locus is fully silenced. The authors postulate that this could explain why previous ChIP studies could not show BCL11A binding to the γ-globin gene proximal promoters. Introducing HPFH-associated mutations at the sites at −200 and −115 bp resulted in loss of binding activity and increased HbF expression in comparison to wild-type cells, mimicking HPFH. The independent binding sites of BCL11A and ZFTB7A lend support to an earlier study that showed that *ZFTB7A* and *BCL11A* double-knockout cells had greater increases in HbF than *ZFTB7A* or *BCL11A* single-knockout cells[13].

What is not clear is whether binding by the two repressors is successive or what order is needed to bring about the full chromatin reconfiguration.

Genetic targets for β-hemoglobinopathies

Compelling evidence from the naturally occurring HPFH-associated mutations shows that elevated HbF levels can alleviate the clinical severity of β-hemoglobinopathies, prompting both pharmacological and genomic approaches for therapeutic HbF reactivation. One genetic approach currently being explored is disruption of the erythroid-specific enhancer of *BCL11A* by CRISPR–Cas9 genome editing[14]. However, simulating naturally occurring HPFH-associated mutations may be a more attractive approach, as in a recent proof of principle where deleting 13 bp in the γ-globin gene promoter (a naturally occurring HPFH-associated mutation) in primary human erythroid progenitor cells led to relatively increased HbF expression[15]. Martyn and colleagues[6] used CRISPR–Cas9-mediated genome editing of a human erythroid cell line to recapitulate various naturally occurring HPFH-associated mutations in the γ-globin gene promoter and successfully disrupted binding of the two transcription factors. The findings confirm these sites as potential DNA targets for genome-editing-mediated therapy of β-hemoglobinopathies and provide support for autonomous silencing of the γ-globin gene in hemoglobi n switching. ❐

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Competing interests
The authors declare no competing interests.

CANCER GENETICS

Evaluating tumor-suppressor gene combinations

In vivo verification of tumor suppressors and their interactions with each other has required complex experiments. A report in this issue uses a novel CRISPR–Cas9 technology with barcodes to test, in parallel, the tumorigenic potential of functional loss of multiple tumor-suppressor genes in the context of a genetically engineered mouse model of lung adenocarcinoma with mutant *Kras*.

James Kim and John D. Minna

Understanding of the genetic landscape of human lung cancers has dramatically progressed owing to the availability of sequence data from thousands of lung cancers, with sequencing now an important part of clinical care to help select targeted therapy, chemotherapy and potentially immunotherapy for lung cancer[1–6]. Several oncogenic driver mutations have been identified and are usually 'mutually exclusive' with one another. However, a greater number of genetic aberrations have been identified (~50–100 per tumor) that appear to cause loss of function. These aberrations need to be validated as functionally important tumor-suppressor genes (TSGs) and for their impact in the context of other mutations. In this issue, the Petrov and Winslow laboratories report the use of a new approach (Tuba-seq, for tumor barcoding) to functionally test the tumor-suppressive capabilities of 11 genes in the context of $Kras^{G12D}$ genetically engineered mouse models (GEMMs) in pairwise combinations with either *Trp53* or *Stk11* (*Lkb1*) loss. They use a novel method that induces genetic loss of multiple tumor suppressors simultaneously via CRISPR–Cas9 technology[7,8]. This method uses a lentiviral construct encoding one barcode that tracks each single guide RNA (sgRNA) targeted against a TSG and another barcode that tracks each individual tumor, allowing measurement of tumor cell number, and thus tumor size, resulting from loss of multiple different TSGs within the same mouse.

Interaction of tumor suppressors

GEMMs have been critical for uncovering fundamental aspects of lung cancer biology; however, elucidation of oncogene and tumor-suppressor biology in GEMMs is time-consuming and expensive, requiring germline alterations and subsequent breeding to obtain the desired genotypes (Fig. 1a). Recently, CRISPR–Cas9 technology has been used to accelerate this process[9,10]. Lentiviruses encoding Cre recombinase, Cas9 nuclease and sgRNA are delivered by intratracheal instillation into the lungs of mice to initiate lung adenocarcinomas with, for example, expression of mutant $KRAS^{G12D}$ alone or in combination with *Trp53* loss. Cas9 induces indels in genes targeted by the sgRNAs, producing out-of-frame mutant transcripts of these genes that are degraded (Fig. 1b). A key advantage of in vivo CRISPR–Cas9 technology is the time and cost savings. Generation of desired genotypes may take

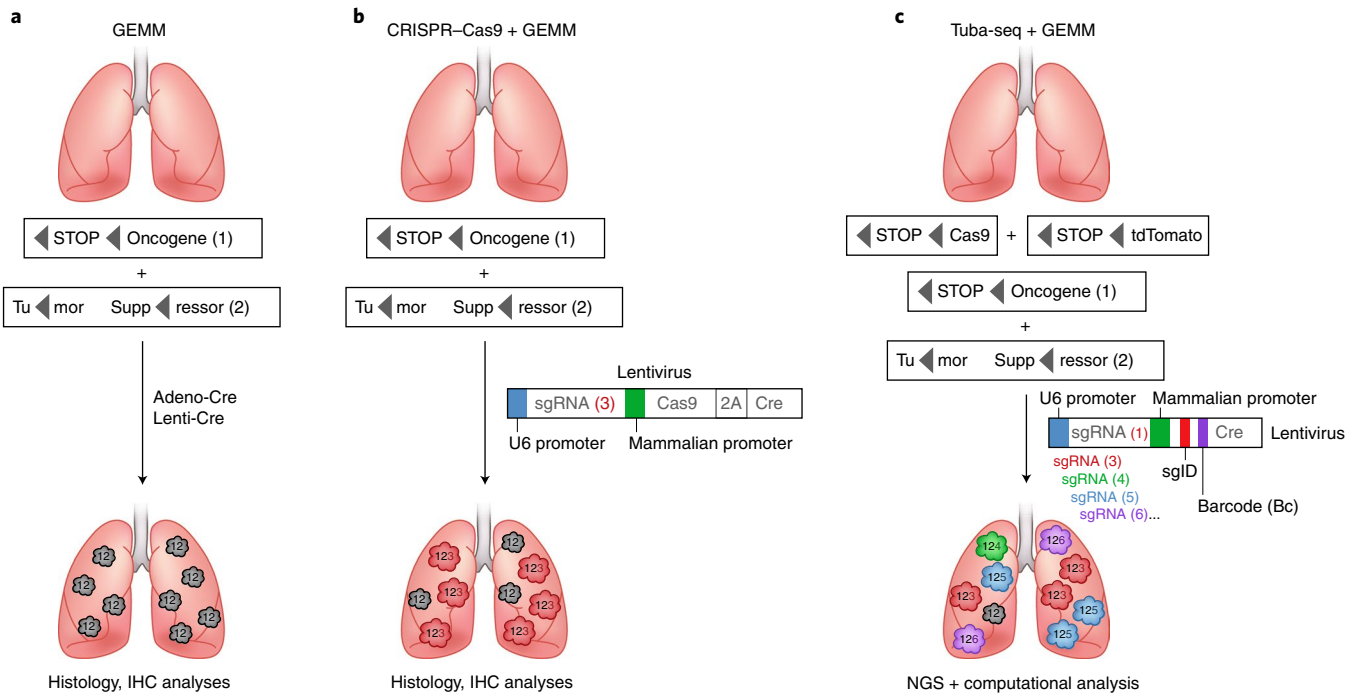

Fig. 1 | Tumor-suppressor evaluation in GEMMs. a, In a traditional conditional GEMM, Cre-mediated recombination of *loxP* sites (triangles) generates tumors (12) that express an oncogene (1) with tumor suppressor loss (2). **b**, CRISPR–Cas9 with sgRNA against a tumor suppressor (3) is used with a GEMM to generate tumors (123) with loss of two tumor suppressors. **c**, Tuba-seq generates tumors (123, 124, etc.) with multiple tumor-suppressor deletions simultaneously via sgRNAs (3, 4, etc.) against tumor suppressors. IHC, immunohistochemistry; NGS, next-generation sequencing.

a year or more with traditional GEMMs, whereas similar results can be accomplished within months using CRISPR–Cas9. However, phenotypic quantification of tumor-suppressor loss still requires measurement of total tumor area, reliable antibodies for immunohistochemistry to assess loss of the targeted protein and large cohorts of mice for each tumor suppressor studied.

Rogers et al.[7] advance the study of in vivo tumor-suppressor loss by using Tuba-seq[8] in lung cancer GEMMs. Tuba-seq is a novel method of CRISPR–Cas9 that induces indels using identifiers and unique barcodes for sgRNAs that allow for precise quantification of the number of cells in individual tumors with loss of the targeted TSG through next-generation sequencing. To generate tumors, lentiviruses encoding Cre recombinase, sgRNA, an sgRNA identifier and a unique barcode are delivered intratracheally into the lungs of mice expressing Cas9, thus ensuring efficient indel formation by Cas9 and recombination at *loxP* sites by Cre in the infected cells. The barcoding and quantification system allows for the dissection of entire lungs from a mouse, with next-generation sequencing performed to simultaneously identify individual tumors, determine their cell number and quantify the specific TSG targeted by the sgRNA within the same mouse, overcoming many of the previous hurdles (Fig. 1c).

Rogers et al.[7] employed sgRNAs against 11 genes with established tumor-suppressor provenance in the context of mouse lung adenocarcinomas with mutant $Kras^{G12D}$. Indels in 6 of the 11 genes led to increased tumor growth, with *Setd2* and *Lkb1* loss having the greatest effect. They also determined which combinations of the 11 TSGs gave larger tumors. Two main comparisons were of mice with wild-type *Trp53* versus *Trp53*-null mice and of mice with wild-type *Lkb1* versus *Lkb1*-null mice; the effect on tumor size from disruption of the paired TSGs varied dramatically depending on the *Trp53* versus *Lkb1* TSG background. When they compared their mouse GEMM data to human lung adenocarcinoma data from The Cancer Genome Atlas (TCGA) and the AACR Genomics Evidence Neoplasia Information Exchange (GENIE), there was general concordance between the potency of tumor growth due to loss of different TSG combinations in mice and the mutation frequency of the TSG combinations that occurred in the human datasets. Thus, they demonstrated that 'context' (for example, mutant or wild-type *Trp53*) was important for the effect from the loss of the targeted TSG. This context led to marked differences in 'fitness' (size of the tumors) with different potencies of the test TSGs, which in turn resulted in great intertumor heterogeneity (a 'rugged landscape') even within an individual mouse. In the context of mutant $Kras^{G12D}$ and *Trp53* loss, *Setd2* and *Lkb1* loss induced the greatest tumor growth. However, *Smad4*, *Arid1a* and *Atm* were also now identified as TSGs, although they did not show any tumor-suppressive effects with $Kras^{G12D}$ alone. In $Kras^{G12D}$ mice with *Lkb1* loss, surprisingly, only *Rb1* and *Apc* emerged as tumor suppressors. Furthermore, *Rb1* and *Apc* were the only genes that had tumor-suppressive effects in all three mutant mouse strains (mutant *Kras* alone, mutant *Kras* + *Trp53* loss, and mutant *Kras* + *Lkb1* loss).

Several insights come from this study: the order of TSG loss (for example, *TP53* and *LKB1*) in pathogenesis can be important; *Setd2* is confirmed as a potent lung cancer tumor suppressor[10]; and the potency of tumor suppression does not correlate with mutation frequency. While *TP53* is the most commonly mutated gene in all major lung cancer histologies[1,2,4], its tumor-suppressive potency is relatively mild, suggesting that other mechanisms must govern mutation frequency. Ultimately, the interactions of tumor suppressors are unpredictable and need to be functionally determined.

Potential future applications

The study by Rogers et al.[7] with Tuba-seq[8] presents a powerful new tool for the study of tumor biology, including applications to clinically relevant situations. These include comparing the functional roles of different combinations of TSG loss in primary versus metastatic lesions, in 'field effects' (for example, hyperplasias and adenomas versus invasive cancers), with other driver oncogenes besides mutant *Kras*, after treatment with chemotherapy, targeted therapy, radiotherapy or immunotherapy, in cancer metabolism, and in cancer stem cell biology, as well as determining their influence on the tumor microenvironment and role in immune surveillance. Finally, while patient-derived xenografts and organoids are being used as 'avatars' to test therapy for individual patients, the study by Rogers et al.[7] raises the possibility of using Tuba-seq and CRISPR–Cas9 GEMM technology to take sequencing information from individual patients and model this in mice, which could then receive the same treatment as the patient. The oncogenotype of surviving tumor cells would in turn allow selection and development of therapies to target tumor cells resistant to the initial therapy. ❐

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Competing interests

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