Genome wide conditional mouse knockout resources

C. Kaloff[1], K. Anastassiadis[2], A. Ayadi[3], R. Baldock[4], J. Beig[1],
M.-C. Birling[3], A. Bradley[5], S.D.M. Brown[6], A. Bürger[1], W. Bushell[5],
F. Chiani[7], F.S. Collins[8], B. Doe[5], J.T. Eppig[9], R.H. Finnell[10,11],
C. Fletcher[12], P. Flicek[13], M. Fray[6], R.H. Friedel[38,39], A. Gambadoro[7],
H. Gates[6], J. Hansen[1], Y. Herault[3,14,15,16,17], G.G. Hicks[18],
A. Hörlein[40], M. Hrabé de Angelis[19], V. Iyer[5], P.J. de Jong[20],
G. Koscielny[41,42], R. Kühn[1,43,44], P. Liu[5], K.C.K Lloyd[21],
R.G. Lopez[22], S. Marschall[19], S. Martínez[22], C. McKerlie[23],
T. Meehan[13], H. von Melchner[24], M. Moore[25], S.A. Murray[9],
A. Nagy[26], L.M.J. Nutter[23], G. Pavlovic[3,14,15,16], A. Pombero[45],
H. Prosser[5], R. Ramirez-Solis[5], M. Ringwald[9], B. Rosen[5,46],
N. Rosenthal[9], J. Rossant[27,47], P. Ruiz Noppinger[28], E. Ryder[5],
W.C. Skarnes[5], J. Schick[48], F. Schnütgen[24], P. Schofield[29],
C. Seisenberger[1], M. Selloum[3], D. Smedley[13,30],
E.M. Simpson[31,32,33,49,50], A.F. Stewart[2], L. Teboul[6],
G.P. Tocchini Valentini[7], D. Valenzuela[34], A.P. West[5],
W. Wurst[1,35,36,37,*]

[1]Institute of Developmental Genetics, Helmholtz Zentrum Muenchen, D-85764 Neuherberg, Germany
[2]Biotechnology Center (BIOTEC) of the Technische Universität Dresden, 01307 Dresden, Germany
[3]Institut Clinique de la Souris (ICS), CELPHEDIA, PHENOMIN, CNRS, INSERM, University of Strasbourg, 1 rue Laurent Fries, F-67404 Illkirch, France
[4]MRC Human Genetics Unit, Institute of Genetics and Molecular Medicine, University of Edinburgh, College of Medicine and Veterinary Medicine, Edinburgh, Scotland EH4 2XU, UK
[5]The Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB101HH, UK
[6]Mammalian Genetics Unit and Mary Lyon Centre, MRC Harwell, Oxfordshire OX110RD, UK
[7]Monterotondo Mouse Clinic, Italian National Research Council (CNR), Institute of Cell Biology and Neurobiology, Monterotondo-Scalo, I-00015 Rome, Italy
[8]National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892, USA
[9]The Jackson Laboratory, Bar Harbor, ME 04609, USA
[10]Dell Pediatric Research Institute, The University of Texas at Austin, Austin, TX 78712, United States

[11]University of Texas at Austin, Austin, TX 78712, USA

[12]National Institutes of Health, Bethesda, MD 20205, USA

[13]European Bioinformatics Institute (EBI), Hinxton, Cambridge CB101ST, UK

[14]Institut de Génétique et de Biologie Moléculaire et Cellulaire, Université de Strasbourg, 1 rue Laurent Fries, 67404 Illkirch, France

[15]Centre National de la Recherche Scientifique, UMR7104, Illkirch, France

[16]Institut National de la Santé et de la Recherche Médicale, U964, Illkirch, France

[17]Université de Strasbourg, 1 rue Laurent Fries, 67404 Illkirch, France

[18]University of Manitoba, Manitoba Institute of Cell Biology, Winnipeg, MB R3EOV9, Canada

[19]Institute of Experimental Genetics, Helmholtz Zentrum Muenchen, D-85764 Neuherberg, Germany

[20]University of California, San Francisco Benioff Children's Hospital Oakland Research Institute, Oakland, CA, 94609, USA

[21]Mouse Biology Program, School of Medicine, University of California, Davis, CA 95616, USA

[22]Institute of Neurosciences, Miguel Hernández University, Spanish National Research Council, San Juan Campus, 03550, Sant Joan d'Alacant, Alicante, Spain

[23]The Centre for Phenogenomics, Toronto, Ontario, Canada

[24]Department of Molecular Haematology, University Hospital Frankfurt, Goethe University, 60590 Frankfurt am Main, Germany

[25]IMPC, San Anselmo, CA, USA

[26]Samuel Lunenfeld-Tanenbaum Research Institute, Sinai Health System, Toronto, ON, M5G1X5, Canada

[27]Program in Developmental and Stem Cell Biology, The Hospital for Sick Children, Toronto, ON M5G2L3, Canada

[28]Metanomics Health GmbH, 10589 Berlin, Germany; Medizinische Fakultät Carl Gustav Carus, Technische Universität Dresden, 01307 Dresden, Germany

[29]New Product Introduction, Global Manufacturing and Supply, GlaxoSmithKline, Harmire Road, Barnard Castle DL12 8DT, UK

[30]William Harvey Research Institute, Queen Mary University of London, Gower Street, London WC1E 6BT, UK

[31]Centre for Molecular Medicine and Therapeutics at the Child and Family Research Institute, University of British Columbia, Vancouver, BC, V5Z 4H4, Canada

[32]Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia, Canada

[33]Department of Psychiatry, University of British Columbia, Vancouver, British Columbia, Canada

[34]Velocigene Division, Regeneron Pharmaceuticals Inc., Tarrytown, NY 10591, USA

[35]Deutsches Zentrum für Neurodegenerative Erkrankungen e. V. (DZNE), Standort München — Feodor-Lynen-Str. 17, 81377 München, Germany

[36]Munich Cluster for Systems Neurology (SyNergy), Feodor-Lynen-Str. 17, 81377 Munich, Germany

[37]Technische Universität München-Weihenstephan, Lehrstuhl für Entwicklungsgenetik, c/o Helmholtz Zentrum München, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany

[38]Fishberg Department of Neuroscience, Friedman Brain Institute, Icahn School of Medicine at Mount Sinai, New York, New York, 10029, USA

[39]Department of Neurosurgery, Friedman Brain Institute, Icahn School of Medicine at Mount Sinai, New York, New York, 10029, USA

[40]Institute of Epidemiology II, Helmholtz Zentrum Muenchen, D-85764 Neuherberg, Germany

[41]Open Targets, Wellcome Genome Campus, Hinxton, Cambridge, CB10 1SD, UK

[42]GSK, Medicines Research Center, Gunnels Wood Road, Stevenage, SG1 2NY, UK

[43]Transgenic Core Facility, Max Delbrück Center for Molecular Medicine, Robert-Rössle-Str. 10, Berlin-Buch, Germany

[44]Berlin Institute of Health, Kapelle-Ufer 2, Berlin, Germany

[45]Intituto Murciano de Investigación Biosanitaria (IMIB)-Arrixaca, University of Murcia, Murcia, Spain

[46]AstraZeneca Darwin Building (Unit 310), Cambridge Science Park, Milton Road, Cambridge, CB4 0WG, United Kingdom

[47]Department of Molecular Genetics, University of Toronto, Toronto, Canada

[48]Institute of Molecular Toxicology and Pharmacology, Helmholtz Zentrum Muenchen, D-85764 Neuherberg, Germany

[49]Genetics Graduate Program, University of British Columbia, Vancouver, BC, V6T 1Z2, Canada

[50]Department of Ophthalmology and Visual Science, University of British Columbia, Vancouver, BC, V5Z 3N9, Canada

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 6000 mutant mouse strains have been generated by the IKMC in collaboration with the International Mouse Phenotyping Consortium (IMPC). In addition, a cre-driver resource was established including 250 C57BL/6 cre-inducible mouse strains. Complementing the cre-driver resource, a collection comprising 27 rAAVs expressing cre in a tissue-specific manner has also been produced. All resources are easily accessible from the IKMC/IMPC web portal (www.mousephenotype.org). The IKMC/IMPC resource is a standardized reference library of mouse models with defined genetic backgrounds enabling the analysis of gene-disease associations in mice of different genetic makeup and should therefore have a major impact on biomedical research.

*Corresponding author: W. Wurst (wurst@helmholtz-muenchen.de)

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 a similar genome organisation, 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 because 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 its impact on human disease. To address 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 ES cells harboring mostly conditional mutations in nearly 18,500 genes in a C57BL/6N genetic background using a combination of gene trapping and targeting complemented recently by designer endonuclease mediated genome editing [5–12]. The ES cell-based mutations are typically converted into mice by blastocyst injection and phenotypic alterations analyzed in heterozygous and/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 also 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 temporally restricted mutations by crossing the mutant strains to flpe and/or cre site-specific recombinase expressing deleter mice [20].

Fig. 1 shows the FlipRosaβgeo conditional gene trap vector used by the IKMC. It contains a typical gene trap cassette (splice acceptor (SA)-reporter (βgeo)-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
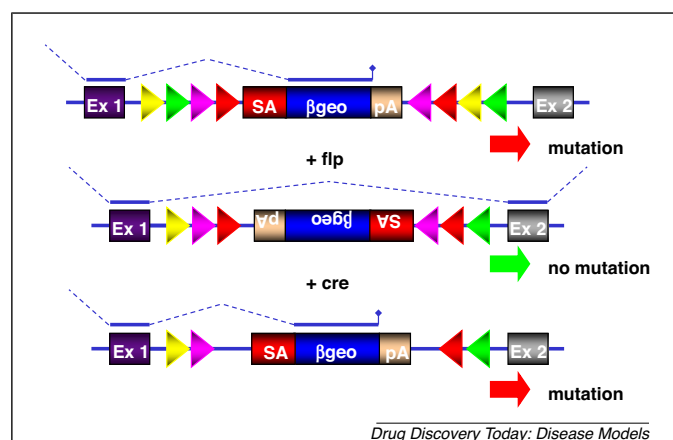

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Fig. 1. Conditional gene inactivation by a SAβgeopA cassette. A. The SAβgeopA cassette flanked by recombinase target sites (RTs) in a FlEx configuration is illustrated after integration into an intron of an expressed gene. Transcripts (shown as gray 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 SA βgeopA cassette and terminate prematurely at the gene trap's polyadenylation (pA) site thereby causing a mutation. Exposure to flpo inverts the SAβgeopA 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 SAβgeopA cassette back onto the coding strand and re-induces the mutation. Arrows indicate primer positions within FlipRosaβgeo 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; βgeo, b-galactosidase-neomycin-phosphotransferase fusion gene; pA, polyadenylation sequence.

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

successive delivery of flpe and cre [21,22]. Gene trap muta-genesis can be easily scaled-up so that thousands of muta-tions 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 defi-nitely more precise, in some cases involving the disruption of multiple isoforms, conditional gene trapping proved superior [21]. Collectively, the IKMC has generated gene trap muta-tions 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 129 Sv ES cells allowing the comparative analysis of the same mutation in different genetic back-grounds.

Unlike gene trapping, gene targeting by homologous re-combination in ES cells is a precise technology enabling the induction of 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 ho-mologous recombinants which is quite 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 con-ditional gene trap vectors enable spatial and temporal control of gene activity which is essential for distinguishing between pleiotropic gene functions during embryogenesis and adult-hood [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-βgal-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; Fig. 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) (Fig. 2A). 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 muta-tions in ES cells (Table 2). While most of the conditional targeting vectors successfully mutated the respective genes, a set of around 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 proj-ect 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 suitable targeting vectors and sgRNAs (www.eummcr.org/crispr/search). Most of the 13,000 conditional targeting vectors of the IKMC resource have protospacer (PAM) sequences suit-ably located for Cas9-assisted targeting, suggesting that the conditional mutant resource can now be readily completed [31]. In addition, lacZ tagged gene deletions have been gen-erated by Regeneron using Velocigene technology utilizing a bacterial artificial chromosome (BAC)-based strategy (Fig 2B, Table 2) [26]. Furthermore, over 6,000 mutant mouse strains generated from the IKMC ES cell resource together with their primary phenotypes are presently available at www.mousephenotype.org [14–16]; see also article in this issue.

Cre driver transgenic mouse strains

Full exploration of the IKMC conditional loxP alleles requires a complementary resource of inducible cre recombinase expressing transgenic mouse strains (cre-drivers) [32]. Ideally, cre-driver mice 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 endogenous loci [34] were used to provide high fidelity cre expression.

About 30% of all null mutations lead to embryonic lethali-ty and therefore temporal control of gene inactivation is also required for the analysis of postnatal gene function. Tempo-ral 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) that 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
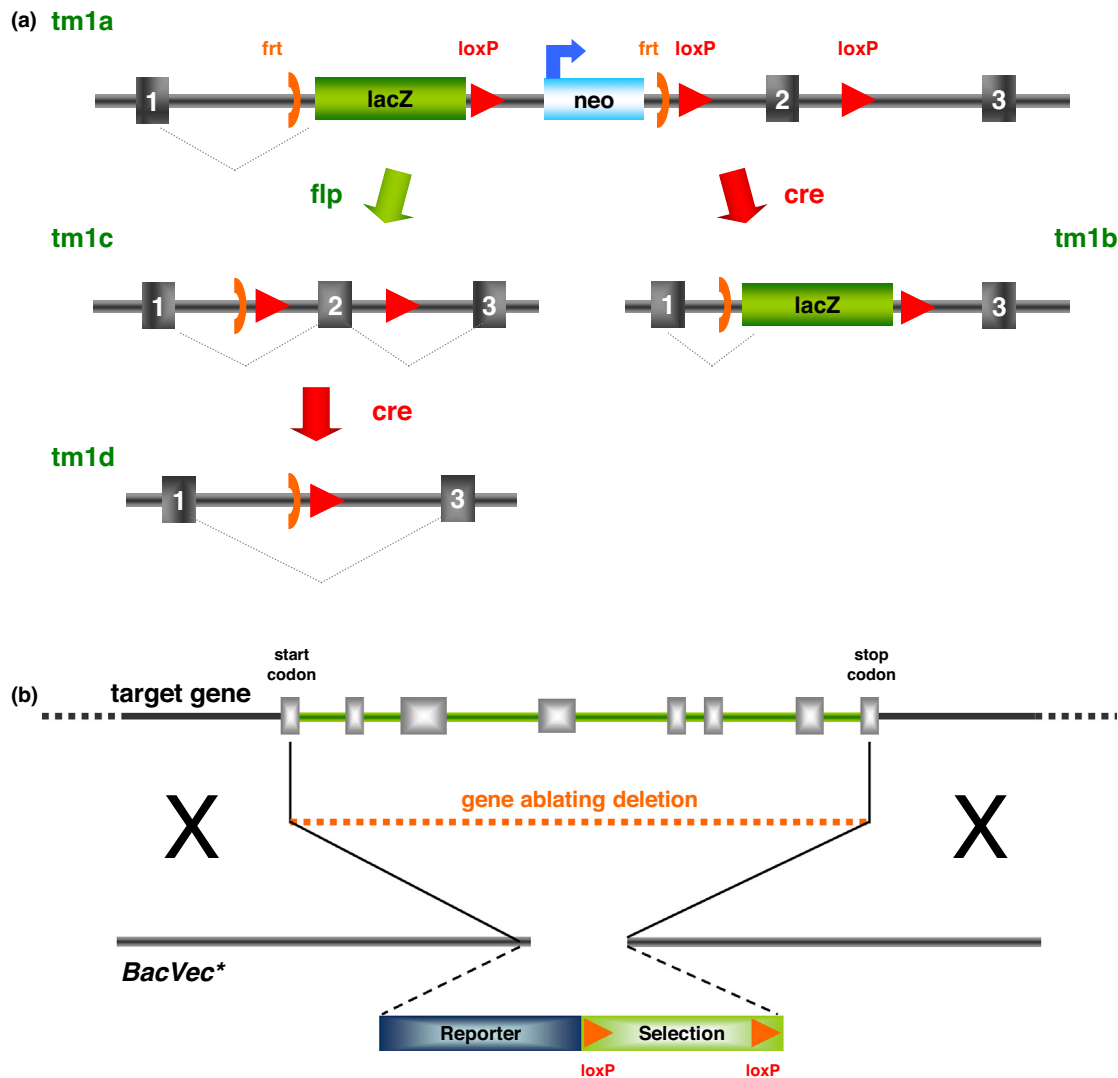
Fig. 2. IKMC gene targeting strategies. A) Knock-out first conditional ready targeting strategy. The targeting vector contains a splice-acceptor carrying lacZ reporter and neomycin selector cassette flanked by frt and loxP sites followed by a critical exon flanked by loxP site. After integration into 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.

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

Table 2. Targeted mutations in ES cells generated by the IKMC consortium

	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

Note: the NorCOMM allele represents a null allele convertible into a conditional allele (see NorCOMM web site).
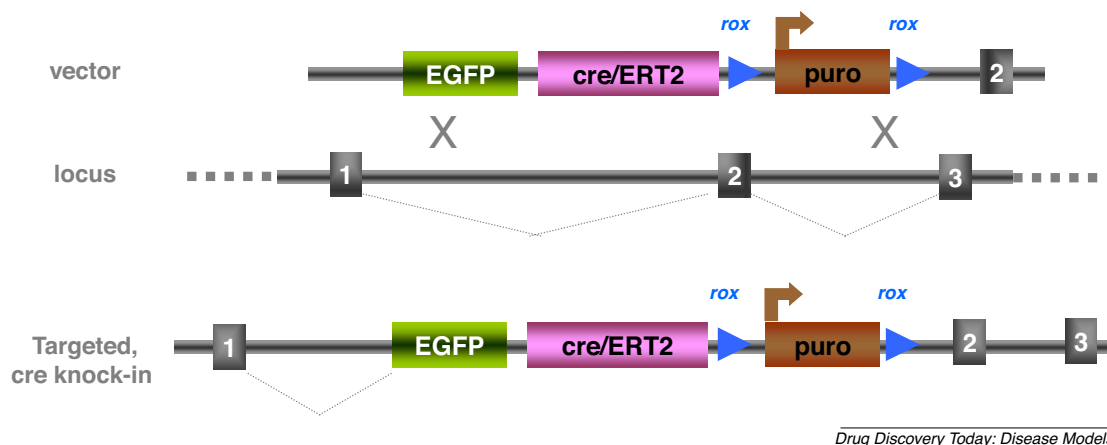
Fig. 3. EUCOMMTOOLS cre/ERT2 targeting vectors. The targeting vectors contain green fluorescent protein and cre/ERT2 separated with a T2A peptide sequence. The selection cassette is flanked with rox sites and is removable using dre recombinase.

specific cre-drivers was estimated in an internet-based large scale survey of the scientific community's needs and based on this 639 candidate genes chosen.

Fig. 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-lox-PAi14Tdtomato 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 Figs. 4 and 5, respectively.

All recovered expression patterns have been integrated into an anatomical ontology atlas with cellular resolution illustrating 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) generated over 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 presently performing transposon-based enhancer trapping 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
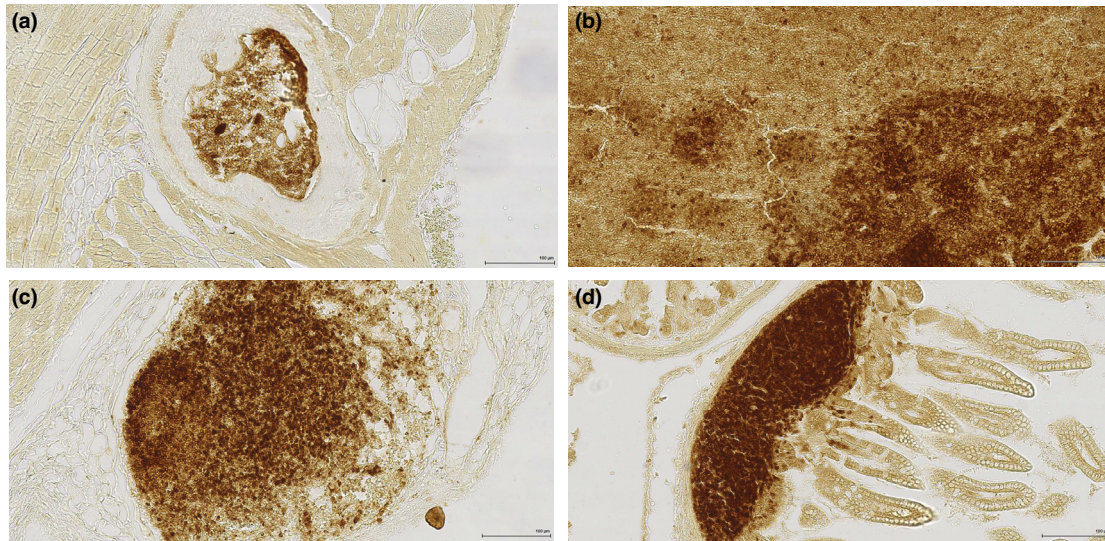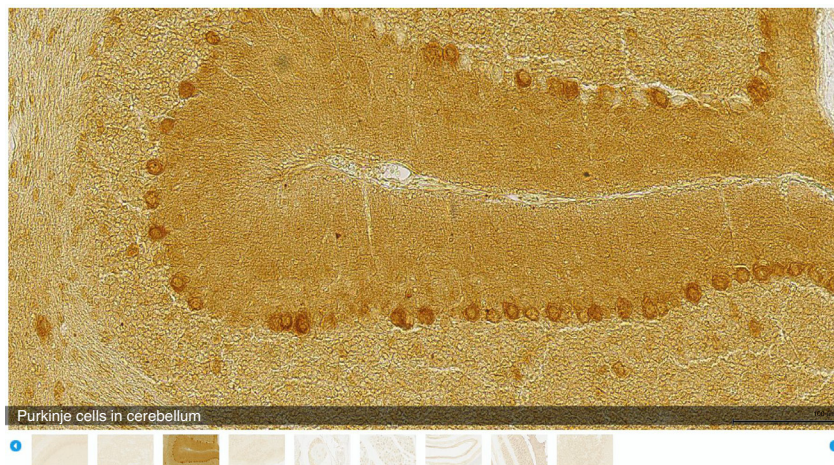
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Fig. 4. Cre-activity annotation of PbPrcap promoter driving cre-recombinase strain in the bone marrow (A), thymus (B), lymph node (C) and the peyer's patch of the midgut (D) in P14 mice. The expression was detected in the entire lymphatic system from 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.

maintained as live colonies, implemented a web resource documenting the characteristics of the 100 most frequently used cre-driver strains (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 (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 expanded by using CRISPR/Cas9 based approaches (Murray, personal communication).

IKMC/IMPC and cre-driver web portal

With the extension of the IMPC phenotyping activities the IKMC/IMPC web portals have been merged to create a single 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

Purkinje cells in cerebellum

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Fig. 5. Cre-activity of the TRpv1 promoter cre-driver strain in Purkinje cells of the cerebellar cortex of P14 mice.

product can be ordered (see repository article in this issue). The gene trap alleles are displayed at www.informatics.jax.org/allele. 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 on 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 protein-coding gene and made these available to the scientific community via internet-based 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), MMRRC (www.mmrrc.org), 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 at large (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 (1800 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 6000 mutant mouse strains have been produced from the IKMC resource and are presently subjected to phenotyping 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 of 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 re-stricted manner. As the majority of IKMC alleles are conditional simple crossing to cre-driver strains will yield the desired answers. 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 such as insertion of reporter genes for live cell imaging, optogenetic gene cassettes for light sensitive gene regulation, Ca^{2+}-sensors for monitoring neuronal activity, site-specific recombinases for tissue specific expression, 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 the re-use of existing targeting vector intermediates for making new alleles as exemplified by the EUCOMMTOOLS cre knock-ins. Most importantly, the IKMC alleles enable the examination of patient-specific sequence variants in the context of an entire organism by simply inserting the disease gene variant into the mouse ortholog by RMCE or alternatively, by CRISPR/Cas9 mediated genome editing in one-cell embryos [50,51].

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.

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.

Acknowledgements

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