mutant mouse strains have been produced by the IKMC and mostly the International Mouse Phenotyping Consortium (IMPC). In addition, a cre-driver resource was established including 250 inducible cre-driver mouse strains in a C57BL/6 background. Complementing the cre-driver resource, a collection of comprising 27 cre-driver rAAVs has also been produced. The resources can be easily accessed at the IKMC/IMPC web portal (www.mousephenotype.org). The IKMC/IMPC resource is a standardized reference library of mouse models with defined genetic backgrounds that enables the analysis of gene-disease associations in mice of different genetic makeup and should therefore have a major impact on biomedical research.

Introduction

Comparative analyses of mouse and human genomes have provided in depth knowledge on gene and genome organization and, in combination with disease specific genetic alterations discovered by genome wide association studies (GWAS), exome sequencing and entire genome sequence, paved the way for generating mouse models of human disease. As mice and humans share many physiological and pathological features and have similar genome structures mouse models are favorite tools for the functional annotation of the human genome. In most cases disease underlying genetic alterations are missense mutations, splice site mutations or deletions. Corresponding mutations can now be easily copied into the mouse genome to study their molecular and phenotypic consequences. However, functional gene annotation requires a null mutation first to reveal the phenotypic consequences of a complete loss of function in the context of an entire organism. On top of this, as many common diseases occur later in life due to localized tissue specific gene dysfunctions and about one third of null mutations result in embryonic lethality conditional mutations enabling spatially and temporally restricted gene inactivation are essential to fully understand gene function and it impact on human disease. To address

Conflict of interest:

EMS, and the University of British Columbia have filed for US patent protection on a subset of the MiniPs used in cre-driver strains developed by Pleiades/CanEuCre.

this challenge, a comprehensive international project aimed at inactivating and archiving all 20,000 protein coding genes in ES cells was launched in 2003 [1,2]. Up to now, the International Knockout Mouse Consortium (IKMC, www.knockoutmouse.org) comprising the European Conditional Mouse Mutagenesis Program (EUCOMM), the Knockout Mouse Project (KOMP), the North American Conditional Mouse Mutagenesis Project (NorCOMM) and the Texas A&M Institute for Genomic Medicine (TIGM) [1–4] has created a repository of mutant ES cells, most of them conditionals in a C57BL/6N inbred background for nearly 18,500 genes using a combination of gene trapping and targeting complemented more recently by designer endonucleases [5–12]. The ES cell-based mutations are typically converted into mice by blastocyst injection and phenotypic alterations analyzed in heterozygous or homozygous offspring [13–15]. In parallel, the International Mouse Phenotyping Consortium (IMPC, see www.mousephenotype.org) was established to produce mutant mice from the IKMC ES cell resource and lately by direct nuclease mediated mutagenesis in zygotes for large scale primary phenotyping. Thus far, for the systematic analysis of gene function, the establishment of a large collection of mouse mutants by high-throughput reverse genetics turned out to be the most productive [14–16].

High throughput knockout resources

Because at the time when the IKMC was established it was uncertain whether gene targeting by homologous recombination could be successfully up-scaled, gene targeting and trapping were performed in parallel. The ease of establishing mutant gene trap libraries paved the way for the generation of a large collection of gene trapped ES cell lines carrying mutations in most protein-coding genes expressed in ES cells. Gene trapping introduces random insertional mutations throughout the genome and is performed with either viral or plasmid gene trap vectors [6–10]. Their integration into an intron or exon simultaneously disrupts and reports the expression of a trapped gene [17] and provides a molecular tag enabling easy identification of the disrupted gene by various PCR strategies [18,19]. By employing the Flip-Excision (FlEx) system classic gene trap vectors were converted into conditional vectors enabling the induction of spatially and temporary restricted mutations by crossing the mutant strains to Flpe and/or Cre site-specific recombinase expressing deleter mice [20].

Figure 1 shows the FlipRosabgeo conditional gene trap vector used by the IKMC. It contains a typical gene trap cassette (splice acceptor (SA)-reporter (bgeo) –polyadenylation sequence (polyA) flanked by heterotypic flpe and cre recombinase target sites that enable directional cassette inversions by the corresponding flpe and cre recombinases. Thus, gene trap mutations can be repaired and re-induced by the successive delivery of flpe and cre recombinases [21,22]. Gene trap mutagenesis can be easily scaled-up so that thousands of mutations can be generated rapidly in one experiment. However, gene trap mutagenesis is generally biased towards expressed genes and genes with longer reading frames [23]. While targeted mutagenesis does not have these biases and is definitely more precise, in some cases involving the disruption of multiple isoforms, conditional gene trapping proved superior [21]. Collectively, the IKMC has generated gene trap mutations in more than 11.000 ES cell genes (Table 1, www.genetrap.org, www.informatics.jax.org/allele). As there is some overlap within the IKMC gene trap resource allelic variants can be established. Moreover,

about 4.600 mutations are also available in 129Sv ES cells allowing the comparative analysis of the same mutation in different genetic backgrounds.

Unlike gene trapping, gene targeting by homologous recombination in ES cells is a precise technology enabling the induction any desired mutation ranging from single base-pair substitutions to large deletions [5]. However, in contrast to random gene trapping, gene targeting requires targeting vectors for each individual gene and screening for rare homologous recombinants which is laborious and time consuming. Nonetheless, the IKMC succeeded to develop a high throughput gene targeting strategy by combining serial BAC recombineering for vector generation with high throughput gene targeting in C57BL6/N ES cells [24–29]. Most importantly, IKMC targeting vectors induce reporter tagged conditional ready null mutations which like the conditional gene trap vectors enable spatial and temporal control of gene activity which is essential for distinguishing between pleiotropic gene functions during embryogenesis and adulthood [27,29]. IKMC's most commonly employed targeting vector is the knock-out first conditional ready vector [29,30]. It consists of homology arms embedding a gene trap (SA-bgal-pA) and selectable marker gene (pgk-neo-pA) cassette flanked by frt sites and a critical exon flanked by loxP sites whose deletion causes a frameshift mutation (tm1a allele; Figure 2). Following homologous recombination, the reporter is spliced into the endogenous transcript resulting in a fusion transcript from which a truncated endogenous protein fused to the reporter is translated (tm1a). Removal of the gene trap and selectable marker cassettes with FLPe converts the knockout-first tm1a allele into a conditional allele (tm1c) (Figure 2). Subsequent deletion of the critical exon from the tm1c allele induces a null mutation by frameshift-mediated nonsense-mediated mRNA decay (tm1d). With these "knockout first conditional ready" targeting vectors the IKMC generated about 13.000 conditional gene mutations in ES cells (Table 2). While most of the conditional targeting vectors successfully mutated the respective genes, a set of about 1,800 genes were not amenable to targeting even after several attempts, possibly due non-permissive chromatin conformation. These genes were pursued further within the framework of EUCOMMTOOLS (a successor project of EUCOMM) by employing CRISPR/Cas9 genome editing technology. We could demonstrate that Cas9-assisted targeting combined with the IKMC conditional targeting vectors resource successfully mutated most genes previously inaccessible to conventional targeting [31]. To accelerate the adoption of Cas9 assisted conditional mutagenesis, we implemented a new search function within the EUMMCR website (www.eummcr.org) enabling direct access to Cas9-amenable vectors and matching sgRNAs (www.eummcr.org/crispr/search). Most of the 13,000 conditional targeting vectors of the IKMC resource have suitably located protospacer (PAM) sequences for Cas9-assisted targeting, suggesting that the conditional mutant resource can now be readily completed [31]. Furthermore, more than 6,000 mutant mouse strains generated from the IKMC ES cell resource by the IKMC and IMPC and their primary phenotypes are available at www.mousephenotype.org [14–16]; see also article in this issue).

Cre driver transgenic mouse strains

To take full advantage of the IKMC conditional loxP alleles, a complementary resource of inducible cre recombinase expressing transgenic mouse strains (cre-drivers) is required [32]. Ideally, cre expressing transgenic mouse strains should be at hand for every adult cell type

in order to dissect pleiotropic gene functions related to human disease. To achieve spatial control of cre expression either entire bacterial artificial chromosomes [33], or cre knock-ins into the endogenous loci [34] were used to provide highest fidelity of cre expression.

About 30% of all homozygous null mutations lead to embryonic lethality and therefore temporal control of gene inactivation is required for the analysis of postnatal gene function. Temporal control of cre activity is achieved by employing fusion proteins of cre with modified versions of the ligand binding estrogen receptor domain (cre/ER or cre/ERT2) which also binds the synthetic estrogen receptor antagonist -tamoxifen [35–37]. Without ligand cre/ER or cre/ERT2 are sequestered in the cytoplasm in an inactive state. After ligand binding following exposure to tamoxifen cre/ER translocates to the nucleus and excises floxed sequences from the genome. There are various sophisticated approaches available for generating Cre-driver mice; all of which have advantages and disadvantages. EUCOMMTOOLS exploited a knock-in strategy based on past experience with large-scale vector production and on the existing IKMC vector resource as well as previously characterized cre-knock-ins faithfully mirroring endogenous gene expression.

EUCOMMTOOLS produced 223 inducible cre-driver lines in a C57BL/6N genetic background. The demand for cell type specific cre-drivers was determined in a large scale survey of the scientific community and based on this 639 candidate genes for chosen.

Figure 3 illustrates the knock-in strategy employed by EUCOMMTOOLS which takes advantage of gene cassettes encoding EGFP-Cre/ERT2 separated by a T2A polyprotein cleavage sequence where EGFP provides a convenient reporter for tracking endogenous gene expression. After excluding haploinsufficient genes, a total of 459 Cre knock-in vectors and 374 recombinant C57BL/6 ES cell clones were produced of which 223 were converted into mice. Cre-driver lines were validated by crossing to Rosa26-loxPlacZ, or Rosa26-loxPAil4Tdtomato reporter mice [38,39] and monitoring tamoxifen induced cre activity in progeny tissue samples by histochemistry at 14 (P14) and 56 (P56) days after birth. Cre expression was annotated to various cells and tissues by monitoring EGFP together with lacZ or Tdtomato-reporter gene expression. Presently the following 74 cre-driver strains have been annotated: *Tns1, Krt17, Acan, Mb, Tshb, Prdm16, Cpne, Ucp1, Gpx3, Heatr8, Tle6, Trpv1, Smyd3, Hoxa2, Tac2, Pou2f1, Rbpjl, Spp2, ROSAL2/WT, Ptprcap, Cd3e, Atp6v1g2, Ccl25, Hes5, Myo1a, Insl3, Csk/Csktm1, Dmbx1, Mia, Spink8, Entp5, Acsm4, Dbx1, Cdx2, Gnai2, Pkd2l1, Sfrp1, Cnp, P2rx7, Pdgfrb, Gast, Cd63, Cpne6, Nefh, Amhr2, Rbfox3, Wnt9a, Barhl1, Zfp819, cldn7, Sgca, Pbx2, Zbtb32, A830010M20F, Lztfl1, Ttl25, Sds, Aqp3, Eomes, Spic, Zfp629, Slc26a5, Fga, Tbx2, Tff1, Cmtm5, Ttr, Myh4, aldh3a2, Igj, Slc26a4, Mog, Cdh2, and Sost.* All brain and body annotations as well as selected images of tissue specific cre expression can be found at www.imib.es/AnotadorWeb/public.jsf. Representative images of whole body and brain cre-activity annotations are shown in Figures 4 and 5, respectively.

All recovered expression patterns have been integrated into an anatomical ontology atlas with cellular resolution l which illustrates the body and brain structures of P14 and P56 mice (www.imib.es/AnotadorWeb/public.jsf). Based on the Allen Brain Atlas (www.brain-map.org) ontology, regional brain annotation was performed at cellular

resolution. For whole body annotation, EUCOMMTOOLS developed its own ontology tree for each organ at cellular resolution. This novel cre-driver resource significantly increases the value of the IKMC resource as it provides mice amenable to tamoxifen-inducible cre expression in a wide range of organs, tissues and cell types. In this context it is of note that the CanEuCre (successor project of Pleiades Promoters program in cooperation with EUCOMMTOOLS and IKMC) produced more than 27 Cre/ERT2-driver strains mostly targeting the brain and 27 recombinant adeno-associated viral iCre constructs for in vitro and in vivo applications (www.jax.org/mouse-search?searchTerm=icre%20Simpson, www.addgene.org/Elizabeth_Simpson [40]). Furthermore, the successor of the NorCOMM project, NorCOMM2LS is performing transposon-based enhancer trapping strategy to identify novel loci suitable for spatially restricted cre-recombinase expression (www.norcomm2.org).

The IKMC cre-driver program and other international large-scale cre-driver initiatives are jointly aiming to assemble a complete cre-driver zoo. Accordingly, the Allen Institute for Brain Science is generating brain-specific cre-driver strains by taking advantage of their comprehensive atlas of brain-specific expression patterns [38]. Moreover, the GENSAT (Gene Expression Nervous System Atlas) program produced and characterized 289 nervous system specific cre-driver strains mainly for the forebrain (www.gensat.org/cre.jsp). However, with few exceptions [33,41] the majority of these cre driver lines express Cre constitutively precluding a temporal control. An additional neural cre-driver resource was established by the Neuroscience Blueprint Cre-Driver Network which produced and validated over 100 cre-driver strains ([42], www.credrivermice.org). The JAX Cre Repository, which currently distributes over 365 cre-driver strains, many of which are maintained as live colonies, implemented a web resource documenting the characteristics of over 100 highly-used cre-driver strains distributed by the JAX Repository (www.jax.org/research-and-faculty/tools/cre-repository). All data generated in this pipeline are routinely updated and disseminated through Mouse Genome Informatics (MGI's) Creportal resource (www.informatics.jax.org/home/recombinase; [43], see below). Finally, cre-driver efforts have also been recently described by Murray et al. [44], and are currently being extended by using CRISPR/Cas9 based approaches (Murray, personal communication).

IKMC/IMPC and cre-driver web portal.

With the expansion of IMPC phenotyping activities the IKMC/IMPC web portals have been merged to create one stop and shop site for genetic and phenotypic data. The public IMPC/IKMC web portal (www.mousephenotype.org) provides updated information on the conditional genetic resources including detailed description of targeting vectors, targeted alleles, ES cell clones, mouse mutants and mutant phenotypes [45,46]. For each product there is a link directing users to the appropriate repository from which the desired product can be ordered (see repository article in this issue). The gene trap alleles are displayed at www.informatics.jax.org/ahele. The EUCOMMTOOLS' Cre driver resource is accessible from the IMPC web portal (www.mousephenotype.org/data/order/creline, www.imib.es/AnotadorWeb/public.jsf) and partly from MGI www.informatics.jax.org/home/recombinase. These portals contain comprehensive information about cre-drivers, including constructs,

promoters, strategies for cre-driver strain establishment and anatomical annotation with images.

IKMC, other repositories and distribution

Large scale international projects have provided mutant alleles for almost every proteincoding gene and made them available to the scientific community via public repositories. The gene trapping and targeting vectors as well as mutant ES cells are freely available via EuMMCR (www.eummcr.org) or KOMP (www.komp.org), and the mutant mice produced from IKMC material by Infrafrontier (www.infrafrontier.eu) CMMR (www.phenogenomics.ca) and KOMP (www.komp.org) for a nominal fee. In addition, the large scale cre-driver projects such as EUCOMMTOOLS, GENSAT, Allen Brain Institute and Blueprint Cre Driver Network deposited all their cre-driver strains in public repositories (www.infrafrontier.eu, www.mmrrc.org, and www.jax.org). Moreover, the International Mouse Strain Resource (IMSR; www.informatics.jax.org/home/recombinase) contains records for more than 2.700 cre-driver strains produced by the scientific community (see repository article in this issue).

Future perspectives

1. The IKMC vector- and ES cell resource is an unprecedented asset for the seamless generation of mutant mice and for the functional annotation of the mammalian genome. It forms the core for large-scale mutant mouse line production, phenotyping and archiving which is being performed by several mouse phenotyping consortia such as IMPC (www.mousephenotype.org), Infrafrontier (www.infrafrontier.eu) and KOMP2 (commonfund.nih.gov/KOMP2). Although the IKMC resource is quite comprehensive it is not yet complete (1,800 protein coding genes are still missing). However, by combining IKMC conditional gene targeting vectors with CRISPR/Cas9 genome editing whole genome coverage is within reach [31]. More than 6,000 mutant mouse strains have been produced from the IKMC resource and are presently being phenotyped by the IMPC [14–16]. It is conceivable that the IKMC/IMPC mutations in combination with nuclease mediated gene editing will lead to the establishment of a functional encyclopedia comprising the entire mammalian genome within the next decade.

2. The IKMC derived mouse models represent standardized references to which future mutant alleles in different genetic backgrounds can be referred to. This is important because analysis of null alleles in a defined inbred mouse strain is a first step in understanding any *in vivo* gene function. Together with streamlined phenotyping - as performed by the IMPC - the IKMC resource will have a major impact on the characterization of complex genetic traits. Moreover, the IKMC library also provides a guide for the generation of mutations by designer endonucleases in the future. Using the IKMC/IMPC resource as reference, the scientific community will be able to determine the contribution of genes and genetic backgrounds to the various disease-associated phenotypes [47,48].

3. To unravel pleiotropic gene functions it is necessary to inactivate genes in cell type-specific and temporally restricted manner. As the majority of IKMC alleles are conditional simple crossing to cre-driver strains will yield the correct answer. Conditionality is a major advantage of the resource because the generation of conditional alleles by CRISPR/Cas technology is still a major challenge.

4. IKMC products contain multipurpose alleles amenable to recombinase-mediated cassette exchange (RMCE) enabling virtually any locus modifications including the insertion of reporter genes for live cell imaging, optogenetic gene cassettes for cell activation or repression, Ca^{2+} -sensors for monitoring neuronal activity, alternative recombinases such as flp, human orthologues of genes of interest and disease-associated missense mutations for allelic series generation [49,50]. The modular approaches employed by the IKMC for vector construction enable re-use of existing targeting vector intermediates for making new alleles as exemplified by by the EUCOMMTOOLS cre knock-ins. Most importantly in future endeavors would be the examination of patient-specific sequence variants in the context of an entire organism. An amazing number of such variations have been already identified. Thus, by inserting disease gene variants into mouse orthologs by RMCE or alternatively, by oligonucleotide-mediated CRISPR/Cas9 mediated insertion into one-cell embryos [51] their characterization could be seamlessly achieved.

5. Finally, IKMC conditional mouse ES cells combined with CRISPR/Cas9 technology could be used to identify genes responsible for ES cell pluripotency and lineage differentiation by generating inducible homozygous mutant ES cell lines.

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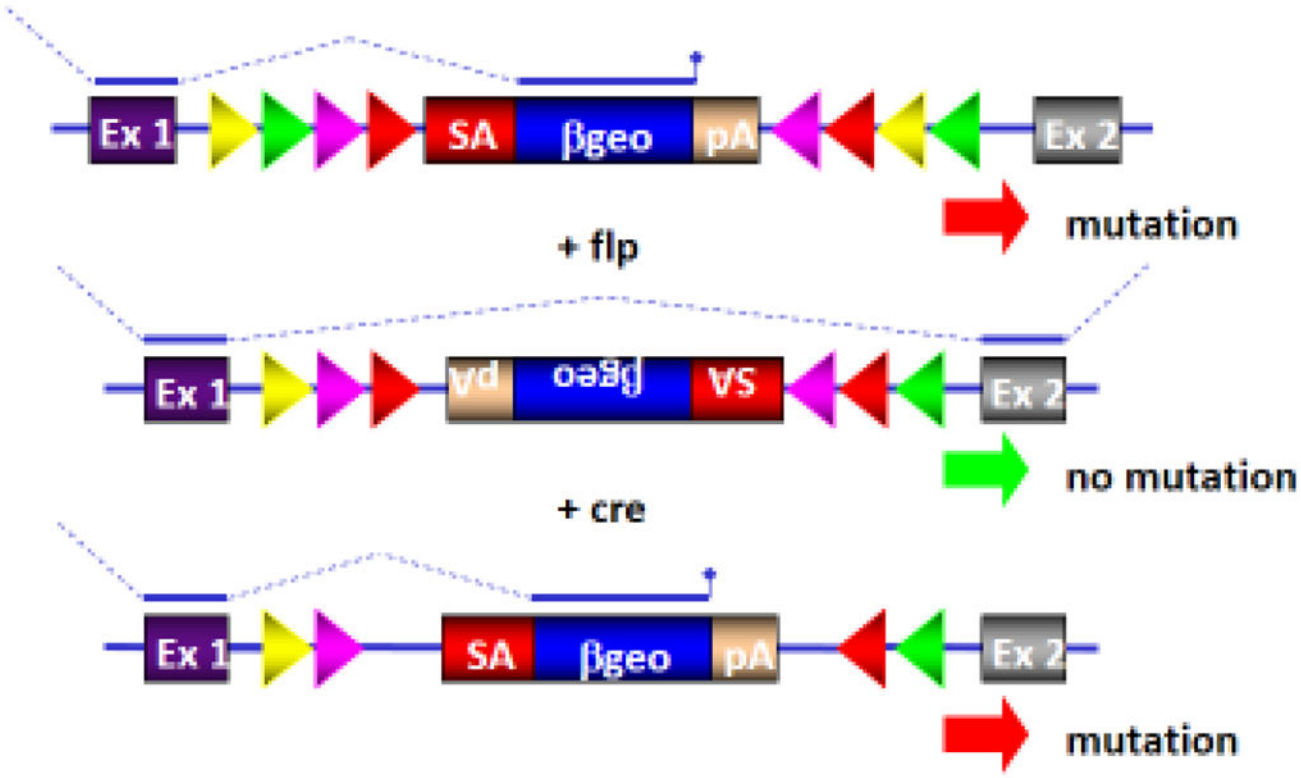

Figure 1:

Conditional gene inactivation by a SAbgeopA cassette. **A.** The SAbgeopA cassette flanked by recombinase target sites (RTs) in a FlEx configuration is illustrated after integration into an intron of an expressed gene. Transcripts (shown as gray arrows) initiated at the endogenous promoter are spliced from the splice donor (SD) of an endogenous exon (here exon 1) to the splice acceptor (SA) of the SAbgeopA cassette and terminate prematurely at the gene trap's polyadenylation (pA) site thereby causing a mutation. Exposure to flpo inverts the SAbgeopA cassette onto the non-coding strand, which reactivates normal splicing. This repairs the mutation by restoring normal gene expression. A second, Cre mediated re-inversion positions the SAbgeopA cassette back onto the coding strand and re-induces the mutation. Arrows indicate primer positions within FlipRosabgeo used to diagnose inversions. Frt (yellow triangles) and F3 (green triangles), heterotypic target sequences for the flpe- and flpo-recombinases; loxP (purple triangles) and lox5171 (red triangles), heterotypic target sequences for the cre-recombinase; SA splice acceptor; bgeo, b-galactosidase-neomycin-phosphotransferase fusion gene; pA, polyadenylation sequence.

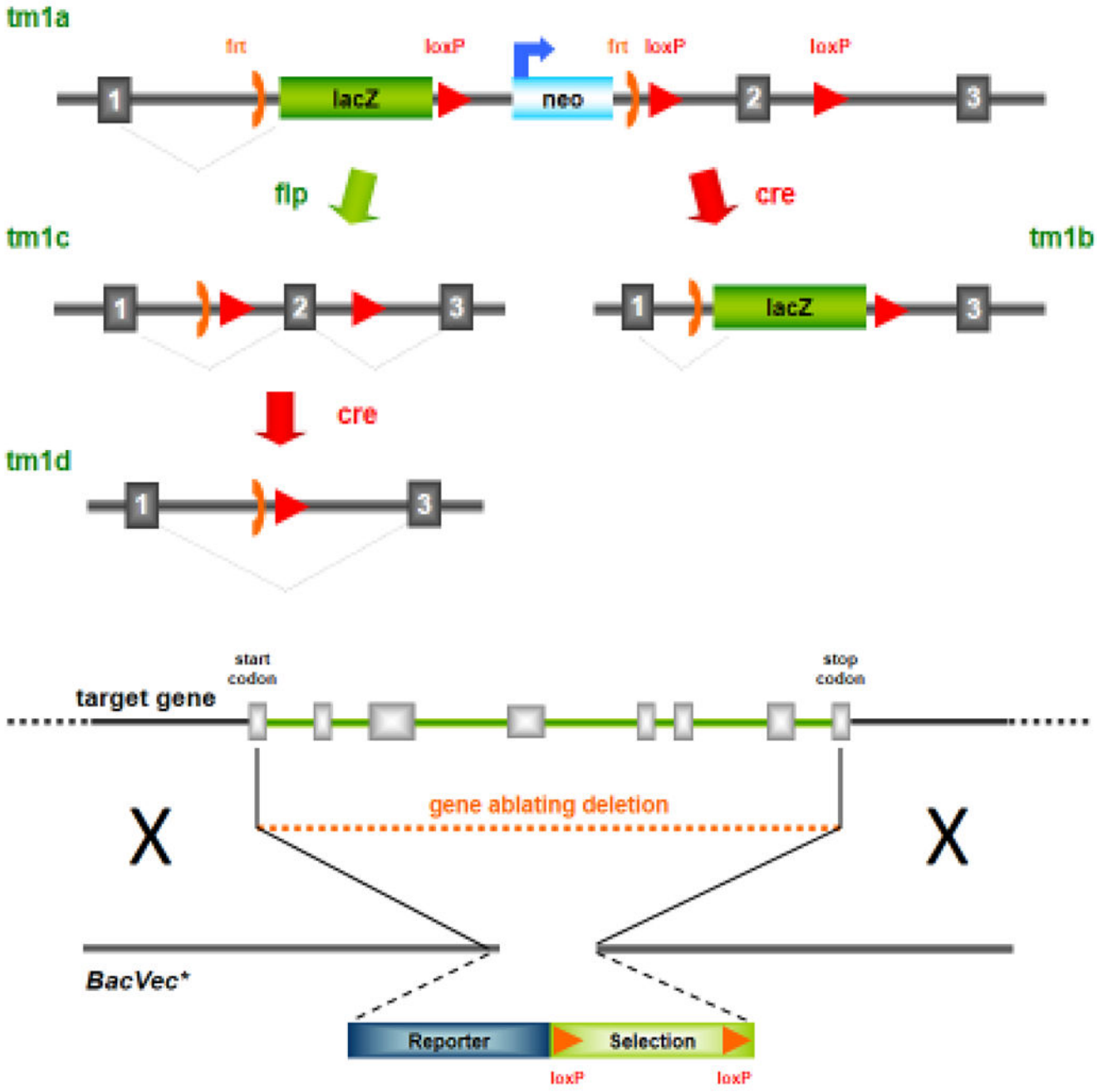

Figure 2:

IKMC gene targeting strategies. **A)** Knock-out first conditional ready targeting strategy. The targeting vector contains a splice-acceptor carrying lacZ reporter and neomycin selector cassette flanked by frt and loxP sites followed by a critical exon flanked by loxP site. After integration into the genome this allele is named tm1a allele. Exposure to flp recombinase deletes the reporter and selector cassette resulting in a wild type conditional floxed allele (tm1c). The critical exon can be deleted by cre recombinase expression (tm1d). Exposure of the tm1a allele first to cre recombinase deletes neomycin cassette and the critical exon

leading to a reporter null allele (tm1b). **B)** Represents the generation of entire gene deletions using large BAC targeting vectors integrating a reporter and loxP flanked selector cassette.

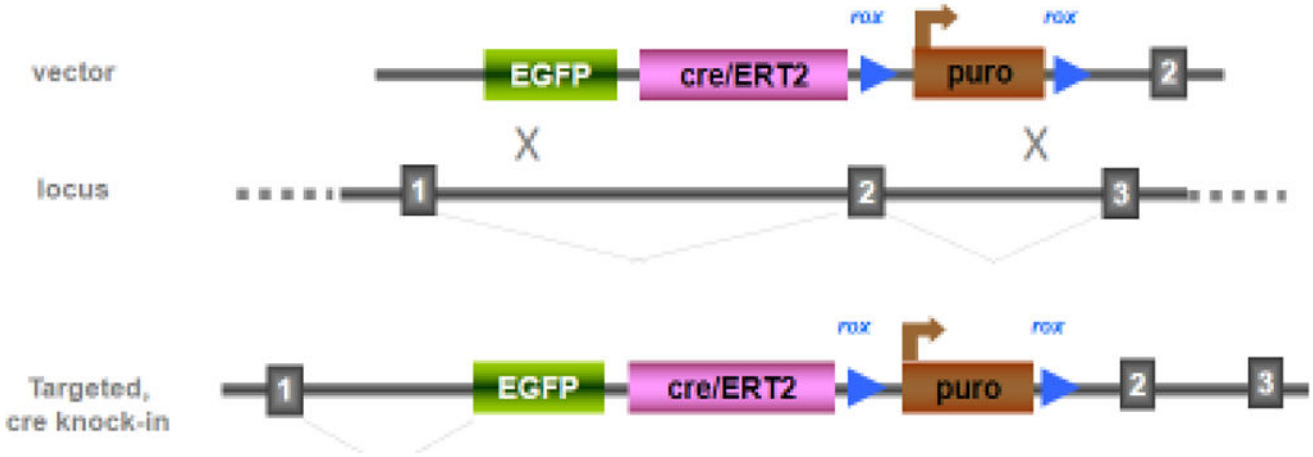

Figure 3:

EUCOMMTOOLS cre/ERT2 targeting vectors. The targeting vectors contain green fluorescent protein and cre/ERT2 separated with a T2A peptide sequence. The selection cassette is flanked with rox sites and is removable using dre recombinase.

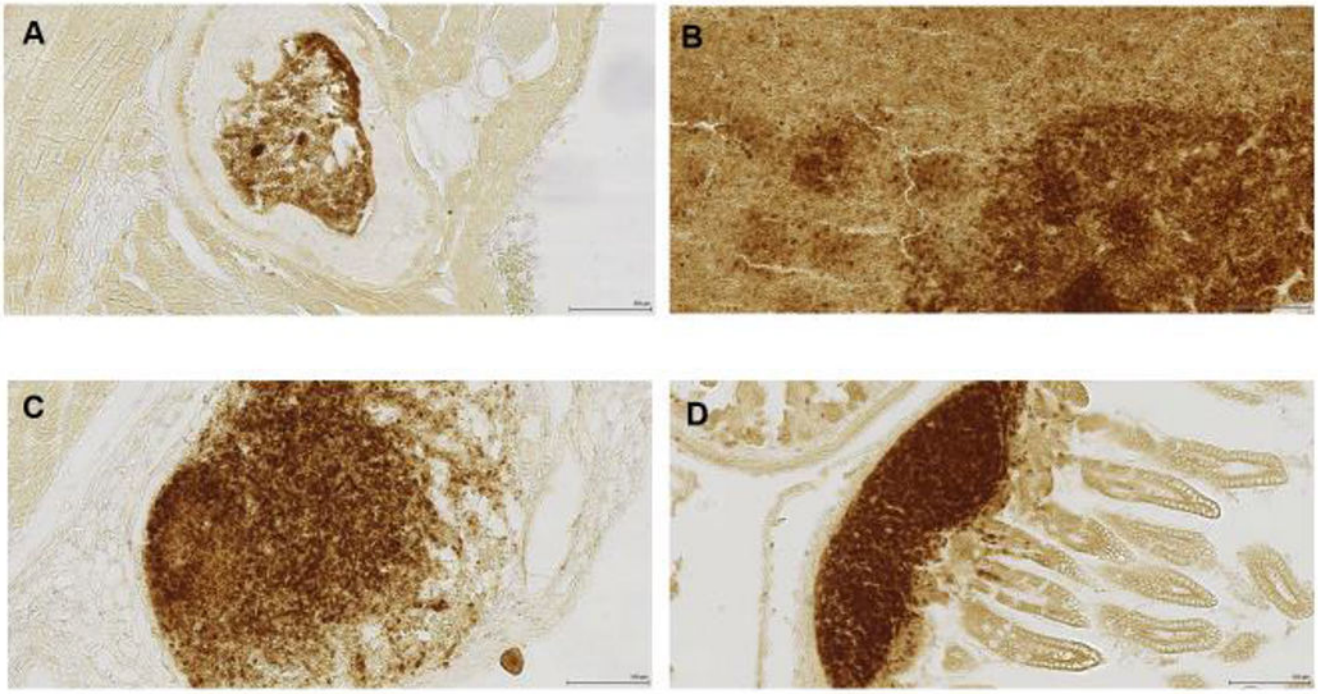

Figure 4:

Cre-activity annotation of PbPrcap promoter driving cre-recombinase strain in the bone marrow (A), thymus (B), lymph node (C) and the peyer's patch of the midgut (D) in P14 mice. The expression was detected in the entire lymphatic system from lymphoblastic precursors in the bone marrow to mature lymphocytes in the lymphatic nodes. Therefore, the inducible cre-driver system will allow gene inactivation at different stages of lymphocyte differentiation.

Figure 5:
Cre-activity of the TRpvl promoter cre-driver strain in Purkinje cells of the cerebellar cortex of P14 mice.

Table 1:

Gene trap mutations generated by the IKMC consortium.

	EUCOMM/EUCOMM-TOOLS	KOMP-CSD	KOMP-REGN	NorCOMM	TIGM	total
Gene trap mutations	4.412			3.832	9.882	11.390

Table 2:

Targeted mutations in ES cells generated by the IKMC consortium (*Note: the NorCOMM allele represents a null allele convertible into a conditional allele (see NorCOMM web site).

	EUCOMM/EUCOMM-TOOLS	KOMP-CSD	KOMP-REGN	NorCOMM	TIGM	Total
Targeted conditional mutations	8.333	4.252		32		12.406
Targeted deletion mutations	325	1.197	4.134	569		5.829
						14.975