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Genetically-engineered mouse models of small cell lung cancer: the next generation

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Abstract

Small cell lung cancer (SCLC) remains the most fatal form of lung cancer, with patients in dire need of new and effective therapeutic approaches. Modeling SCLC in an immunocompetent host is essential for understanding SCLC pathogenesis and ultimately discovering and testing new experimental therapeutic strategies. Human SCLC is characterized by near universal genetic loss of the *RB1* and *TP53* tumor suppressor genes. Twenty years ago, the first genetically-engineered mouse model (GEMM) of SCLC was generated using conditional deletion of both *Rb1* and *Trp53* in the lungs of adult mice. Since then, several other GEMMs of SCLC have been developed coupling genomic alterations found in human SCLC with *Rb1* and *Trp53* deletion. Here we summarize how GEMMs of SCLC have contributed significantly to our understanding of the disease in the past two decades. We also review recent advances in modeling SCLC in mice that allow investigators to bypass limitations of the previous generation of GEMMs while studying new genes of interest in SCLC. In particular, CRISPR/Cas9-mediated somatic gene editing can accelerate how new genes of interest are functionally interrogated in SCLC tumorigenesis. Notably, the development of allograft models and precancerous precursor models from SCLC GEMMs provides complementary approaches to GEMMs to study tumor cell-immune microenvironment interactions and test new therapeutic strategies to enhance response

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to immunotherapy. Ultimately, the new generation of SCLC models can accelerate research and help develop new therapeutic strategies for SCLC.

INTRODUCTION

Small cell lung cancer (SCLC) accounts for ~15% of all lung cancer cases and is characterized by rapid growth, early metastasis, and an increased propensity for resistance to multiple therapies. Despite extensive research efforts and a large number of clinical trials, the 5-year survival rates for SCLC patients have remained disappointingly low over the years, hovering around 8–10%, with a median overall survival of 10–12 months [1–4]. The implementation of more effective therapeutic strategies against SCLC will likely require a better understanding of the molecular and cellular mechanisms underlying the biology of SCLC, including how SCLC cells interact with other cells in their microenvironment and how these cancer cells gain a high level of plasticity allowing them to reach therapeutically-resistant cell states.

Historically, it has proven challenging to investigate SCLC in patients. In large part, this is because patients with SCLC are often diagnosed with metastatic disease, and surgical resections are rarely performed, thereby limiting the quantity and number of samples that can be obtained and precluding the collection of longitudinal samples from the same patients. In recent years, there have been several breakthroughs in the field, including the use of circulating tumor cells (CTCs) to generate patient-derived xenograft (PDX) models, so-called CTC-derived xenografts (CDXs) [5–8], and advances in molecular analyses at the RNA and protein levels using paraffin sections from archival SCLC tumors (e.g., spatial transcriptomics and proteomics) [9, 10]. Moreover, a notable conceptual shift has taken place in acknowledging the heterogeneity among SCLC patient tumors. Traditionally considered a homogeneous disease, there is now a growing appreciation for the molecular and cellular heterogeneity within SCLC. A recent proposal introduces the classification of SCLC cells into three main groups, SCLC-A, SCLC-N, and SCLC-P, based on expression of lineage-defining transcription factors: ASCL1, NEUROD1, and POU2F3, respectively [9]. Alternatively, these subtypes can be divided into neuroendocrine (SCLC-A/-N) and non-neuroendocrine (SCLC-P). An “inflamed” subtype of SCLC has also been proposed, with low neuroendocrine features and a gene signature displaying elevated expression of genes involved in immune signaling along with robust immune infiltration [10]. Expression of other transcription factors, such as YAP1, which is enriched in inflamed SCLC [11], and ATOH1 [12] have also been proposed to reflect distinct subsets of SCLC. Understanding the clinical implications of this molecular classification for personalized therapy in SCLC patients is crucial. It is imperative to investigate the cellular and molecular mechanisms underlying each subtype. However, the scarcity of patient samples remains a significant challenge in comprehensively studying intertumoral heterogeneity, which has been a primary motivation for considering animal models as alternative avenues for research.

Preclinical models can serve as valuable tools to study SCLC; they allow researchers to investigate the biological underpinnings of the disease in controlled experimental settings and provide a platform for hypothesis-testing, drug-screening, and target validation. Cell

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lines and PDX/CDX models have been used traditionally and have proved valuable in understanding numerous aspects of SCLC biology (discussed elsewhere [13, 14]). However, limitations in the ability of these models to recapitulate the tumor microenvironment and immune landscape of SCLC tumors highlight the need for autochthonous tumor models established in an immunocompetent host. Meanwhile, genetically-engineered mouse models (GEMMs) have emerged as indispensable tools in SCLC research, offering opportunities to study the disease in a controlled and physiologically-relevant environment, including temporal and spatial progression with a fully competent immune system. Therefore, this review will focus on SCLC GEMMs and syngeneic models derived from the GEMMs. The first GEMM of SCLC was developed by Meuwissen et al. 20 years ago [15]. This model was based on the near-ubiquitous inactivation of both the *TP53* and *RB1* tumor suppressor genes observed in human SCLC (see below). Since then, GEMMs of SCLC have been instrumental in elucidating critical molecular pathways in SCLC, revealing potential therapeutic vulnerabilities and guiding the development of targeted therapies; these models have also facilitated investigations into drug resistance mechanisms and the development of combination therapies to improve treatment outcomes [16–43].

One major limitation of this first generation of SCLC GEMMs has been the requirement for complicated crosses (slow and costly), which has hampered the utilization of these GEMMs by a broader community of researchers. Here we discuss recent advances in the field allowing for faster, cheaper, and more broadly accessible models.

Modeling SCLC in mice: the first generation

In the first GEMM of SCLC, the lungs of adult mice with conditional mutant alleles for *Rb1* and *Trp53* where the genes are flanked by *lox* sites (“flox”) ($Rb1^{flox/flox};Trp53^{flox/flox}$ mice, or *RP* mice) were infected with an adenoviral vector expressing Cre recombinase under the broadly-expressed CMV promoter (Ad-CMV-Cre) (Fig. 1A). Deletion of the two tumor suppressors ($Rb1^{\Delta/\Delta};Trp53^{\Delta/\Delta}$) in this context leads to the development of tumors that closely resemble human SCLC; these tumors also metastasize to multiple tissues, similar to human SCLC. Notably, loss of 3 out of 4 alleles ($Rb1^{flox/\Delta};Trp53^{\Delta/\Delta}$ or $Rb1^{\Delta/\Delta};Trp53^{flox/\Delta}$) was insufficient to generate SCLC [15]. Thus, the *RP* model conclusively demonstrated the requirement for complete loss of function of both *Rb1* and *Trp53* for SCLC initiation.

One limitation of the *RP* model is that few tumors develop (1–5) with a long median latency of 210 days in this initial report. Additional models with faster-growing and more abundant tumors have since been developed and used by several groups in the community. Based on some evidence that the levels of the p130 protein (encoded by the *RBL2* gene) are low in human SCLC tumors (likely from a combination of mutations, silencing, and protein degradation [31, 44]), the *Rb1;Trp53;Rbl2* (*RPR2*) model was developed. The *RPR2* is also known as the triple knockout (TKO) model (Fig. 1B) [30]; we propose a standardized naming system for GEMMs in Table 1. Using similar doses of Ad-CMV-Cre, these *RPR2* mice develop ~10–20 times more tumors in approximately half the time as *RP* mice, confirming the tumor suppressive role of p130/*Rbl2* in SCLC. Tumors in the *RP* and *RPR2* models express high levels of the ASCL1 transcription factor, representing the largest subtype of human SCLC tumors. In contrast, the $Rb1/Trp53/Myc^{T58A}$ (*RPM*)

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model, with inducible expression of an oncogenic form of MYC (MYC^{T58A}) at the time of *Rb1* and *Trp53* inactivation, develop NEUROD1-high SCLC, the second most frequent SCLC subtype, that can progress to a $YAP1^{+}$ state (Fig. 1C) [25, 26]. Tumors in the *RPM* model initiate rapidly within 4–10 weeks using multiple Cre viruses targeting distinct cells of origin [22], validating MYC as a potent oncogene in SCLC.

Investigating candidate genes in mouse models of SCLC

The *RP*, *RPR2*, and *RPM* models have been used in the past few years to investigate mechanisms of SCLC initiation, progression, and metastasis. The use of these models to investigate the cell types in the lungs from which SCLC may originate has been reviewed recently [45]: briefly, these studies in mice support a model in which neuroendocrine cells (or a subset of neuroendocrine cells [46]) serve as a cell-of-origin for SCLC; however, other cell types in the lung epithelium can be reprogrammed toward a neuroendocrine phenotype and initiate SCLC upon genetic alterations, particularly in the context of oncogenic MYC [22, 45, 47, 48].

In addition to p130, suspected tumor suppressors such as PTEN [38, 49] and $p16^{INK4A}$ [50] have been validated in loss-of-function experiments in mouse models of SCLC. In addition to MYC^{T58A} [26], overexpression of wild-type MYC [47] and other MYC family members (N-MYC and L-MYC) rapidly accelerates SCLC in inducible gain-of-function mouse models and drives resistance to chemotherapy [20, 21, 35]. MYC family member overexpressing GEMMs are ideal tools to study therapy resistant SCLC. The MYC partner MAX was shown to be a tumor suppressor in the *RP* model, functioning to repress the expression of key metabolic programs, but MAX was required for tumor growth in the same *RP* model when L-MYC was overexpressed [20]. L-MYC was also shown to be required for proper tumor growth in a knockout experiment in the *RPR2* model [16]. Overexpression of NFIB in the *RP* model revealed its role as an oncogene in SCLC [17, 35]. Moreover, ASCL1 inactivation in the *RPR2* model, which nearly exclusively expresses ASCL1, demonstrated that ASCL1 is required for the development of SCLC [43]; ASCL1 loss in the *RPM* model delayed tumor initiation and completely blocked neuroendocrine fate [22]. Similar to ASCL1, SOX2 is required for tumor initiation in *RPR2* mutant mice [51]. Finally, the oncogenic role of FGFR1 was assessed in the *RP* model using a constitutively active point mutant ($FGFR1^{K656E}$), which indicated that FGFR1 activation can promote SCLC development from tracheobronchial-basal cells but not from neuroendocrine cells [41]; conversely, FGFR1 was required for tumor development in the *RPR2* model (but not in the *RP* model), and the proposed mechanism of this genotype-specific impact of FGFR1 is that loss of p130 derepresses FGFR1 expression and results in dependency on the receptor signaling [40]. The chromatin regulators CREBBP and EP300 were validated as tumor suppressors in SCLC by loss-of-function studies in the *RP* and *RPR2* models, respectively [19, 52], while the SWI/SNF component BRG1 (also known as SMARCA4) is required for tumor growth in the *RP* model [53]. Finally, the role of developmental signaling pathways has been assessed in mouse models of SCLC, including an oncogenic role for Hedgehog signaling [39], TGFβ signaling [54], and the WNT5A ligand in the non-canonical WNT pathway (with no effect of β-catenin loss in the same context) [55], and a tumor suppressive role for Notch signaling [31].

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These experiments (summarized in Table 1) have provided extremely informative insights into the biology of SCLC, including mechanisms controlling heterogeneity and plasticity as well as response to treatment in immunocompetent mice where tumors develop in the native immune microenvironment of the lung. However, it is important to note that only about ~30 candidate genes have been functionally investigated in the 20 years since the publication of the *RP* model. This is in part due to our initial poor understanding of genes and pathways implicated in SCLC development and response to treatment, but also due to the time and cost of generating mice with combinations of mutations using classical mouse genetics.

Recent novel insights into the biology of human SCLC from genomic studies

A number of recent genomic analyses of human SCLC tumors (e.g., whole genome sequencing, exome sequencing, or targeted sequencing) confirmed near universal loss of *RB1* and *TP53* [31, 56–59], validating the relevance of the *RP* model to human SCLC. However, these studies have also revealed numerous other genetic alterations that co-occur with *RB1* and *TP53* loss, nominating several candidate tumor suppressor genes and relatively fewer candidate oncogenes. While some of these candidate drivers have already been tested in SCLC GEMMs using classical mouse genetics, roles for many of these candidates have yet to be explored. In addition, RNA sequencing (RNA-seq) and immunohistochemistry (IHC) from human tumors have uncovered unsuspected levels of inter- and intra-tumoral heterogeneity in SCLC, which has led to the nomination of molecular SCLC subtypes based on expression of lineage-related transcription factors. These SCLC molecular subtypes broadly consist of: (1) ASCL1 (60–70%); (2) NEUROD1 (20–30%); (3) POU2F3 (10–15%); and (4) Inflammatory, with generally low expression of ASCL1/NEUROD1/POU2F3 that sometimes expresses YAP1 (10–15%) [9–11, 60–64]. IHC data from human SCLC and single-cell RNA-seq analyses have also uncovered intra-tumoral heterogeneity of molecular subtypes with ASCL1 and NEUROD1 expression either being co-expressed or expressed in a mutually exclusive manner in a significant fraction of SCLC tumors [25, 61, 65–67]. In contrast, cells in the *RP* and *RPR2* SCLC models homogenously express ASCL1 in nearly all tumors [26, 43]. The *RPM* model expresses NEUROD1 and can evolve to become non-neuroendocrine and express YAP1 demonstrating MYC drives plasticity between SCLC subtypes [25]. The *RPM* mutant mice can also develop POU2F3-positive tumors but only when an unknown cell of origin is targeted with Ad-CMV-Cre [22, 25]. Therefore, current SCLC GEMMs only partially encompass the transcriptional subtype diversity and intra-tumoral heterogeneity observed in human SCLC, with efficient GEMMs of POU2F3-positive SCLC and Inflammatory-SCLC particularly lacking.

This additional level of complexity is challenging to address with classical SCLC GEMMs, as incorporating additional floxed alleles (for candidate tumor suppressors) or transgenes (for candidate oncogenes) is slow and costly. Thus, it is important for the field to develop new models with lower costs and easier access to a larger group of investigators.

CRISPR/Cas9 mouse models of SCLC

The development of the first CRISPR/Cas9 transgenic mouse models has allowed for the opportunity to develop SCLC GEMMs more rapidly through somatic gene editing using CRISPR/Cas9, creating homozygous null alleles for tumor suppressor genes of interest.

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Differences between “classical” GEMMs and the new generation of models described below are summarized in Table 2.

The first Cas9 mouse was engineered by placing the Cas9 transgene in the *Rosa26* locus where Cas9 was either ubiquitously expressed or could be conditionally expressed through Cre-mediated recombination of a *Lox*-Stop-*Lox* (LSL) cassette (referred to hereafter as *Rosa26-LSL-Cas9* mice) [68]. Adeno-associated viruses (AAVs) encoding sgRNAs targeting *Trp53, Stk11*, and *Kras* with a homology directed repair (HDR) template encoding the $Kras^{G12D}$ mutation and Cre recombinase were intratracheally (IT) instilled into *Rosa26-LSL-Cas9* mice. Mice developed lung adenocarcinomas demonstrating the feasibility of introducing a single AAV vector into Cas9 mice to make GEMMs of cancer entirely using CRISPR/Cas9 [68]. This approach allows for inclusion of multiple sgRNAs of interest into a single AAV and bypasses the requirement for time-consuming breeding to develop GEMMs. Shortly thereafter, another Cas9 transgenic mouse model where the Cas9 transgene was inserted into the *H11* locus (*H11-LSL-Cas9* mice) was developed [69]. Using this model, lentiviruses that encode Cre recombinase and a single sgRNA were injected via retrograde pancreatic ductal injection to generate pancreatic ductal adenocarcinomas [69].

In 2019, *LSL-Cas9* mice were utilized to make an *RPR2*-equivalent SCLC GEMM using a single AAV. AAV was engineered to encode sgRNAs against *Rb1, Trp53*, and *Rbl2* (referred to as *RPR2*) and Cre recombinase driven by an *EFS* promoter. Intratracheal instillation of this AAV into *LSL-Cas9* mice yielded autochthonous lung tumors with histology consistent with SCLC and most tumors spontaneously metastasized to the liver. CRISPR amplicon sequencing confirmed complete loss of the *Rb1* and *Trp53* alleles in all tumors with *Rbl2* loss in at least one allele [70]. However, with AAV, there were a significant fraction (~25%) of tumors with histology consistent with histiocytic sarcoma. Subsequently, adenovirus encoding *RPR2* sgRNAs and Cre recombinase driven by a *CMV* promoter was used instead of AAV and autochthonous tumors formed in nearly 100% of mice intratracheally instilled with this single adenovirus [70]. The histology of all tumors was consistent with SCLC and the majority of mice had spontaneous liver metastases. Of note, the latency of both AAV and adenovirus CRISPR/Cas9 models was longer (8–10 months) than observed with the *RPR2* SCLC GEMM using classical mouse genetics (4–6 months), which could suggest different cell types of origin and/or differences in the efficiency of Cre and Cas9 to induce mutations in this context. This all-in-one adenovirus was then modified to include an additional sgRNA of interest (referred to hereafter as “sgT” for target sgRNA) (Fig. 2A). Initially the histone demethylase KDM5A was selected for targeting as *KDM5A* was necessary for SCLC proliferation and ASCL1 neuroendocrine differentiation in SCLC cell lines [70]. Tumor formation was significantly delayed in mice that received the sg*Kdm5a-RPR2* adenovirus compared to mice that received non-targeting sgRNA (sgControl)-*RPR2* adenovirus leading to an increase in overall survival in sg*Kdm5a-RPR2* mice. These results show that KDM5A is important for SCLC tumorigenesis and demonstrate the feasibility of somatic gene editing using CRISPR in *LSL-Cas9* mice to generate SCLC GEMMs with at least four inactivating mutations (sgT + *RPR2*) all encoded in a single adenovirus (Fig. 2B) [70]. This approach allows for testing candidate targets of interest in SCLC GEMMs without the requirement for time-consuming breeding, and only requires the investigator to maintain *LSL-Cas9* mice and engineer adenoviruses using traditional and gateway recombination cloning. The

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adenoviral vectors from the original study described above were subsequently modified so that a target gene sgRNA (sgT) could be easily cloned into the vector using traditional restriction enzyme cloning (available on Addgene, #209060). This is followed by gateway recombination cloning to generate the final adenoviral vectors. The adenovirus vector is referred to hereafter as sgT_*RPR2*_CMV-Cre, and may be employed in both *Rosa26-LSL-Cas9* or *H11-LSL-Cas9* mice. Subsequently, this all-in-one adenoviral vector approach has been used to study the role of inactivating candidate target genes of interest including *Notch1*, *Notch2*, *Ascl1*, and *Kdm6a* [71, 72]. Consistent with the study showing that ASCL1 was essential for SCLC tumor formation in *RPR2* mice using classical mouse genetics [43], inclusion of an *Ascl1* sgRNA in the *RPR2* CMV-Cre adenovirus similarly blocked tumor formation in nearly all mice. Interestingly, there were a few tumors that did form in sg*Ascl1-RPR2* mice and amplicon sequencing showed in-frame insertion/deletions (indels) at the *Ascl1* sgRNA binding site, demonstrating strong selective pressure to maintain ASCL1 for SCLC tumorigenesis in the *RPR2* model [72]. Moreover, these results suggest that this approach can be used to understand the consequences of deleting genes that are necessary for SCLC tumor formation with the caveat that some tumors arising in the model may escape homozygous deletion of the targeted gene. Inactivation of the epigenetic modifier *Kdm6a*, which is mutated at a low frequency in human SCLC (3–4%) [31, 73], yielded SCLC neuroendocrine tumors with intra-tumoral heterogeneity of ASCL1 and NEUROD1 expression [71]. Heterogenous expression of ASCL1 and NEUROD1 is frequently observed in human SCLC (35–40%) [61] and hence the sg*Kdm6a-RPR2* is a SCLC GEMM (along with *RPM* mice) that can be used to model this ASCL1 and NEUROD1 intra-tumoral heterogeneity. Moreover, it provides evidence that this approach to make SCLC GEMMs with additional cooperating mutations could yield insights into genetic drivers of SCLC molecular subtypes and eventually provide the field with more diverse SCLC GEMMs that capture the transcriptional heterogeneity seen in human SCLC.

Another recent study used a complementary approach that bypasses the need for Cas9 transgenic mice with an adenovirus encoding a CRISPR/Cas9/Cre hybrid vector developed by genetically fusing the CRISPR system (sgRNA and Cas9 endonuclease) to Cre recombinase through a self-cleaving T2A peptide derived from *Thosea asigna virus* (Fig. 2C) [52]. The resulting vector expresses the sgRNA and Cas9-2A-Cre fusion protein. Self-cleavage of the 2A peptide releases Cas9 and Cre that in turn induce sgRNA-assisted mutation and deletion of floxed *Rb1* and *Trp53* alleles, respectively, within the same lung epithelial cell. This CRISPR/Cas9/Cre hybrid system, delivered in a single adenoviral vector, accomplishes the same goal as the other systems without requiring expensive, time-consuming genetic crosses and allows simultaneous characterization of multiple mutations. The vector has been used to test the impact of recurrent inactivating mutations affecting histone acetyltransferase domain (HAT) in *Ep300* by targeting the coding sequences in vivo [52]. This is particularly significant because existing mouse strains with conditional alleles are designed for complete knockout of *Ep300* and a conditional allele for the sequence encoding the HAT domain is not available. This CRISPR/Cas9 approach to generate SCLC GEMMs is tractable, inexpensive, and easily expandable for testing additional mutations. Moreover, these CRISPR/Cas9 somatic engineering approaches are readily applicable to

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other cancer models that are receptive to adenoviral gene delivery including non-small-cell lung cancers.

Variations of the CRISPR/Cas9-gene-editing approach to SCLC GEMMs have been described in other studies to interrogate the function of candidate SCLC drivers. One study utilized an adenoviral vector (named Ad5-USEC) to express sgRNA and Cre in *RP; Rosa26-LSL-Cas9* mice and to test the impact of inactivating *Rbl1* (*p107*) or *Rbl2* (*p130*) on tumor development (Fig. 2D) [37]. This study validated the tumor suppressor role of p107 and p130 and also showed differential impacts of inactivating the *Rb1* homologs on SCLC development in mice. This functional heterogeneity among the closely related tumor suppressors highlights the need for more accurate modeling of the SCLC genome and supports the somatic engineering approach.

In another study, CRISPR-mediated somatic engineering was also used to model a recurrent gene fusion in *RPR2; LSL-Cas9* mice [24]. *RLF-MYCL* gene fusion is detected in ~5% of ASCL1-positive human SCLC tumors [57]. To model the gene fusion in the mouse genome, double-strand DNA breaks were generated in the first introns of *Rlf* and *Mycl* by infecting *RPR2; Rosa26-LSL-Cas9* mice with a lentiviral vector that express both Cre and gRNAs targeting the intron of *Rlf* and *Mycl* [24]. This targeting approach, first used to model lung adenocarcinoma [74], successfully resulted in the gene fusion comparable to that in patient tumors and accelerated SCLC development and metastasis in mice.

Increasing the throughput of gene targeting

A major limitation of the first generation of GEMMs for SCLC is the requirement for multiple crosses to combine loss of *Trp53* and *Rb1* with other alleles of interest. The recent development of Tuba-seq (tumor barcoding with ultradeep barcode sequencing) has enabled the functional investigation of multiple putative cancer drivers simultaneously in a pooled format in a quantitative manner [75, 76]; this approach was first developed in GEMMs of lung adenocarcinoma but could be applied to any cancer type. Briefly, the Tuba-seq approach consists of transducing lung epithelial cells with a lentiviral vector that expresses both Cre recombinase (to delete genes that are flanked by *lox* sites) and sgRNA molecules (to mutate targets of interest in cells expressing Cas9). A key aspect of this approach is that the Lenti-sgRNA/Cre contains both a clonal identifier (i.e., a random DNA barcode, BC) and a vector-specific identifier (i.e., sgRNA-ID or sgID). Sequencing of harvested lungs allows for a quantitative measure of tumor growth for each sgRNA tested. One issue in the implementation of Tuba-seq to SCLC has been that the lentiviral vectors used by the majority of investigators do not initiate tumors in GEMMs as efficiently as adenoviral vectors. This could be because the VSV-G envelope protein for lentiviral vectors that is commonly used does not interact efficiently with its receptor on the main cell type(s) of origin for SCLC, but other pseudotyping systems have not been tested. Another difference between lentiviral and adenoviral vectors that can explain differences in tumor initiation is that titers are often lower with lentiviral vectors. This study found that pre-treatment of mice with naphthalene, which damages the lung epithelium, significantly increased the efficiency of tumor initiation with Lenti-Cre vectors. Naphthalene is particularly toxic to club and ciliated cells and can lead to expansion of basal, neuroendocrine, and/or

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variant club cells [77, 78]. While the mechanisms by which naphthalene promotes SCLC initiation upon Lenti-Cre infection are not well understood, it could be because tissue repair is accompanied by cell proliferation of particular cell types, which may facilitate viral integration and transgene expression. Combining naphthalene pre-treatment and the Tuba-seq platform, *RPR2; H11-LSL-Cas9* mice were infected with a Lenti-sgRNA/Cre library encoding sgRNAs to quantitatively assess the impact of 40 candidate genes on the initiation and growth of SCLC in a relatively low number of animals [79]. This work highlights the role of the PI3K-AKT-mTOR pathway in SCLC development, including the TSC1/2 tumor suppressors.

This Lenti-sgRNA/Cre approach can significantly accelerate the testing of candidate genes for SCLC growth. It only requires the investigator to obtain *RPR2; H11-LSL-Cas9* and *RPR2* mice as controls (or other GEMMs with and without inducible Cas9). It is also not necessary to use the Tuba-seq platform and one can simply test genes individually with sgRNAs (similar to the lentiviral platform described above with the *Rlf-Mycl* fusion). One limitation is that we still do not know what cell type(s) initiate tumors from lentiviruses. Also, while broad inactivation of *Rb1/Trp53* throughout the lung leads predominantly to SCLC, as additional driver genes are perturbed, including those important for other cancer types, there is potential for broader tumor types to emerge. Thus, it is important to validate screen hits using single sgRNAs to test the impact on tumor growth and histopathology. Another limitation is the size of the DNA fragments that can be used with lentiviral vectors, which limits the promoter regions that can be used to direct transgene expression to specific cell populations. AAVs are also limited in the size of the DNA fragments that can be used and, in contrast to lentiviruses, AAVs do not integrate into the genome. Adenoviruses, on the other hand, can accommodate significantly more genetic material than lentiviruses or AAVs, but also suffer the disadvantage of not integrating into the genome. See Table 3 for viral approaches used to make SCLC GEMMs. We note that in the *RPM* model, tumors can be efficiently generated with lentiviral vectors even without naphthalene pre-treatment [79], consistent with increased oncogenic competence in this model [22, 47].

Syngeneic mouse models as an orthogonal approach to study SCLC in an immunocompetent host

SCLC GEMMs are autochthonous models where tumors develop in the native lungs of immunocompetent mice allowing investigators to study SCLC pathogenesis at its earliest stages. Moreover, SCLC GEMMs spontaneously metastasize to distant organs (e.g., liver) recapitulating the highly metastatic nature of human SCLC. As described above, SCLC GEMMs can be made using classical mouse genetics or with CRISPR/Cas9-mediated somatic engineering. An alternative strategy is to develop syngeneic mouse models, or allografts, using cell lines or organoids derived from these autochthonous SCLC GEMMs (Fig. 3A). In SCLC, this has been achieved by multiple laboratories using cell lines derived from SCLC GEMMs using classical mouse genetics [80–85], and from CRISPR/Cas9 SCLC GEMMs [81, 86]. Most of these studies subcutaneously injected SCLC GEMM-derived cell lines into the flanks of host mice, but allografts can be generated in any organ site. For example, transthoracic injection generates SCLC allografts in the lungs of C57BL/6 mice [81], tail vein injections lead to liver metastases [87], and intracranial injections mimic

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brain metastases [88] (Fig. 3B). Some advantages of allografts are that: (1) large cohorts of immunocompetent mice can be transplanted to study the interplay of the tumor immune microenvironment with SCLC tumor cells; and (2) tumors have a shorter latency compared to most autochthonous SCLC GEMMs. Thus far, allografts have been commonly used to study the combination of immune checkpoint blockade with another targeted agent of interest. For example, SCLC allografts were recently used to discover that LSD1 inhibition cooperates with PD-1 immune checkpoint blockade in two independent studies [82, 83], which has inspired clinical trials testing the combination of LSD1 inhibitors with PD-L1 checkpoint blockade. Allograft experiments can also be utilized to ask basic questions about SCLC tumor immunology. For example, non-neuroendocrine derivatives of the original GEMM-derived SCLC neuroendocrine cell line *RPR2* 631 had restored surface expression of MHC-I and were immunologically rejected when transplanted back into host mice [86]. Moreover, these SCLC GEMM-derived cell lines can be genetically manipulated ex vivo prior to transplantation to determine how activating or inactivating a candidate gene of interest impacts anti-tumor immunity or the response to immunotherapy. This offers the opportunity for more high-throughput CRISPR/Cas9 in vivo screening to unbiasedly identify targets that synergize with immunotherapy as previously explored in other syngeneic mouse models [89–91].

SCLC syngeneic models utilize cell lines derived from established SCLC GEMMs. Therefore, they cannot be used to study the early events of SCLC pathogenesis nor to identify genes required for full oncogenic transformation. To dissect the early events required for SCLC tumorigenesis, a precancerous cell (preSC)-based model of SCLC development was developed (Fig. 3C). This was established using cells isolated from microscopic neuroendocrine lesions developed in the lungs of $Rb1^{flox/flox};Trp53^{flox/flox};Rbl2^{flox/+}$ mice infected with adeno-CMV-Cre [16]. This identification of preSCs itself is significant as the preneoplasia of SCLC in human patients, unlike those identified for lung squamous cell carcinoma and adenocarcinoma, remains elusive. Importantly, the fact that these mutant neuroendocrine cells are immortalized and maintain key features of premalignancy rationalizes an approach to define an oncogenic driver for its ability to transform cells to full-blown tumor. This cellular system is particularly well suited for assessment of candidate SCLC driver gene function. For example, lentiviral over-expression of the MYC family members, FGFR1, or WNT5A in preSCs increased cellular proliferation and drove transformation [35, 40, 55]. CRISPR/Cas9-mediated targeting of *Crebbp* or *Ep300* was sufficient to drive tumorigenic progression of preSCs [19, 52]. CRISPR-driven formation of the *Rlf-Mycl* gene fusion accelerated transformation and preSC proliferation [24]. These preSC models have served as a complementary experimental system to the GEMMs not only for functional validation of the recurrent SCLC drivers but also for elucidating the mechanism of SCLC development. Comparative profiling of preSCs and tumor cells permitted identification of the molecular pathways and vulnerabilities associated with tumor progression. For instance, defining key oncogenic pathways driven by L-MYC, FGFR1, and WNT5A led to a preclinical test of existing drugs that inhibited the growth of human and mouse SCLC tumors [16, 40, 55]. The tractability of this preSC-based model allows for functional interrogation of a large number of candidate driver mutations found in the SCLC genome, increasing the likelihood of identifying key oncogenic drivers. The

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preSC system has also been used for genome-scale functional screens to systematically identify genes with tumor suppressor activity [20]. Furthermore, since the preSCs can be implanted in immunocompetent C57BL/6 mice, it can serve as an experimental system for studying the role of candidate regulators involved in the co-evolution of SCLC precursors and the immune microenvironment during tumor development.

A similar approach isolating primary cells from the earliest stages of tumor formation has been applied to the *RPM* model [25]; in this case, MYC-driven cells transition in vitro from ASCL1$^+$ to NEUROD1$^+$ to YAP1$^+$, providing a system to study subtype plasticity, including genes and factors that may alter this trajectory. For example, pharmacological Notch inhibition blocked cell progression to the YAP1$^+$ state [25], consistent with the ability of Notch signaling to promote non-neuroendocrine SCLC [28]. Finally, organoids can be generated from normal tracheal basal cells of GEMMs, and then administered Cre recombinase to induce genetic alterations, or organoids can be generated from established tumors; transformed organoids form allograft tumors in immune-deficient mice that recapitulate their primary tumor counterparts (T.G.O., unpublished data). Approaches such as these can be coupled with CRISPR-based approaches as described above to study tumor-initiating mechanisms and enable functional studies. A mouse lung organoid approach was recently used to interrogate the consequences of deleting the methyltransferase *Kmt2c* [92]. Here, organoids were generated from C57BL/6 lungs and lentiviral vectors expressed sgRNAs toward *Rb1*, *Trp53* and *Kmt2c* and also overexpressed MYC and Cas9. Orthotopic injection of genetically perturbed organoids into the lungs of C57BL/6 mice revealed roles for KMT2C in tumor suppression and metastasis. Studies have identified differences in cell fate and other cancer properties when cells are grown in two-dimensional culture as opposed to three-dimensional organoid settings ([93–95] and T.G.O., unpublished observations); each condition may select for a specific tumor cell fate, which highly influences tumor behavior. Thus, it is critical when using these approaches to evaluate and understand the lineage identity of cells in their culture conditions.

Limitations of the current approaches using CRISPR/Cas9-mediated inactivation and future opportunities

Although there are several advantages to utilizing CRISPR/Cas9 to study the role of candidate drivers more rapidly in SCLC tumorigenesis, there are limitations to the current approaches. The all-in-one single vector approach and the CRISPR/Cas9/Cre hybrid approach uses adenoviruses that do not integrate into the host genome and hence can only be used to create loss-of-function alleles at tumor initiation. Moreover, the current CRISPR/Cas9-based models cannot be used to express an oncogenic variant, as is done with transgenes using classical mouse genetics; nor can they be used to test the consequences of genetically inactivating a target gene in established tumors, as this would require a virus that integrates into the host genome (e.g., lentivirus) with an inducible Cas9 system.

It is often desirable to understand the consequences of genetically inactivating a candidate gene in established tumors (e.g., tumor maintenance) because this more closely mirrors pharmacological inhibition of a candidate therapeutic target. This has been achieved using inducible systems (tamoxifen induction or doxycycline responsive promoters) with classical

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mouse genetics [96, 97]. This is not achievable with the current adenoviral approaches for CRISPR/Cas9 somatic engineering in SCLC as this requires a lentivirus to integrate into the host genome. Lentiviral infection is feasible given the recent success of using CRISPR/Cas9 somatic engineering with lentiviruses to make SCLC GEMMs [24, 79]. One approach to achieve inducibility could be to engineer a lentivirus that encodes Cre recombinase, an inducible sgRNA, and an rtTA, and then infect a SCLC GEMM Cas9 mouse (e.g., *LSL-Cas9-RP*, *LSL-Cas9-RPR2*, or *LSL-Cas9-RPM* mice) with the lentivirus. Using this approach, sgRNA expression is induced with doxycycline after tumor formation. Alternatively, one could utilize doxycycline-inducible Cas9 transgenic mice [98] crossed with *RP, RPR2*, or *RPM* mice and then inject a lentivirus encoding Cre recombinase and an sgRNA. Both approaches are worthy of investigation as it would be valuable to genetically inactivate a candidate therapeutic target with temporal control during tumor maintenance to test gene driver function.

Conversely, it is also desirable to activate a candidate driver gene (e.g., MYC family members or FGFR1) and study its function during SCLC tumorigenesis. Classically, this has been achieved with transgenic mice, but now could possibly be achieved using CRISPR/Cas9 with CRISPR activation (CRISPRa). For CRISPRa, a variant of Cas9 that lacks endonuclease activity (dCas9) is fused to a transcriptional activator. This Cas9 variant is expressed with an sgRNA that recognizes the transcription start site of a gene of interest promoting gene activation [99–101]. Recently transgenic CRISPRa mice have been developed where dCas9 is fused to the p300 activator and placed in the *Rosa26* locus with an LSL cassette for inducible dCas9-p300 expression [102]. Other CRISPRa mice have since been developed [103]. These mice could be used to attempt to activate candidate driver genes in SCLC GEMMs. A lentiviral approach for sgRNA transduction would be necessary as viral integration into the genome would allow for continual activation of the driver gene during SCLC tumorigenesis.

In contrast to NSCLC, human SCLC infrequently harbors recurrent mutations in oncogenic drivers and therefore most SCLC mouse models rely on loss-of-function mutations in tumor suppressors and have not used gain-of-function oncogenic variants, apart from the modeling of MYC family members and FGFR activation [21, 25, 26, 40, 41, 47]. However, many cancers including NSCLC are driven by oncogenic variants, and GEMMs for these cancers utilize transgenes with point mutations in oncogenic variants (e.g., KRAS, EGFR, BRAF) [104–106]. For SCLC, there is utility in introducing mutations into endogenous alleles to model candidate rare oncogenic variants or to understand drug resistance mechanisms. Therefore, newer approaches are emerging to precisely engineer point mutations in endogenous alleles using CRISPR/Cas9 somatic engineering. An all-in-one CRISPR/Cas9 approach using AAV was attempted to positively select for oncogenic $KRAS^{G12D}$ point mutations to create $KRAS^{G12D}$-mutant lung adenocarcinomas [68]. This was achieved using an AAV encoding sgRNAs against *Kras*, *Trp53*, and *Stk11* and an HDR template to introduce G12D with the expectation that the G12D HDR template would be positively selected [68]. However, introducing point mutations using sgRNAs to create double-strand breaks for HDR repair is highly inefficient, and although $Kras^{G12D}$ mutations were observed, there were several tumors that had loss of *Trp53* and *Stk11* without $Kras^{G12D}$ point mutations. Newer, more efficient CRISPR/Cas9 engineering approaches

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have been developed including base editing and prime editing [107, 108]. Mice capable of CRISPR/Cas9 prime editing [109] and CRISPR/Cas9 base editing [110] were developed in 2023. Prime editing utilizes Cas9 with a reverse-transcriptase to precisely and efficiently engineer mutations of interest. It can be utilized to introduce hotspot mutations or loss-of-function mutations. In the proof-of-principle study, hotspot mutations for *Kras* and *Trp53* were somatically introduced into the lung and pancreas [109]. Base editing can utilize either cytosine or adenine base editors. The base editing transgenic mouse utilizes a Cas9 cytosine base editor where the transgenic allele is under control of a tetracycline responsive element and therefore base editing can be inducibly controlled with doxycycline [110]. Both prime editing and base editing mice allow for precise somatic engineering of point mutations into endogenous alleles, which can facilitate the development of GEMMs with oncogenic variant alleles, allow the study of specific consequences of point mutations within a domain of a tumor suppressor gene, and/or engineer point mutations to study drug resistance.

The current CRISPR/Cas9 models described above utilized up to four sgRNAs to simultaneously delete four genes of interest to generate SCLC GEMMs. Human SCLCs have a high mutational burden nearly always harboring more than four potential driver mutations. It is possible that the current approaches could allow for cloning additional sgRNAs into the adenoviral vector for CRISPR/Cas9 somatic engineering to test a larger combination of mutations more closely resembling the complex patterns of driver mutations found in human SCLC. However, there is likely a limit to how many target genes can be altered using these current approaches. Recently a Cas12a transgenic mouse model was developed [111]. Cas12a has advantages over Cas9 in that it does not require a separate TracrRNA for each CRISPR RNA (crRNA), and thus it is easier to multiplex sgRNAs in a single vector. Hence, Cas12 transgenic mice may offer an opportunity for multiplexing sgRNAs for generation of more complex genetic models to better understand the combinatorial effects of driver mutations in SCLC. Moreover, some mutations in SCLC are truncal (*RB1* and *TP53*) and therefore present in the vast majority of tumor cells, while others are likely subclonal. Given that the PAM recognition site of Cas9 and Cas12a are distinct, there is an opportunity to utilize both transgenes (Cas12a and Cas9) together in a single mouse for temporal control of gene inactivation. This could achieve somatic gene editing to inactivate drivers at tumor initiation (e.g., *Rb1* and *Trp53*) and other drivers in established tumors yielding an enhanced understanding of SCLC tumor evolution. As we learn more about the mutual exclusivity and co-occurrence of mutations found in human SCLC, this combinatorial mutation approach offers opportunities to model the functional relationships of genetic alterations in a temporally controlled manner.

In conclusion, the new approaches to modeling SCLC in mice will accelerate functional characterization of SCLC-mutated genes including complex combinations of alterations for roles in tumor suppression, metastasis, drug response/resistance, and tumor-immune-stromal cell interactions. To that end, a rational integration of the models is important and should be based on information gathered from the advanced profiling of fresh patient tumors, circulating tumor cells, and PDX/CDXs. We anticipate that future GEMMs will better capture key subsets of human SCLC such as POU2F3-positive and low-neuroendocrine, inflamed SCLC. Ultimately, the approaches to modeling SCLC in mice could evolve toward

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mimicking the major biologically distinct subsets of patient tumors, enabling discovery of specific vulnerabilities and testing of personalized therapies.

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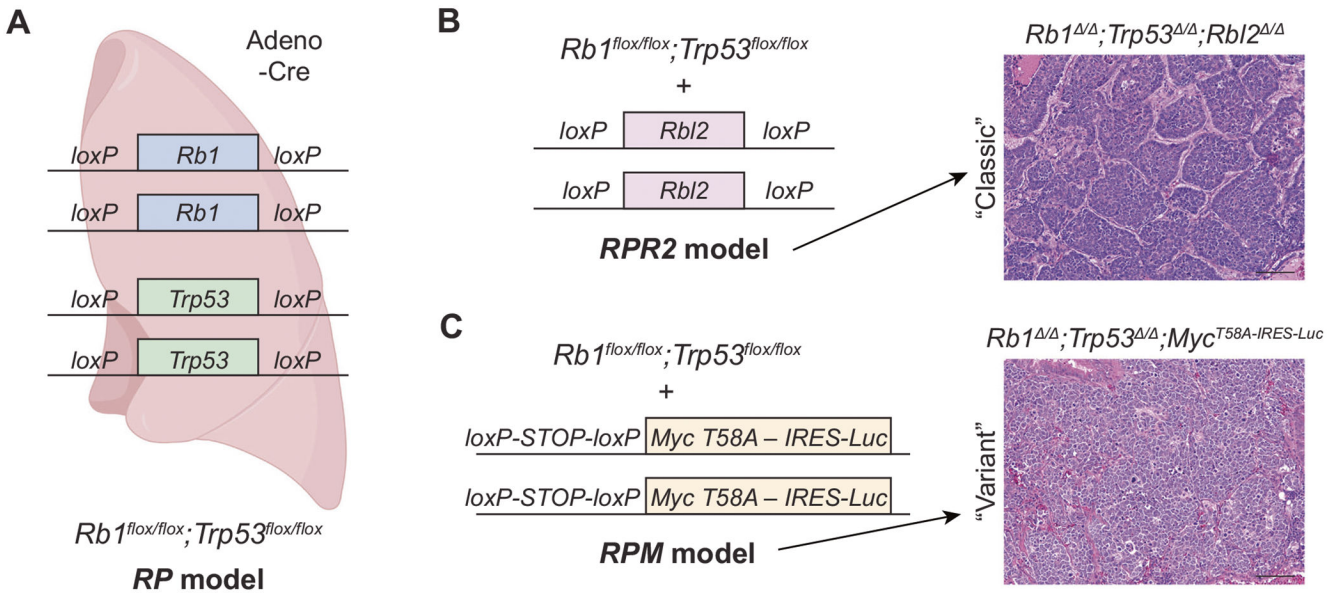

Fig. 1. Frequently used SCLC GEMMs developed using classical mouse genetics.

A The *RP* model is generated by intratracheally injecting adeno-Cre into the lungs of mice that harbor homozygous *Rb1*$^{flox/flox}$ and *Trp53*$^{flox/flox}$ alleles. Intranasal delivery is an alternative route for delivery, but can induce olfactory tumors in some contexts, so intratracheal injection is preferred. **B** The *RPR2* model is generated by intratracheally injecting adeno-Cre into the lungs of mice that harbor homozygous *Rb1*$^{flox/flox}$, *Trp53*$^{flox/flox}$, *Rbl2*$^{flox/flox}$ alleles. The "classic" SCLC histology of the *RPR2* GEMM is shown on the right (hematoxylin and eosin counterstain). **C** The *RPM* model is generated by intratracheally injecting adeno-Cre into the lungs of mice that harbor homozygous *Rb1*$^{flox/flox}$, *Trp53*$^{flox/flox}$ alleles, and transgenic *lox*P-Stop-*lox*P *Myc*^{T58A}-*Ires-Luciferase* knocked into the *H11b* locus. The creators and subsequent investigators have noted that while the *Myc* allele harbors the gene coding for luciferase, this allele has not proven reliable for quantitative bioluminescent imaging; however, the *Luc* transcript can be utilized in single-cell transcriptomics and potentially for other applications. The "variant" SCLC histology of the *RPM* GEMM is shown on the right. However, the *RPM* GEMM has histological variation from "classic" in situ lesions toward more invasive tumors. Scale bar, 100 μm.

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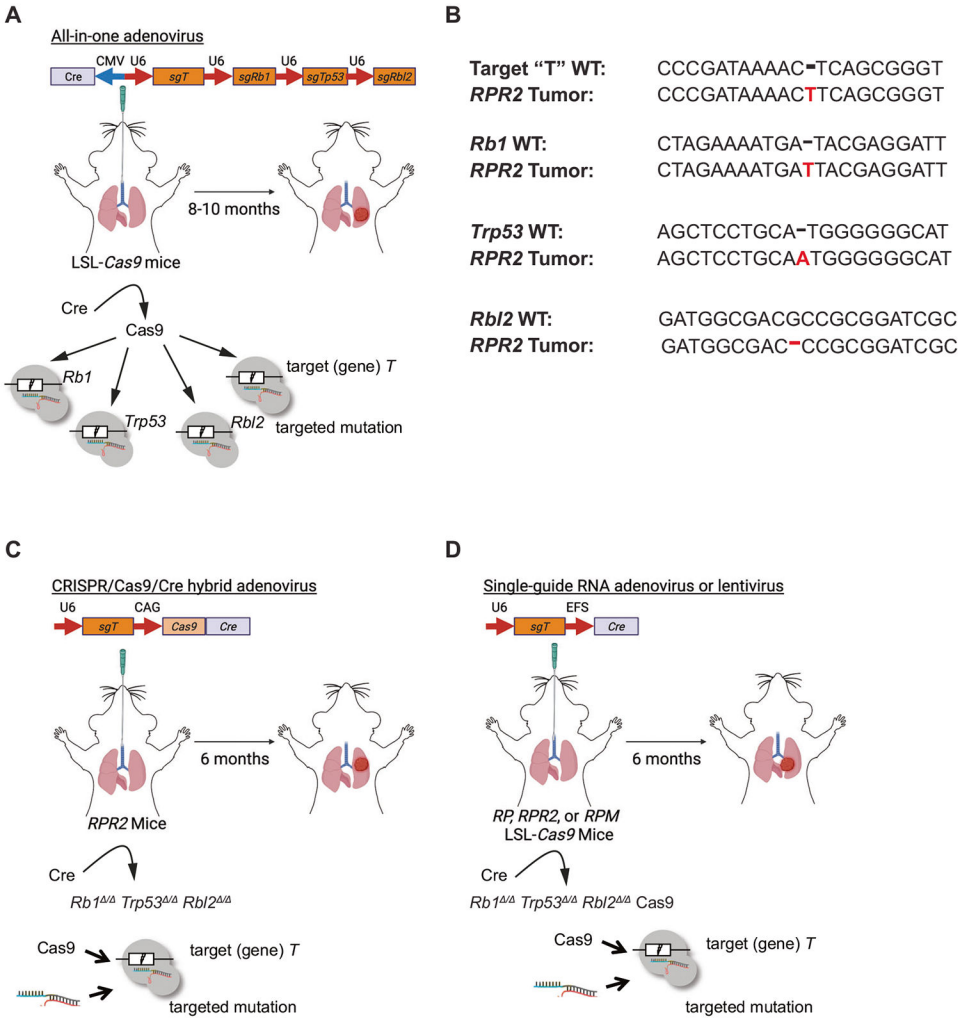

Fig. 2. Approaches to make the "Next Generation" of SCLC GEMMs using CRISPR/Cas9 somatic engineering.

A The all-in-one adenoviral approach to make CRISPR/Cas9 SCLC GEMMs using *LSL-Cas9* mice where the adenovirus contains sgRNAs targeting *Rb1*, *Trp53*, *Rbl2*, and a gene of interest (sg*T*) as well as CMV-Cre. **B** Examples of insertion or deletions (indels) in *Rb1*, *Trp53*, *Rbl2*, and sg*T* (*Kdm5a*) from mouse SCLC lung tumors. **C** The CRISPR/Cas9/Cre hybrid adenoviral approach to make CRISPR/Cas9 SCLC GEMMs using *RPR2* mice where the adenovirus contains sg*T*, *Cas9*, and *Cre*. **D** The CRISPR/Cas9 adenoviral or lentiviral approach to make CRISPR/Cas9 SCLC GEMMs using *RP, RPR2*, or *RPM LSL-Cas9* mice where the adenovirus contains sg*T* and Cre. **A**, **C**, and **D** were created with BioRender.com.

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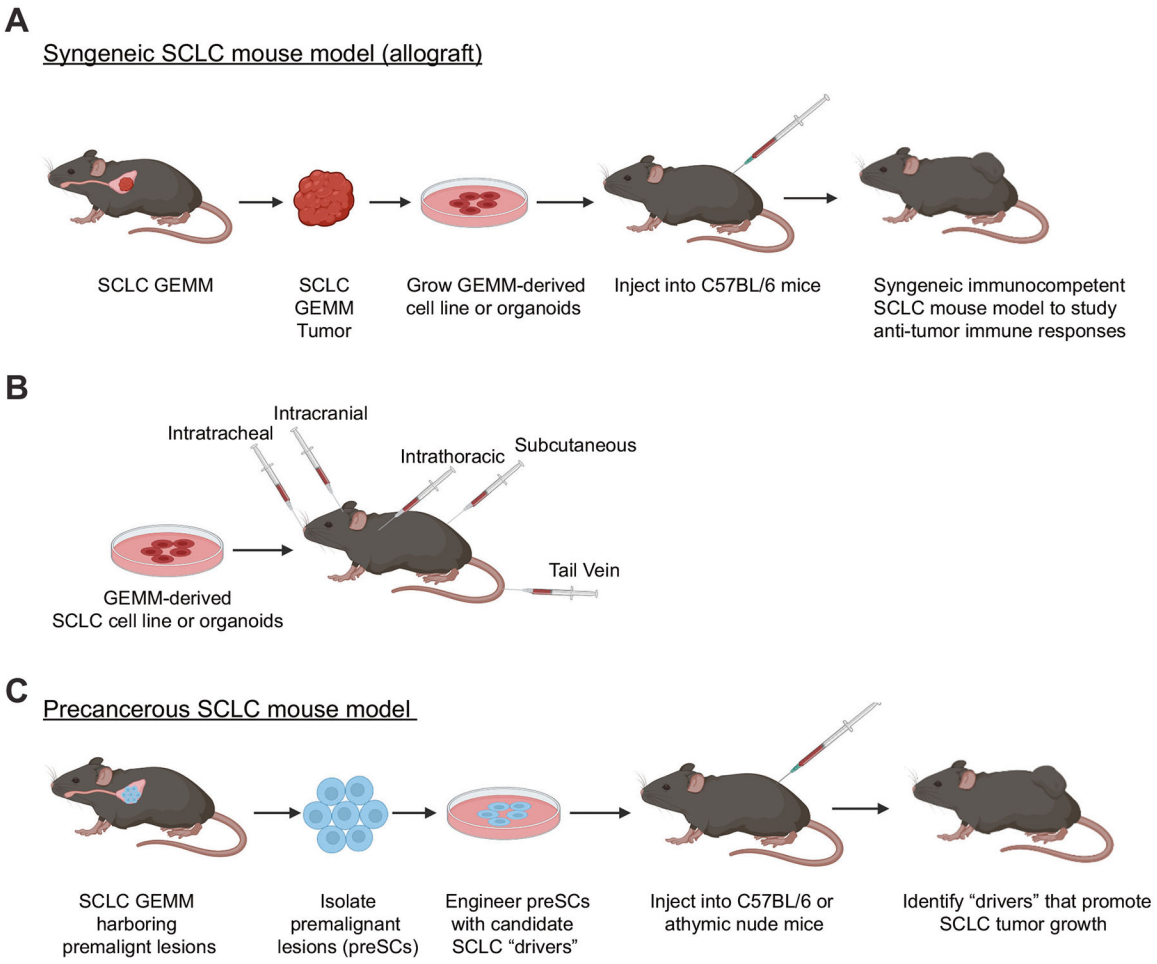

Fig. 3. Approaches to generate immunocompetent syngeneic mouse models of SCLC.

A Schematic showing the process of developing syngeneic immunocompetent mouse models of SCLC (allografts) from autochthonous SCLC GEMMs. **B** Possible injection sites to grow syngeneic SCLC models are shown including intracranial, intrathoracic, intratracheal, subcutaneous, and tail vein. **C** Schematic showing the process of developing precancerous SCLC mouse models (preSCs) using SCLC GEMMs with premalignant lesions. Note that while C57BL/6 mice are often used for immunocompetent models, some of the historical *Rb1* and *Trp53* alleles were bred into the 129Sv/J background; cancer cell lines derived from these models can often be grown in F1 mice from C57BL/6 and 129Sv/J parents. **A**, **B**, and **C** were created with BioRender.com.

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Table 1.

Testing gene function in mouse models of small cell lung cancer.

Cre-driven models of SCLC			
Gene(s) of interest	**Mouse model (proposed abbreviation for frequently-used models)**	**Impact on tumorigenesis (SCLC subtype modeled[a])**	**PMID reference**
Rb1 and *Trp53*	*Rb1*$^{fl/fl}$; *Trp53*$^{fl/fl}$(***RP* model**)	Loss of RB and p53 initiates SCLC in mice using Ad-CMV-Cre[b] (SCLC-A)	14522252
Rbl2	*RP; Rbl2*$^{fl/fl}$(***RPR2* model**)	RBL2 (p130) loss using Ad-CMV-Cre or Ad-CGRP-Cre increases the number of tumor and shorten the tumor latency compared to RP mutant mice (SCLC-A)	20406968
Myc	*RP; H11b-MycT58A-IRES-Luc*$^{LSL/LSL}$(***RPM* model**)	Expression of a stable form of MYC in lung neuroendocrine cells using Ad-CGRP-Cre enhances tumor development and alters neuroendocrine differentiation (SCLC-A/N/Y with plasticity among subtypes); Ad-CMV-Cre promotes a similar phenotype	28089889 32473656
Myc, Cas9	*RPM; Rosa26-Cas9-Ires-GFP* (*RPM*-Cas9)	Similar phenotype as RPM model above, except with the presence of Cas9- Ires-GFP allele	32473656
Myc	*Rosa26*$^{LSL\text{-}MYC\text{-}IRES\text{-}hCD2}$ *and RP; Rosa26*$^{LSL\text{-}MYC\text{-}IRES\text{-}hCD2}$	Expression of MYC in lung epithelial cells is sufficient to initiate SCLC lesions; additional loss of RB and p53 leads to aggressive, metastatic disease	34675406
Mycn	*RP; Mycn tg*	Ectopic expression of N-MYC enhances tumor development in *RP* mutant mice (SCLC-A, some SCLC-N)	32820040 Unpublished, DM lab
Mycl	*RP; Mycl tg*	Overexpression of L-MYC enhances tumor development in *RP* mutant mice (SCLC-A)	27373156
	RPR2; Mycl$^{fl/fl}$	L-MYC is required for tumor development in *RPR2* mutant mice	27298335
Max	*RP; Max*$^{fl/fl}$ *RPR2; Max*$^{fl/fl}$	MAX loss enhances tumor development in *RP* mutant mice but is required for tumor growth in *RP; Mycl tg* mutant mice	32470392
Trp53	*Rb1*$^{fl/fl}$; *Trp53*$^{XTR/XTR}$; *Rbl2*$^{fl/fl}$	p53 re-expression in the equivalent of *RPR2* mutant tumors results in senescence or necrosis	37479684
Nfib	*RP; Nfib tg*	NFIB is an oncogene in *RP* mutant mice	27613844
Nfib	*RP; Nfib tg*	NFIB is an oncogene in *RP* mutant mice; NFIB may be more pro-metastatic than L-MYC (SCLC-A)	27373156
Crebbp	*RP; Crebbp*$^{fl/fl}$ *RPR2; Crebbp*$^{fl/fl}$	CREBBP loss enhances tumor development in both *RP* and *RPR2* mutant mice (SCLC-A)	30181244 Unpublished, KSP lab
Pten	*RP; Pten*$^{fl/fl}$	PTEN loss enhances tumor development in the *RP* model; tumors initiated by Ad-CMV-Cre can be SCLC (SCLC-A) and/or NSCLC	24482365, 24630729
Ep300	*RPR2; Ep300*$^{fl/fl}$ *RPM; Ep300*$^{fl/fl}$	Complete loss of EP300 inhibits tumor development in both *RP* and *RPR2* mutant mice	35171684
Smarca4	*RP; Smarca4*$^{fl/fl}$ *RPR2; Smarca4*$^{fl/fl}$	Loss of SMARCA4 inhibits tumor development in both *RP* and *RPR2* mutant mice (SCLC-A)	35967587 Unpublished, KSP lab
Ascl1	*RPR2; Ascl1*$^{fl/fl}$	Loss of ASCL1 inhibits tumor development in *RPR2* mutant mice	27452466

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Cre-driven models of SCLC			
Gene(s) of interest	**Mouse model (proposed abbreviation for frequently-used models)**	**Impact on tumorigenesis (SCLC subtype modeled[a])**	**PMID reference**
Ascl1	*RPM; Ascl1*$^{fl/fl}$	Loss of ASCL1 delays tumor development in RPM mutant mice and leads to SOX9+ neural-crest-like state preceding osteogenic differentiation	34016693
Neurod1	*RPR2; Neurod1*$^{fl/fl}$	Deletion of NEUROD1 did not impact tumor development in *RPR2* mutant mice (SCLC-A)	27452466
Cracd	*RPR2; Cracd*$^{ko/ko}$	Germline knockout of CRACD enhances tumor development in *RPR2* mutant mice (SCLC-A)	36824957
Sox2	*RPR2; Sox2*$^{fl/fl}$	Deletion of SOX2 inhibits tumor development in *RPR2* mutant mice (SCLC-A/N)	34593608
Nkx2-1	*RP; Nkx2-1*$^{fl/fl}$	Deletion of NKX2–1 leads to the development of tumors with “non-classical” histology but without induction of MYC (SCLC-A)	35848993
Hh	*RP; Hh*$^{fl/fl}$	Hh is required for efficient tumor development in *RP* mutant mice	28581526
Hh	*RP; Hh tg*	Ectopic Hh expression enhances tumor development in *RP* mutant mice	28581526
Smo	*RPR2; Smo*$^{fl/fl}$	SMO is required for efficient tumor development in *RP* mutant mice (SCLC-A)	21983857
Notch1/2	*RPR2; Notch1 ICD*^{LSL}	Overexpression of the active form of NOTCH1 or NOTCH2 is tumor suppressive in *RPR2* mutant mice	26168399
Rbpj	*Cgrp*^{CreER}*; RP; Rbpj*$^{fl/fl}$	Loss of RBPJ does not influence tumor development in *RP* mutant mice using *Cgrp* promoter-driven CreER	34597132
	RPR2; Rbpj$^{fl/fl}$	Loss of RBPJ does not affect overall tumor burden but results in a significant reduction in HES1^{+} and CC10^{+} cells in the tumors (SCLC-A)	35577801
Rest	*RPR2; Rest*$^{fl/fl}$	Loss of REST does not affect tumor development in *RPR2* mutant mice (SCLC-A)	35577801
Tgfbr2	*RP; Tgfbr2*$^{fl/fl}$	TGFβ signaling is required for tumor growth in RP mice	34373217
Smad4	*RP; Smad4*$^{fl/fl}$	TGFβ signaling is required for tumor growth in RP mice	34373217
Fgfr1	*RP; Fgfr1 K656E*^{LSL}	FGFR1 induction is detrimental to SCLC growth in in lung neuroendocrine cells but can acts as an oncogene in basal cells in *RP* mutant mice	32187553
Fgfr1	*RP; Fgfr1*$^{fl/fl}$ *RPR2; Fgfr1*$^{fl/fl}$	*Fgfr1* expression is controlled by RBL2 and FGFR1 is required for tumor growth in *RPR2* mutant mice	32973083
Ctnnb1	*RP; Ctnnb1*$^{fl/fl}$ *RPR2; Ctnnb1*$^{fl/fl}$	β-Catenin is not required for tumor development in both *RP* and *RPR2* mutant mice	36102736
Wnt5a	*RPR2; Wnt5a*$^{fl/fl}$	WNT5A is required for tumor development in *RPR2* mutant mice	36102736
Cdkn2a (p16)	*Cdkn2a*$^{fl/fl}$*; Trp53*$^{fl/fl}$	Loss of p16 can promote in situ SCLC development in *Trp*53-mutant mice (SCLC-A)	35577980
Yap1 *Yap1/Wwtr1* (*Taz*)	*Cgrp*^{CreER}*; RP; Yap1*$^{fl/fl}$ *Cgrp*^{CreER}*; Yap1*$^{fl/fl}$*; Wwtr1*$^{fl/fl}$	Loss of YAP alone does not influence tumor development in *RP* mutant mice using *Cgrp* promoter-driven CreER; however, deletion of both YAP and TAZ inhibits tumor development (SCLC-A)	34597132
CRISPR/Cas9-driven models of SCLC			

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Cre-driven models of SCLC			
Gene(s) of interest	**Mouse model (proposed abbreviation for frequently-used models)**	**Impact on tumorigenesis (SCLC subtype modeled[a])**	**PMID reference**
Gene(s) of interest	**Virus and mouse models**	**Impact on tumorigenesis (SCLC subtype modeled[a])**	**PMID reference**
Kdm5a in addition to *Rb1 Trp53, Rb12*	Ad5-CRISPR (expressing sgRNAs) infected *H11 LSL-Cas9*	Deletion of KDM5A inhibits tumor development in *RPR2* mutant mice (SCLC-A)	31727771
Notch1, Notch2, or *Ascl1* in addition to *Rb1, Trp53, Rbl2*	Ad5-CRISPR (expressing sgRNAs) infected *R26 LSL-Cas9*	Loss of NOTCH1 or NOTCH2 modestly accelerates tumor development in *RPR2* mutant mice. Loss of ASCL1 inhibits tumor development in *RPR2* mutant mice (SCLC-A)	34810201
Kdm6a in addition to *Rb1 Trp53, Rbl2*	Ad5-CRISPR (expressing sgRNAs) infected *R26 LSL-Cas9*	Loss of KDM6A promotes the development of tumors that heterogeneously express ASCL1 and NEUROD1 in *RPR2* mutant mice (SCLC-A/N)	37591951
Rbl1/Rbl2	Ad5-USEC (expressing Cre and sgRNA) infected *RP; R26 LSL-Cas9*	Loss of either RBL1 (p107) or RBL2 (p130) alone enhances tumor development and metastasis in *RPR2* mutant mice	31871154
Rlf-Mycl	Ad5-USEC (expressing Cre and two sgRNAs) infected *RPR2; R26 LSL-Cas9*	Generation of RLF-MYCL fusion protein enhances tumor development and metastasis in *RPR2* mutant mice (SCLC-A)	34344693
Ep300	Ad5-CRISPR/Cas9-Cre (expressing Cre, Cas9, sgRNA) infected *RPR2*	Unlike complete loss of EP300, generation of EP300 DHAT enhances tumor development in *RPR2* mutant mice (SCLC-A)	35171684
40 genes identified in the meta-analysis of candidate pathways in SCLC	Lenti-sgRNA-Cre (expressing sgRNA and Cre) infected *RPR2; R26 LSL-Cas9/RPR2; H11 LSL-Cas9*	PTEN and TSC1 in the PI3K-AKT-mTOR pathway were identified as robust tumor suppressors (SCLC-A)	36640300

A comprehensive table listing the genes of interest that have been tested in genetically-engineered mouse models of SCLC. The table includes the gene(s) of interest, the mouse model used to test the gene(s) of interest, the impact on SCLC tumorigenesis, and PMID reference numbers. Note that abbreviations are proposed for frequently-used models including the $Rb1^{fl/fl};Trp53^{fl/fl}$ *(RP)*, $RP;Rbl2^{fl/fl}$ *(RPR2)*, and *RP;LSL-Myc*^{T58A}*-IRES-Luc* (*RPM*). For other less frequently-used models and when new models are developed, we recommend a nomenclature that involves *Rb1/Trp53 (RP)* plus additional first initial or official gene name of additional genetic alterations. The table is subdivided into classical genetics Cre-driven SCLC models (top) and CRISPR/Cas9-driven SCLC models (bottom).

Tg transgenic mouse, *R26 Rosa26* gene, fl flox.

[a] When known, the corresponding subtype is indicated for each model in this Table (it is likely that, when unknown, models derived from the *RP* and *RPR2* models will be SCLC-A tumor models).

[b] Studies relevant to the various cell types of origin are not included in this Table.

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Table 2.

Advantages and limitations of classical genetics vs. somatic engineering in mouse models of SCLC.

Approach	Advantages	Limitations
Classical mouse genetics (e.g., Cre/Lox)	Validated approach Homogeneous gene alteration in targeted cells Usually large number of tumors (e.g., *RPR2* and *RPM* models), facilitating quantification Feasibility of cell type-specific tumor induction Possibility of combining with a number of genetic approaches (Flp/Frt, Dre/Rox, doxycycline-inducible systems, etc.)	Time-consuming and costly processes of incorporating new alleles of interest into validated models (e.g., *RP, RPR2*, or *RPM*) Not all genes of interest in SCLC have mutant alleles in mice Limited choice of somatic alterations for gene of interest, with often complete (conditional) knockout and rarely more specific relevant alterations
Somatic engineering (e.g., adeno/lentiviral CRISPR)	Relative ease of cloning viral vector and preparing high-titer virus Rapidity of targeting multiple genes individually or in combination Readily applicable to a mouse strain with constitutive or inducible Cas9 (Ad5-sgRNAs) Readily applicable to *RP* mutant mice or variants with constitutive/inducible Cas9 expression (Ad5-sgRNAs) or those without (Ad5-sgRNA-Cas9/Cre) Insertion/Deletions (indels) can yield information about selective pressure for the target gene of interest (e.g., LOF indels vs. in-frame indels)	Fewer tumors develop (e.g., comparing Ad-CMV- Cre infected RPR2 mutant mice and *Ad5-sgRb1/sgTrp53/sgRbl2* mutant mice) Currently unable to target specific cell types of interest in the lung epithelium Heterogeneous gene alterations in targeted cells, including those resulting in complete to no loss of function, and those of unknown significance for the gene of interest—this may make it more difficult to quantify tumor growth and link phenotype (e.g., tumor size) to genotype

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Table 3.

Main features of the different types of viral vectors used to generate genetically-engineered mouse models of SCLC.

Viral approach	Titer	Advantages	Limitations
Adenovirus	1×10^7–4×10^8 plaque-forming units	Can accommodate large sizes of transgenic DNA Easily transduces somatic cells in the lung	Does not integrate into the genome
Adeno-associated virus	1×10^{11} genome copies	Easily transduces somatic cells in the lung	Limited in the size of the transgenic DNA fragment Does not generally integrate into the genome
Lentivirus	2×10^6–2×10^7 plaque-forming units	Stably integrates into the host genome	Limited in the size of the transgenic DNA fragment