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# CRISPR-BASED DRUG TARGET DISCOVERY: IMPLICATIONS FOR CRISPR-BASED THERAPIES

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# CRISPR-based drug target discovery: implications for CRISPR-based therapies

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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Where there is a will there is a way

To my family

# POPULAR SCIENCE SUMMARY OF THE THESIS

Sickle cell disease, beta-thalassemia, hemophilia are examples of genetic, hereditary diseases that run in the family, like a curse. Hereditary diseases are caused by mutations in one or multiple genes, or even by chromosomal abnormalities. People inherit the genetic material, DNA, from their biological parents, half from each. So if there is a hereditary disease on one side of the family, the offspring could also get the same disease.

Previously, mutations in DNA were almost impossible to fix. Recently a new technology, called the CRISPR system, that can be used to correct mutations in DNA has been developed at a dramatically rapid pace. Notably, the technology was awarded the Nobel Prize in 2020. As such, CRISPR is considered to have great potential to be used for clinical gene therapy to cure hereditary diseases, such as sickle cell disease.

However, researchers have discovered some safety issues related to CRISPR system. One of them is that CRISPR may cause changes that could lead to cancer development, but this is not fully understood. Therefore, one of the aims of my thesis was to investigate such safety issue.

In **Paper I**, we discovered that the CRISPR system enriches for cells with mutations in genes of a core CRISPR-p53 tumor suppressor interactome. Tumor suppressor genes are like the guardians for the cells, and cells with mutations in such genes are therefore at risk of becoming cancer cells. CRISPR-treated cells may thus enrich for such mutations and finally have a higher chance to develop into cancer cells. Luckily, we also discovered strategies to inhibit this effect, to make CRISPR safer for clinical gene therapy.

CRISPR is a very powerful method, not only for potential clinical gene therapy applications, but also to be used as a drug target discovery platform. Mechanisms contributing to different diseases have been studied using CRISPR-based methods, and related drug targets have been discovered. Another aim of my thesis was to develop CRISPR-based methods that could be used to identify potential drug targets.

In **paper II**, we presented the Rapid CRISPR Competitive (RCC) assay to discover potential drug targets in the hematopoietic system. In **paper III**, we used a CRISPR-based *in vivo* screen and discovered a novel combinatorial treatment for cancers.

In summary, this thesis addresses safety issues related to CRISPR, and identifies strategies that could make clinical gene therapy safer. In addition, several examples of how CRISPR can be used as a drug target discovery platform are shown, including identifying a novel combinatorial cancer therapy.

# ABSTRACT

The CRISPR system was discovered in prokaryotic cells, and it is now one of the most efficient molecular tools to modify genes in eukaryotic cells. It is widely used to inactivate or modify genes in cell lines, animal models, and is also used in several clinical gene therapy trials. Additionally, CRISPR-based screens have been developed as a high throughput methodology to identify drug targets and mechanisms contributing to diseases. However, safety concerns related to the CRISPR system have been emphasized, which challenges the clinical applications of this molecular tool. The consequence of CRISPR-induced DNA damage is one of the challenges and has so far been less studied.

In **Paper I**, we showed that CRISPR-induced DNA damage enriches for cells with mutations in genes of a core CRISPR-p53 tumor suppressor interactome. Such enrichment may contribute to cancer development and is a potential challenge for clinical CRISPR use. We also discovered that such enrichment could be suppressed by transient p53 inhibition. In addition, we discovered factors affecting the enrichment of *p53* mutated cells from a database of >800 human cancer cell lines. In **Paper II**, we presented the Rapid CRISPR Competitive (RCC) assay, which is a rapid and universal experimental approach to discover potential drug targets. In this, we leverage the genetic heterogeneity induced by CRISPR and use Sanger sequencing to discover how different genes are involved in the studied cellular behavior or phenotype based on the enrichment or depletion of mutations. In **Paper III**, we identified that IL-4 can suppress B16-F10 melanoma tumor model growth by inducing a *Gcn1l1* regulated amino acid deprivation response. We used gene expression analysis, mass spectrometry, and an *in vivo* CRISPR screen to link the potent therapeutic activity of IL-4 to ARG1-mediated arginine depletion and identify *Gcn1l1* as a potential synergistic treatment target.

In conclusion, we extensively studied p53 biology in the context of DNA damage induced by CRISPR, and identified strategies for safer CRISPR use. We also developed a rapid and universal CRISPR-based experimental approach to discover potential drug targets. Finally, we use an *in vivo* CRISPR-based screen approach to discover a novel combinatorial cancer therapy.

# LIST OF SCIENTIFIC PAPERS

I. **Jiang L**, Ingelshed K, Shen Y, Boddul SV, Iyer VS, Kasza Z, Sedimbi S, Lane DP, Wermeling F.
CRISPR/Cas9-induced DNA damage enriches for mutations in a p53-linked interactome: implications for CRISPR-based therapies.
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A rapid CRISPR competitive assay for *in vitro* and *in vivo* discovery of potential drug targets affecting the hematopoietic system.
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III. Kasza Z, Jiménez-Andrade Y, **Jiang L**, Shen Y, Iyer VS, Boddul SV, Malin S, Jain M, Nilsson R, and Wermeling F.
IL-4 can potently suppress tumor growth by inducing a *Gcn1l1* regulated amino acid deprivation response.
*Manuscript*

# RELATED PUBLICATIONS

Not included in this thesis

Original Publications:

I. Panda SK, Boddul SV, Jiménez-Andrade GY, **Jiang L**, Kasza Z, Fernandez-Ricaud L, Wermeling F.
Green listed-a CRISPR screen tool.
*Bioinformatics.* 2017 Apr 1;33(7):1099-1100.
doi: 10.1093/bioinformatics/btw739.

II. Schulz A, **Jiang L**, de Vor L, Ehrström M, Wermeling F, Eidsmo L, Melican K.
Neutrophil Recruitment to Noninvasive MRSA at the Stratum Corneum of Human Skin Mediates Transient Colonization.
*Cell Rep.* 2019 Oct 29;29(5):1074-1081.e5.
doi: 10.1016/j.celrep.2019.09.055.

III. Panda SK, Wigerblad G, **Jiang L**, Jiménez-Andrade Y, Iyer VS, Shen Y, Boddul SV, Guerreiro-Cacais AO, Raposo B, Kasza Z, Wermeling F.
IL-4 controls activated neutrophil FcγR2b expression and migration into inflamed joints.
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Reviews:

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Designing custom CRISPR libraries for hypothesis-driven drug target discovery.
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# LIST OF ABBREVIATIONS

| | |
|---|---|
| 53BP1 | tumor suppressor p53-binding protein 1 |
| ALL | acute lymphocytic leukemia |
| AML | acute myeloid leukemia |
| ANLL | acute nonlymphocytic leukemia |
| ATM | ataxia telangiectasia mutated |
| ATR | ATM and Rad3-related |
| ATRIP | ATR-interacting protein |
| BCL-2 | B cell lymphoma-2 |
| BER | base excision repair |
| BM | bone marrow |
| Cas | CRISPR-associated protein |
| CDK | cyclin-dependent kinase |
| CHEK1 | checkpoint protein 1 |
| CHEK2 | checkpoint protein 2 |
| CLL | chronic lymphocytic leukemia |
| CLP | common lymphoid progenitor |
| CML | chronic myeloid leukemia |
| CMP | common myeloid progenitor |
| CRISPR | clustered regularly interspaced short palindromic repeats |
| CRISPRa | CRISPR activation |
| CRISPRi | CRISPR interference |
| crRNA | CRISPR RNA |
| dCas9 | dead Cas9 |
| DDR | DNA damage response |
| DISC | death-inducing signaling complex |

| | |
|---|---|
| DNA | deoxyribonucleic acid |
| DNA-PK | DNA-activated protein kinase |
| DSB | double-strand break |
| GMP | granulocyte-macrophage progenitor |
| gRNA | guide RNA |
| H2AX | H2A histone family member X |
| HDR | homology-directed repair |
| HSC | hematopoietic stem cell |
| ICE | Inference of CRISPR Edits |
| iCR | immuno-CRISPR |
| InDels | insertions or deletions |
| IR | ionizing radiation |
| KAP1 | KRAB-associated protein-1 |
| KO | knock out |
| mAb | monoclonal antibody |
| MEP | megakaryocyte-erythrocyte progenitor |
| MLKL | mixed lineage kinase domain-like |
| MMR | mismatch repair |
| MRN | mre11-rad50-nbs1 |
| NER | nucleotide excision repair |
| NHEJ | non-homologous end joining |
| NK cell | natural killer cell |
| PAM | protospacer-adjacent motif |
| PIDs | Primary immunodeficiencies |
| pre-crRNA | pre-CRISPR RNA |
| RCC | Rapid CRISPR Competitive |
| RE | response element |

| | |
|---|---|
| RIPK1 | receptor-interacting protein kinase 1 |
| RIPK3 | receptor-interacting protein kinase 3 |
| ROS | reactive oxygen species |
| RPA | replication protein A |
| SCA | stem cell antigen 1 |
| sgRNA | single guide RNA |
| SSB | single-strand break |
| ssRNA | single-stranded DNA |
| TALEN | transcription activator-like effector nuclease |
| TIDE | Tracking of Indels by DEcomposition |
| TNFR | tumor necrosis factor receptor |
| TNFR1 | TNF receptor 1 |
| TopBP1 | topoisomerase II binding protein 1 |
| tracrRNA | trans-activating CRISPR RNA |
| UV | ultraviolet |
| WAS | The Wiskott-Aldrich syndrome |
| WT | wide type |
| ZFN | zinc finger nuclease |

# 1 LITERATURE REVIEW

## 1.1 CRISPR

### 1.1.1 Origin of CRISPR/Cas system

CRISPR is short for clustered regularly interspaced short palindromic repeats and Cas means CRISPR-associated protein. The CRISPR/Cas system was first found in prokaryotic organisms and acts as an adaptive immune system that can target specific genetic regions of infecting bacteriophages or viruses and thereby protect from infections (*1-3*).

Originally, the CRISPR/Cas adaptive immune response includes three important steps: adaptation, expression, and interference (*4*). In the *adaptation* part, the Cas proteins recognize the foreign DNA and bind to the target region, next to a protospacer-adjacent motif (PAM). The target region is then cleaved out to become a protospacer and further inserted into the CRISPR array to become a spacer. In the *expression* part, the CRISPR array containing the specific spacer is transcribed to pre-CRISPR RNA (pre-crRNA) and further processed to become the mature CRISPR RNA (crRNA), which is mediated by different Cas proteins. In the *interference* part, the crRNA finds the protospacer in the invading virus or bacteriophages' genome, and cleaves the targeted sequence with a Cas nuclease or nucleases, such as Cas9 (*4-6*).

### 1.1.2 Classification of CRISPR/Cas system

The CRISPR/Cas system can be classified into two major divisions: Class 1 and Class 2. For Class 1, several Cas nucleases are involved in the crRNA processing and the interference parts. The Class 1 CRISPR/Cas system can be further divided into 3 types: Type I, Type III, and Type IV. The Class 1 system is dominant in the CRISPR/Cas system in prokaryotic organisms, which can target both DNA and RNA. The effector module of Class 2 contains only a single multifunctional Cas protein, such as Cas9 and Cas 12. The Class 2 system is also further divided into 3 types: Type II, Type V, and Type VI. Although the Class 2 system only represents 10% of all the CRISPR/Cas systems, it is the most known CRISPR/Cas system and has been developed most for genome engineering. Cas9, the most famous endonuclease, belongs to the Type II system in Class 2 (*5, 7-9*).

### 1.1.3 Components of CRISPR/Cas9 system

The CRISPR/Cas9 system has been developed and widely applied as a genomic engineering tool. The CRISPR/Cas9 system typically consists of two components; a guide RNA and a Cas9 endonuclease. In the original Type II CRISPR/Cas system, Cas9 is guided by two RNAs. The *crRNA*, which can recognize the target region in the genome, and the trans-activating CRISPR RNA (*tracrRNA*), which links the crRNA to the Cas9 protein as a scaffold, and also mediate the pre-crRNA to be processed into mature crRNA (*10*). During the development of CRISPR

in genomic engineering, a synthetic fusion of both crRNA and tracrRNA has commonly been used, known as a single guide RNA (sgRNA) or guide RNA (gRNA) (*11*, *12*). The most commonly used variant of Cas9 protein, so far, is from *Streptococcus pyogenes*, called SpCas9 (*13*), and new variants of Cas9 have been continuously developed, such as eSpCas9, with enhanced fidelity (*14*). There are two endonuclease domains for Cas9 and its variants: the RuvC nuclease domain and the HNH nuclease domain. The PAM sequence for SpCas9 is 3'-NGG, while e.g. xCas9, which has been developed for increased PAM flexibility, is 3'-NG (*15*).

### 1.1.4 CRISPR/Cas9 mediated genome editing

The sgRNA and the Cas9 protein interact, forming the Cas9/sgRNA complex. The sgRNA then guides the Cas9 nuclease to the target site in the genome, which is ideally specific compared to the rest of the genome and must contain a PAM sequence at the immediate downstream site. Upon binding to the target genomic site guided by the sgRNA, Cas9 undergoes conformational changes and cleaves the target DNA, generating a double-strand break (DSB) (*16*). The cut site is usually located ~3 nucleotides upstream of the PAM sequence. Once DSB forms, it will be repaired by either the non-homologous end joining (NHEJ) pathway or the homology-directed repair (HDR) pathway. The NHEJ pathway is efficient but error-prone with a high probability for the formation of insertions or deletions (InDels), while the HDR pathway is less efficient but can make a precise DSB repair. If the DSB is repaired without the formation of InDels, the target sequence can be recut by the Cas9/sgRNA complex to generate DSB again. So in most cases, DSB will be finally repaired in the NHEJ pathway, which usually leads to a small InDels in the target sequence. As a result, frameshift mutations are generated, and this can cause premature stop codons and finally lead to nonsense-mediated decay. The final result is that loss of function mutations are introduced within the gene, and the target gene is knocked out (*1*, *10*, *17*) (**Figure 1**).

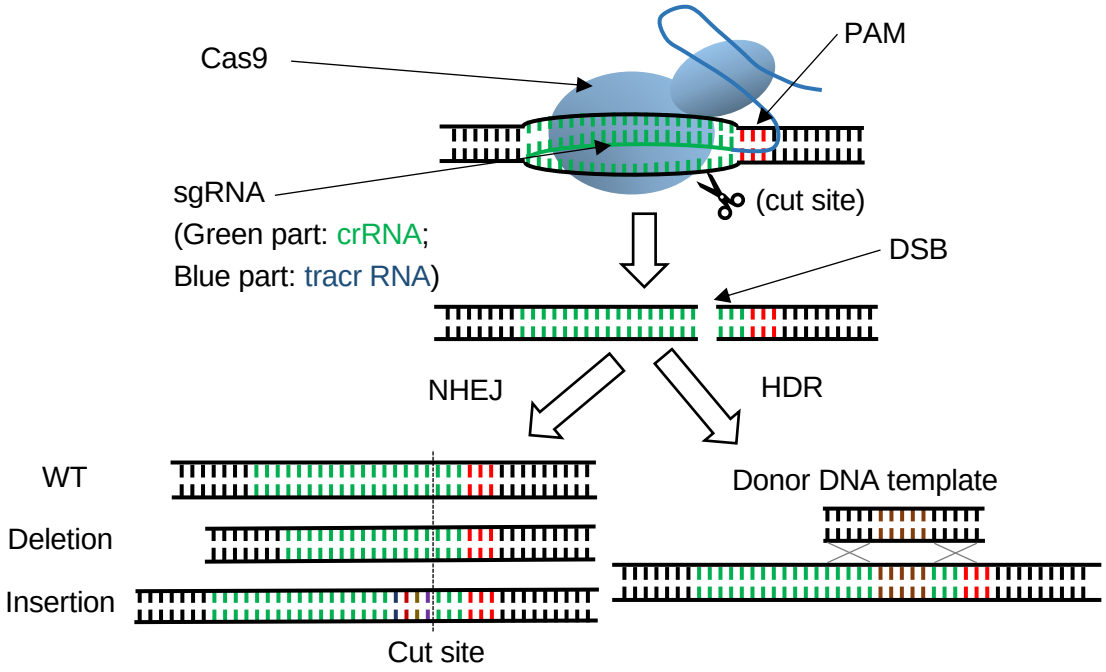


**Figure 1**. The mechanisms of CRISPR/Cas9 mediated genome editing.

CRISPR/Cas9 system can also make precise modifications using HDR (*18*). In order to achieve this, a DNA template must be delivered to the cells together with the Cas9/sgRNA complex. The DNA template should contain the designed insertion sequence, as well as the adaptor homologous sequences of the immediate upstream and downstream of the target cutting site. When DSB is introduced by the Cas9/sgRNA complex as described, the HDR pathway can be activated and finally generates the precise insertion of the DNA template. However, the efficiency is much lower compared to the InDels generated by the NHEJ pathway (*18-20*) (**Figure 1**).

In addition, the CRISPR/Cas9 system can be used to activate or repress the expression of a target gene. When the RuvC nuclease domain and the HNH nuclease domain were rendered inactive by introducing mutations, a dead Cas9 (dCas9) was generated. The dCas9 protein can still bind to the target DNA but loses the function to generate DSB. So when dCas9 binds to the transcription start site of a target gene, the target gene expression can be repressed (CRISPR interference, or CRISPRi). To enhance the repression effect, the dCas9 can also be fused with a repressor, such as KRAB (*21*). If dCas9 is fused with a transcriptional activator, such as VP64, and it binds to the promoter region of the target gene, the target gene will be overexpressed (CRISPR activation, or CRISPRa) (*22-24*).

### 1.1.5 CRISPR-based genetic screens

Screen methods have been broadly used in the pharmaceutical industry to discover drugs aiming to cure disease (*25*). Large-scale high-throughput screens have traditionally been performed by testing (screening) a large number of different small molecular drugs in parallel (*26*). Genetic screening approaches can also be used to study cellular phenotypes linked to disease and, for example, identify underlying genetic causes and potential drug targets related to the studied phenotype (*27*). To perform a genetic genome-wide screen, an extensively genetically heterogeneous cell population needs to be generated and subsequently tested (*28, 29*). Before the widespread use of CRISPR, the common gene-editing tools included restriction enzymes (*30*), zinc finger nucleases (ZFNs) (*31*), and transcription activator-like effector nucleases (TALENs) (*32*). However, those methods are very difficult to use for genome-scale screens because of the low efficiency of generating mutations and the high cost of identifying mutations (*33*). Short hairpin RNA (shRNA) is a small artificial RNA sequence that can silence the expression of a target gene to achieve gene knockdown. ShRNA based genome-wide screen has been developed. However, the expression of a target gene is not always fully silenced by shRNA, and this can lead to bias in the shRNA-based screens (*34*). Following the first study in 2013 using CRISPR as a potent and cost-effective gene-editing tool (*2, 3*), it has been extensively developed, and the first paper describing using CRISPR screens was published already in 2014 (*35*).

For the classic CRISPR-based screen, lentiviral or retroviral delivery of sgRNAs into Cas9 expressing cells is used. In this process, a viral library is initially generated, containing a pool of sgRNAs, targeting the genes of interest. After transducing the viral library into Cas9

expressing cells, a mixed population of heterogeneous mutant cells is thus generated. By design, the sgRNA sequences are integrated from the virus, by the activity of the integrase gene, into the genome of the transduced cells, which subsequently are used as barcodes for sequencing. The generated heterogeneous mutant cell population is subsequently subjected to a selection pressure, depending on the phenotype of interest. By comparing the sgRNA distribution in the cells after selection and before selection, genes affecting the studied phenotype can be identified (*33, 36, 37*) (**Figure 2**).

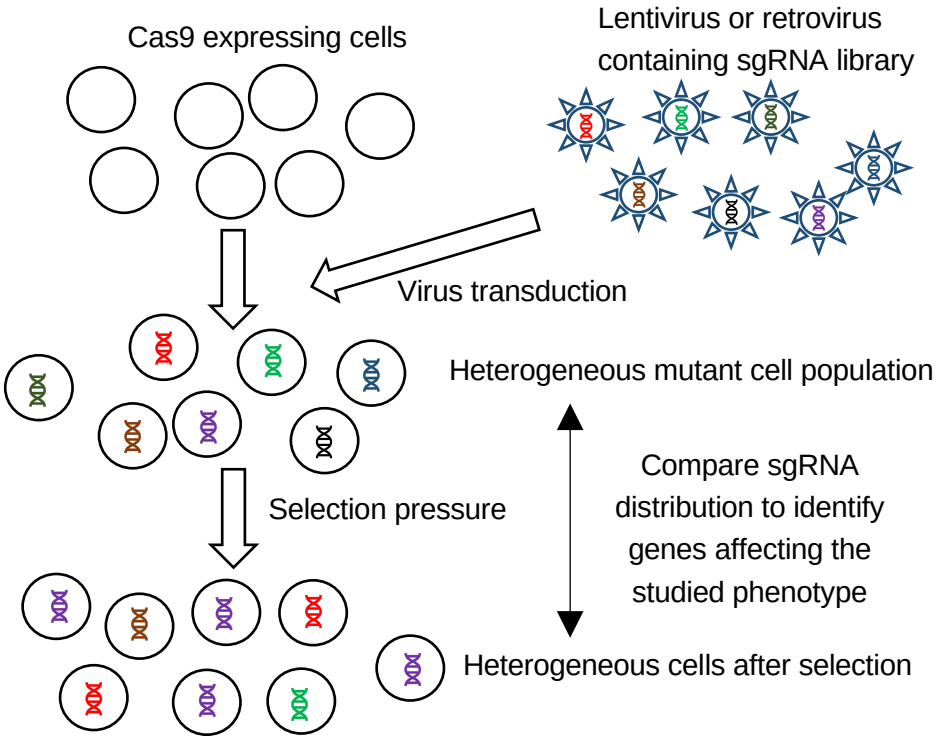


**Figure 2**. The mechanisms of classic CRISPR-based screens.

The library of genes for the a CRISPR screen can vary (*38*). Genome-wide screens need a library targeting all the genes, which are around 20,000 (*39*). Custom screens need a smaller library, targeting a subset of genes that potentially could be involved in the studied phenotype (*40, 41*). Genome-wide screens are unbiased in the design and have the potential to discover all genes related to the phenotype (*42*). However, this approach needs a huge amount of cells, which can be more than 100 million cells. This could be manageable for the *in vitro* screen experiments but very difficult for more complex setting, like *in vivo* screen experiments (*43-45*). The custom screen approach is relatively easy to perform, is suitable for cells that are difficult to work with, and typically has less noise compared to genome-wide screens. On the other hand, custom screens may miss important genes not included in the library (*46-48*).

### 1.1.6 The shortcomings of CRISPR/Cas9 system

The off-target activity is the most studied shortcoming of the CRISPR/Cas9 system. It has been shown that the Cas9 protein can bind to other sites than the intended target sites in the genome and generate DSBs (*49*). The specificity of the sgRNA depends on the 20 nucleotide sequence referred to as the spacer. Mismatches between the target sequence and the sgRNA sequence

can lead to off-target activity (*50*). Notably, mismatches further away from the PAM have a higher chance of resulting in off-target activity compared to the mismatches close to the PAM (*51*). Off-target activity can lead to unwanted mutations in the genome and may lead to cancer development if important genes are affected (*52*). To avoid off-target activity, the main strategy has been to optimize the sgRNA design. As such, low-quality sgRNAs may have more off-targets, and scoring systems for sgRNAs have been developed (*53, 54*). Another strategy to lower the risk is to use modified Cas9 versions with increased fidelity. Several high fidelity Cas9 versions, such as eSpCas9 and eSpCas9-HF1, have been developed to minimize the off-target activities (*14, 55*).

Another shortcoming of the CRISPR/Cas9 system is the DNA damage mediated by the Cas9 nuclease. This has been less studied than the off-target effects and will be discussed in the following section.

## 1.2 DNA Damage

### 1.2.1 DNA and types of DNA damage

DNA, deoxyribonucleic acid, is composed of two polynucleotide chains. The two DNA strands are built of four different nucleotides and store genetic information. Each nucleotide contains one of the four bases: adenine (A), cytosine (C), guanine (G), and thymine (T) (*56*).

As discussed, CRISPR/Cas9 systems are used to generate genetic modifications by inducing DSBs, which trigger cellular DNA damage responses (*2*). Cells are continuously at risk of being exposed to several types of DNA damage beyond CRISPR. *Endogenous sources* of DNA damage include replication mistakes, replicative stress and reactive oxygen species (ROS) (*57, 58*). Ultraviolet (UV) light (*59*), ionizing radiation (IR) (*60*), and chemotherapeutic drugs (*61*) are examples of *exogenous sources* for DNA damage. DNA damage includes double-strand breaks (DSBs), single-strand breaks (SSBs), and also DNA adducts (*61, 62*). To defend against the challenge of DNA damage, organisms have evolved a set of complex signaling networks responding to the DNA damage.

### 1.2.2 DNA damage response (DDR)

DSBs can be sensed by the mre11-rad50-nbs1 (MRN) sensor complex (*63*), which can subsequently recruit the ataxia telangiectasia mutated (ATM) protein. ATM, a central double-strand DNA damage checkpoint kinases, is then activated through auto-phosphorylation, and also recruits H2AX (H2A histone family member X) to the DNA damage region, subsequently phosphorylates H2AX into γH2AX. The activated ATM can also phosphorylate and activate different molecules, including CHEK2 (checkpoint protein 2) downstream, and spread the DNA damage signal to the whole cell by activating a kinase cascade (*64*). In addition, ATM plays an important role in HDR-mediated DNA repair through phosphorylating KAP1 (KRAB-associated protein-1) and recruiting HDR pathway proteins (*65*). Except for MRN, DSBs are

also sensed by the Ku70/80 sensor complex that subsequently recruits the catalytic subunit of DNA-PK (DNA-activated protein kinase), which plays a critical role in NHEJ mediated DNA repair (*66*).

For SSBs, the single-stranded DNA (ssDNA) is detected by RPA (replication protein A). ATR (ATM and Rad3-related) is subsequently recruited followed by ATRIP (ATR-interacting protein) binding to RPA-coated ssDNA. TopBP1 (topoisomerase II binding protein 1) is also necessary to active ATR. Upon ATR activation, CHEK1 (checkpoint protein 1) is phosphorylated and subsequently activates the downstream kinase cascade (*67*).

p53 is a transcription factor and is seen as a central hub for the downstream DNA damage responses. Upon activation of p53, multiple downstream effects can be seen, including cell cycle arrest, DNA repair, and cell death (*68*) (**Figure 3**). More details about p53 will be discussed in Chapter 1.3.

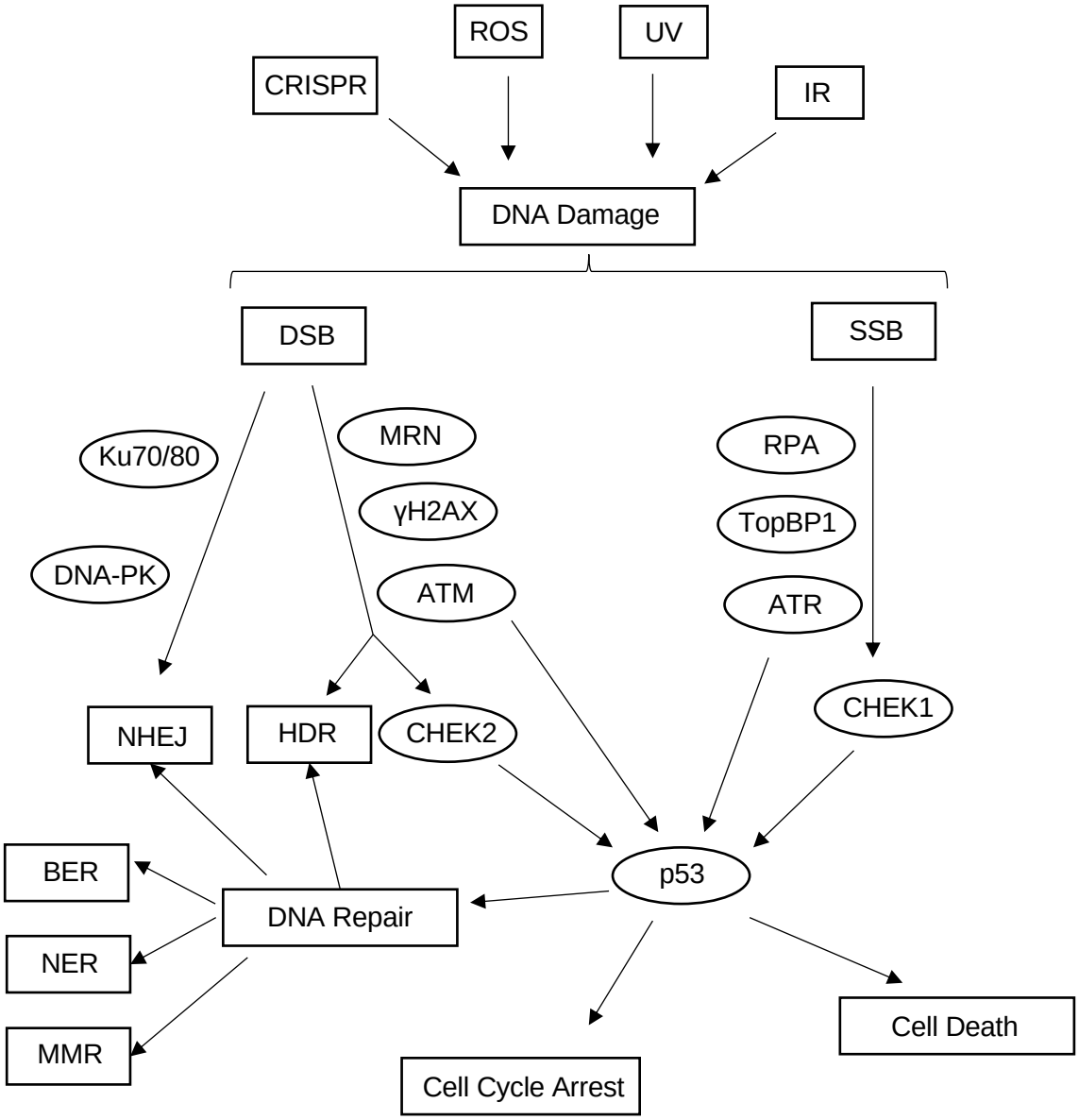


**Figure 3.** Cellular signaling of DNA damage response.

### 1.2.3 DNA damage induces cell cycle arrest

The cell cycle involves a series of events when a cell grows and divides into two new cells. For cells with the ability to divide, there are four phases of the cell cycles: G1 (Gap 1) phase, in which the cells prepare to divide, S (Synthesis) phase, in which the cells replicate DNA, G2 (Gap 2) phase, in which the cells continue the final process before division, M (Mitosis) phase, in which the cells stop growing and divide. Cell cycle checkpoints regulate the cell cycle progression. Three of the four cell cycles phases contain checkpoints: G1 checkpoint (also called restriction checkpoint), G2/M checkpoint, and M checkpoint (also called spindle checkpoint) (*69, 70*).

During DNA damage response, protein kinases CHEK1, CHEK2, and the upstream ATM and ATR, can inhibit the activation of CDK (cyclin-dependent kinase) and subsequently lead to reduced activation of cyclin/CDK complexes (*71*). Different complexes are inhibited in different pathways and involved in different checkpoints. For example, GADD45A is involved in the inhibition of cyclin B/CDK1 complex and further affects G2/M checkpoint (*72*), while p21 is involved in the inhibition of Cyclin E/CDK2 complex and subsequently affects the G1 checkpoint (*73*).

### 1.2.4 DNA damage repair

There are many active DNA repair pathways at different stages of the cell cycles, including base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), HDR, and NHEJ. The first three are mainly responsible for the repair of SSBs, while the latter two are mainly responsible for repairing of DSBs (*74*).

BER is active in the G1 phase and mainly repairs small, non-helix-distorting base DNA damage. DNA glycosylases are important for the initiation of this pathway (*75*). NER mainly removes bulky lesions caused by UV radiation. The deficiency of NER can lead to a set of syndromes predisposition to skin cancer, such as Cockayne Syndrome (CS) (*76*). MMR is mainly responsible for repairing mismatched Watson-Crick base pairs. This pathway is highly conserved and can rapidly remove nucleotides generated from replication errors (*77*).

DSBs are generally repaired in two principal mechanisms: HDR and NHEJ. The HDR pathway is active at the S/G2 phase, and a repair template sequence with the upstream and downstream identical to the damage site is necessary. Usually, the template sequence is from the sister chromosome. HDR generates precise repair but is less efficient than NHEJ (*78, 79*). NHEJ is, in contrast, efficient and not cell cycle restricted. However, this pathway is error-prone and can lead to InDels. During some cell cycles, such as M and G1, when sister chromatids do not exist for HDR, the NHEJ pathway is generally dominated. In addition, the NHEJ pathway can also be activated by Ku70/80 sensor complex, while 53BP1 (Tumor suppressor p53-binding protein 1), encoded by the *TP53BP1* gene, plays an important role in recruiting NHEJ components; while HDR pathway can also be activated by ATM and phosphorylated KAP1, as discussed in the previous content (*64, 80, 81*).

### 1.2.5 DNA damage induces cell death

DNA damage can induce programmed cell death, including apoptosis and necroptosis (*82*).

The caspase family is central to the apoptosis process. The involved caspases can be divided into two groups: *initiator caspases*, including caspase 2, caspase 8, caspase 9, and caspase 10; and *effector caspases*, including caspase3, caspase 6, and caspase 7 (*83*). The initiation of apoptosis is restricted regulated, and the two best-understood activation mechanisms are the intrinsic and extrinsic pathways (*84*). The *intrinsic pathway* is also called the mitochondrial apoptotic pathway, and the essential initiators belong to the BCL-2 (B cell lymphoma-2) family, including PUMA, BAX, and NOXA, which are transcriptionally upregulated by activation of p53 (*85*). Upon initiation, the mitochondrial function is disrupted, and cytochrome C is released, which subsequentially activates caspase 9. In contrast, the *extrinsic pathway* is also called the death receptor pathway. Death receptors, such as TNF receptor 1 belonging to the TNFR (tumor necrosis factor receptor) superfamily, assemble DISC (death-inducing signaling complex). Upon the binding of ligand, caspase 8 and caspase 10 are recruited and subsequently activate the downstream effector caspases (*86, 87*).

Necroptosis is a type of regulated necrosis and characterized by the involvement of RIPK1 and RIPK3 (receptor-interacting protein kinase 1 and 3) (*88*). Its morphological phenotype is similar to necrosis, but it is regulated and programmed like apoptosis. The term necroptosis is a combination of necrosis and apoptosis. The activation of necroptosis is mediated by TNFR1 (TNF receptor 1). RIPK1 and RIPK3 are trans- and auto-phosphorylated and eventually get activated to finally recruit MLKL (mixed lineage kinase domain-like) and lead to necroptosis (*89-91*).

## 1.3 p53

### 1.3.1 Discovery of p53

The protein p53 is encoded by the *TP53* gene in humans and the *Trp53* gene in mice. p53 was discovered in 1979 as a protein that interacts with the large T antigens, an oncoprotein from the SV40 virus (*92, 93*). At first, *p53* was recognized as an oncogene, based on the data that *p53* cDNAs were able to transform cells together with the *Ras* oncogene (*94*). However, mutations were discovered in the mentioned transforming *p53* cDNA (*95*). Later, it was discovered that patients with a mutant allele of *TP53* developed Li-Fraumeni syndrome, which is characterized by the increased risk of getting cancers (*96*), and that *Trp53* KO mice developed cancers at a very young age (*97*). Based on this, p53 protein is recognized to suppress tumors.

### 1.3.2 Structure of p53

The 393-residue p53 protein, which is mainly defined as a transcription factor, includes five main domains: the transactivation domain (residues 1-63), the proline-rich region (residues 63-92), the central DNA-binding core domain (residues 102-292), the tetramerization domain (residues 320-355), and the negative regulatory domain (residues 356-393) (*98*) (**Figure 4**).

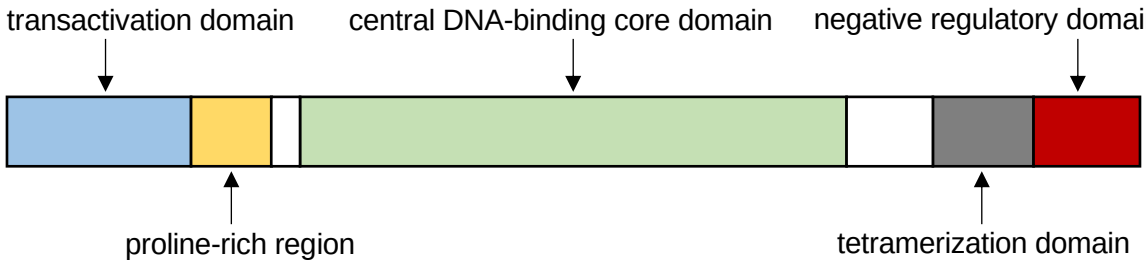


**Figure 4.** Structure of the p53 protein.

The *transactivation domain* contains binding sites to interact with a set of regulatory proteins, including MDM2, which inhibit the activation of p53, as well as the acetyltransferases p300 and CBP, which co-activate p53 and regulate the activation of p53 (*99*).

The *proline-rich region* contains SH3-domain binding motifs (PXXP). The function is not fully understood, but it is required for p53 interactions with chemotherapy drugs and also has a regulatory role in p53-mediated cell growth suppression (*100*).

The *central DNA-binding core domain* specifically binds to the REs (response elements) in the promotor of target genes, to activate or inhibit transcription. The target genes involve functions in multiple processes, such as cell cycle arrest, cell death, DNA repair, and translation control (*101, 102*).

The *tetramerization domain* regulates the oligomerization and DNA binding activity of p53.

The *negative regulatory domain* binds to DNA non-specifically and inhibits the ability of specific DNA-binding of p53 (*103*).

### 1.3.3 Function of p53

The protein p53 generally regulates genes to suppress tumors (*104*). The activation of p53 is usually induced by stress signals, such as DNA damage, activation of an oncogene, and hypoxia (*105*). DNA damage was the first found the most sensitive signal for p53 activation (*106*); even a very minor DNA damage signal is sufficient to active p53 (*107-109*). All these stress signals can increase the level of p53 significantly. However, the increase is not typically due to increased transcription of p53 mRNA, but post-translational regulation (*110*).In addition, the half-life of p53 is also prolonged, and the stability of the protein increased after the stress signal (*111, 112*). Such regulation makes the p53 activity fast and sensitive.

Stress signals can active p53 and lead to a series of cell responses as described in more detail previously. Such responses can protect the genome from instability. Therefore, p53 is also

called "The Guardian of The Genome" (*113*). The transcription factor p53 binds to the target genes, but the binding affinity varies for different target genes. For example, the binding affinity is higher in cell cycle arrest genes compared to the pro-apoptotic genes, which means the transactivation potential could be relatively higher for the cell cycle arrest genes. Further analysis showed that the REs of cell cycle arrest genes are more conserved and more feasible to bind for p53 than the pro-apoptotic genes (*114*).

MDM2 is the most important negative regulator of p53 and forms an autoregulatory loop with p53. The activation of p53 can induce the transcription of MDM2, and MDM2 inhibits the activity of p53 through binding to the transactivation domain of p53 (*115*), exporting p53 out of the nucleus (*116*), and promoting degradation of p53 (*117*).

*p53* is one of the most studied genes related to cancer. The cancer suppressive effect of p53 is not only related to p53 induced cell cycle arrest, DNA repair, cell death, or senescence but also through regulating ROS levels, inducing autophagy or ferroptosis (*118*). Mutations of *TP53* can be found in about half of human cancers (*119*). The two most common types of *p53* mutations in cancer are contact mutations and conformational mutations. The *contact mutations* of p53 protein still has the WT protein conformation but lost the ability to bind to the specific target genes because of the mutations in several specific residues. *Conformation mutations* are also called structural mutations, which lead to structural changes, abnormal folding, and that the conformation is different from the WT proteins (*120*). The mutations can lead to loss of tumor suppressor function or even gain of oncogenic function (*121*). Most of the mutations found in cancers are located at the DNA-binding domain and affect the ability of p53 to bind to the target DNA (*122*).

Based on the common mutation frequency of *p53* in tumors, targeting mutant p53 could be an important strategy for cancer treatment (*123-125*). It has been shown in several animal models that restoration of normal p53 function can inhibit tumors. Such inhibition effect depends on the stage of tumor and the tumor model used, and different pathways, including apoptosis, growth arrest, senescence, or ferroptosis (*126, 127*). Furthermore, pharmacological approaches have been investigated to restore and reactive mutant p53 for cancer treatment, and a set of compounds have been studied and showed promising results. Some of them, such as APR-246, are already at the clinical trials stage (*123, 128*).

## 1.4 Cancer

### 1.4.1 Definition of cancer

The term "cancer" came from the ancient Greek physician Hippocrates, who used the terms karkinos and karkinoma to describe tumors. Those Greek terms were originally used to describe a crab, which Hippocrates believed a tumor with swollen veins resembled (*129*).

Cancer is characterized by uncontrolled cell growth and the ability to invade nearby tissues or even metastasize to other parts of the body far from the cancer (*130, 131*). More than 200 cancer

types have been defined, and more than half of these are derived from epithelial cells. In healthy individuals, cells proliferate when needed by cell division, and when the cells get old, they can undergo cell death and be exchanged by new cells. The normal cell division and cell death are controlled and regulated by a set of genes and multiple molecular mechanisms. When this balanced and regulated process is broken down, healthy cells may develop into malignancies and even threaten the life of the affected individual (*132, 133*).

Cancer is the second leading cause of total death worldwide, just behind cardiovascular disease. More over, the prevalence of cancer is increasing dramatically in the past decades. In males, the most common cancer types are prostate cancer, lung and bronchus cancer, as well as colon and rectum cancer; while in females, they are breast cancer, lung and bronchus cancer, as well as colon and rectum cancer. For children, the most common cancer types are blood cancer, followed by cancer in the brain and lymph nodes (*134, 135*).

### 1.4.2 Cancer genes

Cancer formation and development are driven and modulated by a set of altered genes. More than 700 genes have been implicated in cancer, and they can generally be divided into two main groups: oncogenes and tumor suppressor genes (*136*).

Normally, the proteins encoded by the proto-oncogenes regulate cell growth, differentiation, division and are involved in the inhibition of apoptosis. However, when the proto-oncogenes are altered, they become activated oncogenes, and the original functions are altered. Activated oncogenes can lead to uncontrolled cell division and growth and finally tumorigenesis. For instance, *HOXB8* belongs to proto-oncogenes. In normal cells, it is involved in cell proliferation. When this gene is altered, it may act as an oncogene and can contribute to the development of cancers such as AML (acute myeloid leukemia) (*137-139*).

Tumor suppressor genes are a set of genes that inhibit the growth of cells or even induce cell death. Together with proto-oncogenes, the tumor suppressor genes regulate cell expansion and proliferation, keeping the balance between cell division and cell death in non-transformed cells. When the tumor suppressor genes are inactivated, the balance is broken, and cells will grow and divide in an uncontrolled way. *TP53* is the most famous tumor suppressor gene. Mutations of *TP53* can lead to cancer development, which is why mutations of *TP53* are so frequent in human cancers (*140, 141*).

### 1.4.3 Treatment for cancers

The treatment alternatives for cancers are very limited. The cancer cells generally contain a wide range of mutations, making the cells heterogeneous and hard to target. Mutant cancer cells with resistance to the treatment can be selected during the treatment, and this finally leads to the failure of treatment. In addition, some cancer types can develop significantly fast and affect the normal function of other organs, which increases the difficulty of cancer treatment (*142*).

Traditional cancer treatment includes surgery, chemotherapy, and radiation therapy. Surgery treatment includes the physical removal of cancer tissue and relative tissues. This method can remove a clearly localized tumor tissue, like early-stage breast cancer and lung cancer, but may also lead to metastasis of cancer cells during the surgery process. Surgery treatment is also not appropriate if cancer has metastasized to several different organs. Chemotherapy is the systemic use of anti-cancer drugs to inhibit growth or kill cancer cells. Most cancer drugs can generally lead to a high level of tumor cell death, but the side effect is usually very obvious because of the lack of target specificity. Chemotherapy can injure the cells surrounding the cancer cells and other non-tumor cells with high proliferating speed, such as the hair follicles, digestive tract, and bone marrow. In addition, this method may also select for chemotherapy-resistant cancer cells. Radiotherapy is based on using ionizing radiation to kill malignant cells. This method can be applied to radiosensitive cancers, such as leukemias. However, the radiation may damage normal cells near the tumor cells, and many types of cancer are not sensitive to radiotherapy limiting the application of this treatment (*143, 144*).

A number of novel cancer treatments have been developing in the last decades. One of them is cancer immunotherapy (*145*), where the immune system is activated to fight against the cancer cells. Such treatments include vaccines for cancers, the use of checkpoint inhibitors such as anti-PD-1, and monoclonal antibodies (mAb), such as Rituximab, directly targeting malignant cells (*146, 147*). However, immunotherapy has shown inconsistent results and is tumor type-dependent (*148, 149*). Amino acid starvation is another potential strategy for cancer therapy (*150*). Most studied amino acid deprivation strategies target glutamine, asparagine, and arginine by enzymatic depletion. However, the efficiency of such strategies are tumor type-dependent, and the side effect is generally severe. More research is, thus, needed for better use of this type of therapy (*151-153*).

## 1.5 Hematopoietic stem cells

### 1.5.1 Definition of hematopoietic stem cells

Hematopoietic stem cells (HSCs), also called blood stem cells, can develop and differentiate into all kinds of blood compartments, such as white blood cells, red blood cells, and platelets. HSCs are immature, undifferentiated cells that can both divide to renew themselves and differentiate into mature blood cells, depending on the physiological conditions (*154*).

### 1.5.2 Hematopoiesis

Hematopoiesis, the process by which blood cells are produced, mainly takes place in the bone marrow (BM). The BM HSC niche is a special microenvironment, essential for hematopoiesis, providing protection and the secretion of a set of growth factors and cytokines which regulate the maturation and differentiation of cells from HSC (*155, 156*).

The blood cells, including erythrocytes, granulocytes, monocytes, lymphocytes, and thrombocytes, are differentiated from HSCs in the bone marrow and finally released into the circulation, where they fulfill their functions as mature cells (*157*).

HSCs are at the top of the hematopoietic hierarchy and are responsible for the maintaining of hematopoietic homeostasis. Mouse HSCs are characterized by lacking lineage-specific markers (Lin-) and express CD45, c-kit (CD117), and SCA-1 (Stem Cell Antigen 1). The most used marker for human HSCs is CD34. HSCs divide to renew themselves, and also further differentiate into common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs) (*158*) (**Figure 5**).

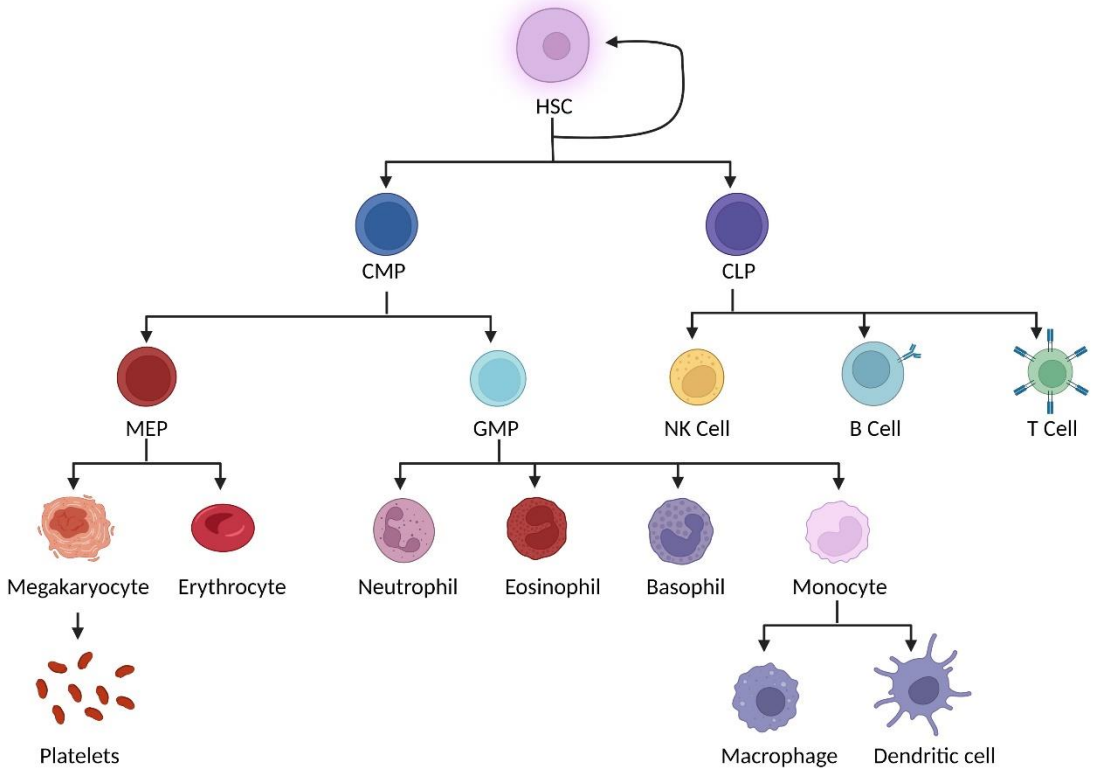


**Figure 5.** Schematic view of hematopoiesis.

Created with BioRender.com.

Erythrocytes, red blood cells, are fully specialized to adapt their function of transporting oxygen from the lungs to tissues and transporting carbon dioxide from tissues to the lung. The mature erythrocytes contain no nucleus but a high amount of hemoglobin. They are shaped like biconcave disks, increasing the surface-volume ratio and facilitating the exchange of oxygen and carbon dioxide. The surface marker for mature erythrocytes are CD235 (Glycophorins A) in humans and TER119 in mice. Similar to erythrocytes, thrombocytes (also called platelets) also contain no nucleus. They are tiny fragments of cytoplasm, derived from megakaryocytes. They have a very important role in hemostasis and contribute to the formation of clots and finally stopping bleeding. Both erythrocytes and megakaryocytes are differentiated from CMPs and megakaryocyte-erythrocyte progenitors (MEPs) (*159, 160*).

Granulocytes include neutrophils, basophils, and eosinophils. Neutrophils are the most abundant type of granulocytes and are essential parts of the innate immune system defense against pathogens. The cytoplasm of mature neutrophils is enriched with multiple granules and secretory vesicles. CD15 and CD16 are examples of surface markers that can be used to define mature granulocytes in humans, and Ly6g and CD11b are used in mice. For monocytes, the surface marker commonly used is CD14 in humans and Ly6C in mice. Monocyte can further be differentiated into macrophages or myeloid dendritic cells. The differentiation of granulocytes and monocytes are very close and share the same progenitors. They are both developed from CMPs and granulocyte-macrophage progenitors (GMPs) (*161*).

Lymphocytes include T cells, B cells, and natural killer (NK) cells. The common surface markers used to detect NK cells is CD56 in humans and, depending on the mouse strain, NKp46 or NK1.1. NK cells are effector lymphocytes in the innate immune system and have several functions, including triggering cell death. T cells express CD3, and mature B cells express CD19. B cells and T cells compose the acquired immune response and play an essential role in protecting against invading pathogens and vaccine responses. All the lymphocytes differentiate from CLPs (*162, 163*).

### 1.5.3 Mutations in HSCs

Mutations in HSCs and progenitor cells can lead to diseases, such as sickle cell disease, primary immunodeficiencies, and leukemia.

Sickle cell disease is characterized by the sickle-like shape of erythrocytes in the patients. This is because of the abnormality of hemoglobin in the red blood cells. In human, there are generally two kinds of hemoglobin: Hemoglobin F, also called fetal hemoglobin, consists of two alpha and two gamma chains, and are mainly found in the fetal red blood cells; Hemoglobin A, also called adult hemoglobin, consists of two alpha and two beta chains, and are the dominant hemoglobin for adults. In sickle cell disease patients, there is a point mutation in the *HBB* gene, which encodes the beta chain, and abnormal hemoglobin S is formed instead of the normal hemoglobin A (*164*).

Primary immunodeficiencies (PIDs) are characterized by developmental and functional disorders of the immune system, including more than 100 different disorders. Most of the disorders are caused by monogenic mutations (*165*). For example, The Wiskott-Aldrich syndrome (WAS) is characterized by immune deficiency and eczema, and it is caused by mutations in the *WASP* gene. The *WASP* gene encoded protein WASP is responsible for transducing signals from surface of blood cells to the actin cytoskeleton (*166*).

Leukemia is a group of blood cancers, which develops from the bone marrow and leads to excessive amounts of abnormal blood cells. Those abnormal blood cells also called leukemia cells, are usually not fully developed and do not function properly (*167*). There are two ways to group leukemia: by how fast the disease develops, and by the type of cells affected. Based on this, leukemia can be divided into acute and chronic. In acute leukemia, the leukemia cells

are in a very immature state and can hardly act their normal function. Those cells proliferate rapidly, and the disease therefore develops very fast to the severe stage. In chronic leukemia, the leukemia cells are relatively more mature and have some normal functions. Such cells proliferate relatively less rapidly compared to acute leukemias, and the disease develops gradually, often over many years. The other way to classify leukemias is by which type of blood cells are affected. Based on this, leukemia can be divided into lymphocytic leukemia and myeloid leukemia, in which the disease affects myeloid cells or myeloid cells, respectively. In combination, the diseases are mainly in four forms: acute lymphocytic leukemia (ALL), which is the most common type of leukemia in children, acute myeloid leukemia (AML), also called acute nonlymphocytic leukemia (ANLL), which occurs in patients at any ages, chronic lymphocytic leukemia (CLL), which is generally diagnosed in adults over 65, and chronic myeloid leukemia (CML), which mainly occurs in adults (*168, 169*).

# 2 RESEARCH AIMS

The aim of this thesis has been to (*i*) investigate the DNA damage response induced by CRISPR, which is a challenge to the CRISPR-based therapies, and to (*ii*) develop experimental CRISPR-based methods that can be used as drug target discovery platforms.

Specific aims:

Paper I: To investigate the DNA damage response induced by CRISPR, and parameters affecting CRISPR-mediated enrichment of cells with *p53* mutations.

Paper II: To develop the rapid CRISPR competitive (RCC) assay, and use it *in vivo* and *in vitro* to identify genes affecting the hematopoietic system.

Paper III: To identify mechanisms behind IL-4 mediated suppression of tumor growth, including using an *in vivo* CRISPR-based screen to discover potential synergistic drug targets related to this treatment.

# 3 MATERIALS AND METHODS

**A detailed description of Materials and Methods used in the constituent papers [*I-III*] are found in the respective "Material and Methods" section.**

**Cells *[I-III]***

The Hox cell line was generated by transducing bone marrow cells of C57BL/6 Cas9+ GFP+ mice with an estrogen inducible retroviral construct expressing Hoxb8. Hox cells are immortalized by estrogen-regulated Hoxb8 (ER-Hoxb8) and display a granulocyte-monocyte progenitor (GMP) phenotype (*47, 139*). *Trp53* KO Hox cells were generated by electroporation of a *Trp53* targeting sgRNA. *[I, II]*

The B16-F10 cell is a mouse melanoma cell line, purchased from ATCC and used at a low passage number. Cas9 expressing cells were generated by transducing B16-F10 cells with lentiCas9-Blast lentiviral particles. *Trp53*, *Il4ra*, and *Gcn1l1* KO version of B16-F10 were generated by transfecting the cells with sgRNA/Cas9 complexes. *[I, III]*

Bone marrow (BM) cells were collected by flushing femurs and tibias with PBS. Lineage negative (Lin-) cells were obtained by depleting lineage positive cells from the BM cells preparation. To differentiate the BM cells *in vitro*, electroporated Lin- cells were switched to indicated cytokines directly after electroporation. *[II]*

PBMCs were derived from buffy coats from consenting healthy donors (Karolinska Hospital Blood Bank) in line with local guidelines. PBMCs were stimulated with anti-CD3/28 beads for T cell expansion. *[II]*

The Jurkat-NF-κB-GFP cell line was generated by transducing Jurkat cells with the pSIRV-NF-κB-eGFP retroviral particles, with the modification to standard protocols that Ecotropic Receptor Booster was added to the cells. *[II]*

**Animals *[I-III]***

8 to 12-week-old, sex- and age-matched mice were used for experiments. All animal experiments were approved by the local Stockholm ethical committee, Sweden. WT C57BL/6 CD45.1 *[I-III]*, C57BL/6 Cas9+ GFP+ *[II]*, and *TCRb* KO mice *[III]* were acquired from the Jackson Laboratory. Homozygous Cas9+ GFP+ CD45.1+ mice were generated by crossing C57BL/6 Cas9+ GFP+ mice and C57BL/6 CD45.1 mice *[II]*. Germline IL-4Rα KO mice were created by breeding IL-4Rα floxed/floxed mice on a C57BL/6 background, backcrossed to C57BL/6 with B6.Rosa26-Cre mice *[III]*.

BM transplantations were performed by i.v. injection of $\sim 10^6$ bone marrow cells into recipient irradiated mice. The BM cells were typically electroporated with a sgRNA<2 h before being injected into the recipient mice. *[II]*

**B16-F10 Cancer model *[I, III]***

$0.5 \times 10^6$ B16-F10 cells were transfected with a *Ccr1* targeting sgRNA or control and directly injected s.c. into C57BL/6 mice, and tumors collected after 21 days. *[I]*

$0.5\text{-}1 \times 10^6$ B16-F10 cells were injected s.c. on one or two flanks (for competitive experiments comparing cancer cells with two different genotypes) of the animals, and the tumor size was followed over time by caliper measurement. *[III]*

**Viral preparation and transduction *[I-III]***

Lentiviral particles were generated by transfecting HEK293T cells with transfer plasmids (lentiCas9-Blast or LentiGuide-Puro-P2A-EGFP_mRFPstuf), as well as pMD2.G, and psPAX2. Retrovirus particles were generated by transfecting HEK293T cells with ER-Hoxb8 *[I]*, or pSIRV-NF-κB-eGFP *[II]* with the EcoPac gag-pol-env. Hox *[I]*, B16-F10 *[I, III]*, or Jurkat *[II]* cells were spin-infected and subsequently selected to remove the non-infected cells.

**sgRNA design, electroporation, and transfection *[I-III]***

sgRNAs were designed using the Green Listed software (*38*, *170*) utilizing sgRNA design from the Doench mouse library (*53*). 2'-O-methyl and phosphorothioate stabilized sgRNAs were ordered from Sigma-Aldrich or Synthego.

For sgRNA delivery, the Neon Transfection System was used for Hox cells *[I and II]*, BM cells *[II]*, PBMCs *[II]*, and Jurkat cells *[II]*, and Lipofectamine 2000 for B16-F10 cells *[I and III]*. The *Trp53* siRNA was typically delivered in the same reaction as the sgRNAs.

**CRISPR KO genotyping *[I-III]***

$1x10^5$ cells were collected for genomic DNA extraction using DNeasy Blood & Tissue Kit. Primers were designed using Primer-BLAST, aiming for a 400-1000 bp amplicon with the sgRNA target in the middle. Amplicons were gel purified and recovered using Zymoclean Gel DNA Recovery Kit. The PCR products were quantified using Nanodrop and sequenced by Eurofins Genomics. The Sanger sequencing data was subsequently analyzed by ICE (Synthego, https://ice.synthego.com). For the IDAA fragment length analysis, genomic DNA samples were sent to COBO Technologies (https://cobotechnologies.com/).

**Cloning of sgRNAs into lentiviral transfer plasmid and CRISPR screens *[I and III]***

sgRNAs with overhangs for the LentiGuide transfer plasmid were designed using the Green Listed software (*38, 170*) using sgRNA design from the Doench mouse library (*53*) and, for intergenic controls, the Wang mouse library (*171*). Individual sgRNAs were ordered from Sigma-Aldrich, and the sgRNA library was ordered from CustomArray as a DNA oligo pool. Cloning was performed using BsmBI cleaved lentiGuide-Puro-P2A-EGFP_mRFPstuf plasmid and the library oligo pool, with NEBuilder HiFi DNA assembly master mix. Endura ElectroCompetent cells were subsequently transformed with the cloned plasmid pool using electroporation. The electroporated cells were combined and seeded on LB agar plates at 37°C overnight. Plasmids were purified using the EndoFree Plasmid Maxi Kit.

The sgRNA cloned lentiGuide-Puro-P2A-EGFP_mRFPstuf was used as a transfer plasmid for lentiviral preparation and transduction. The total amount of transduced cells was calculated based on MOI (0.25 for B16-F10 cells, and 0.05-0.1 for Hox cells), aiming for 1000 transduced cells for each sgRNA.

For the *in vitro* CRISPR screen, cells were exposed to *GFP* targeting sgRNA electroporation with or without *Trp53* siRNA, 0.5 µg/ml Etoposide 8 h pulse stimulation, or 3 µM AMG232 8 h pulse stimulation. *[I]*

For *in vivo* CRISPR screen, B16-F10-Cas9 were subsequently transduced with the lentiviral library and, after selection, injected s.c. ($5x10^6$ cells on two flanks) into WT C57BL/6 mice. *[III]*

After selection, cells were collected for genomic DNA extraction. Genomic DNA was then amplified using Q5 High-Fidelity DNA Polymerase. Sample-specific barcodes and adapters for Illumina Sequencing were introduced at the same time using specific primers. The final PCR products were gel purified and recovered using the Zymoclean Gel DNA Recovery Kit, and quantified with Qubit 4 Fluorometer using the Qubit dsDNA HS Assay Kit, and pooled for next-generation sequencing (Illumina MiSeq v3 run, 2x75bp reads). The raw FASTQ data were analyzed by MAGeCK (*172*).

**Flow Cytometry Analysis *[I-III]***

Single-cell suspensions of BM cells, Hox cells, lymph nodes, spleen, blood, and the tumor was generated and stained with combinations of antibodies, washed and sorted using Sony SH800S, or acquired using BD LSRFortessa, BD FACSVerse, BD Accuri, or Cytek Aurora. Generated FCS files were analyzed by FlowJo version 10 (FlowJo, LLC).

**Analysis of data from the Depmap portal *[I and III]***

sgRNA enrichment (CRISPR Avana Public 20Q4 release), mutation profile (Mutation Public 20Q4 release), drug sensitivity (PRISM Repurposing Primary Screen 19Q4 release), and mRNA expression levels (Expression Public 20Q4 release) was extracted from the Depmap portal (https://depmap.org/portal/) (*173-177*). Correlation analysis was performed with the Depmap data explorer tool. Connectivity maps were generated using the geneMANIA plugin for Cytoscape (*178*, *179*). tSNE plots were made with the Rtsne package (https://github.com/jkrijthe/Rtsne) to analyze the cluster and ggplot2 (https://github.com/tidyverse/ggplot2) to visualize the data. The "ENCODE and ChEA Consensus TFs from ChIP-X" functionality of Enrichr (https://maayanlab.cloud/Enrichr/index.html) (*180*, *181*) was used to identify transcription factor binding to gene sets.

**Competitive co-culture assay *[I]***

*Trp53* KO and WT cells were mixed at a 1:4 ratio and subsequently exposed to different interventions. For hypoxia experiments, Hox cells were cultured in 1% O2 for seven days in a Baker InvivO2 Physiological Cell Culture Workstations. For *in vivo* experiments, B16-F10 cells were transfected with a *Ccr1* targeting sgRNA or control and directly injected s.c. into C57BL/6 mice, and tumors collected after 21 days. The proportion of *Trp53* KO cells was subsequently quantified by sequencing.

**Real-Time PCR *[I]***

RNA was extracted using Direct-zol RNA MiniPrep Kit and was converted into cDNA using High Capacity RNA-to cDNA kit. The expression of indicated genes was quantified with a CFX 384 Real-Time PCR machine (Bio-Rad) using TaqMan gene expression FAM assays for target genes. Expression was normalized by TaqMan gene expression VIC assays for β-actin, and gene expression was quantified using the ddCT method.

**Apoptosis TUNEL assay *[I]***

Cells were collected and fixed by PFA at different time points, and the FlowTAC Apoptosis Detection Kit was used to stain apoptotic cells for analysis by flow cytometry (BD Accuri).

**JAK1/STAT1 activation assay *[I]***

Hox cells were cultured +/- mouse Interferon Beta and the JAK1 inhibitor Solcitinib for seven days. Cells were counted on day seven by flow cytometry (BD Accuri) using CountBright Absolute Counting Beads.

**Macrophage phagocytosis assay *[II]***

The macrophage phagocytosis assay was performed using Phagocytosis Assay Kit. Differentiated macrophages were incubated with the Latex Beads-Rabbit IgG-PE complex. Cells were then washed gently and collected for further analysis.

**RNAseq *[III]***

Tumors were collected from mice two days after i.v. +/- IL-4 complexes were prepared and sequenced as described in (*182*). The data were analyzed using Gene Set Enrichment Analysis (GSEA) (*183*), g:Profiler (*184*), and the GeneMANIA plugin for Cytoscape (*179*).

**Mass spectrometry *[III]***

Tumors were collected from mice treated +/- IL-4 complexes the day after the second injection and analyzed by mass spectrometry.

# 4 RESULTS AND DISCUSSIONS

## 4.1 CRISPR leads to DNA damage response (DDR) and enriches for cells with mutations in *Trp53*. *[Paper I]*

The CRISPR/Cas9 system is a very efficient gene-editing tool and could potentially be used for clinical gene therapy. However, early gene therapy trials have shown the possibility for cancer development, so safety concerns related to the use of CRISPR for clinical gene therapy have been emphasized, including related to the DDR induced by CRISPR. To study this effect, we stimulated Hox cells (transient immortalized mouse hematopoietic cells) with CRISPR and confirmed that CRISPR led to a stereotypical DDR, including delayed cell growth, apoptosis induction, and activation of p53. We also tested pharmacological activation of p53, using Etoposide and AMG232, and found a similar response but with a relatively higher magnitude compared to CRISPR stimulation.

To investigate if the relatively mild DDR phenotype induced by CRISPR was sufficient to select *Trp53* mutated cells, we established a rapid CRISPR competitive (RCC) assay setup, which is future discussed in Paper II (*185*). To this end, *Trp53* KO and WT cells were mixed and exposed to CRISPR stimulation, or pharmacological activation of p53 [pulse stimulation of Etoposide or AMG232 (*186, 187*)], as well as hypoxia, to activate p53 (*188*). We found that the ratio of *Trp53* KO was enriched significantly after CRISPR (including both electroporation and lentiviral delivery), AMG232, Etoposide, or hypoxia stimulations. Similar findings were also observed *in vivo*, when we injected B16-F10 tumor cells (Mixed *Trp53* KO and WT, +/- CRISPR stimulation) into the mice.

To study how the level of CRISPR-induced DDR affected the *Trp53* KO cells enrichment, we tested several sgRNAs with various off-target activity levels. We discovered that higher off-target activity of sgRNAs led to a higher level of DDR, and subsequently higher activation of p53 (measured as transcription of *Cdkn1a*, a target for p53). Importantly, we found that the final enrichment effect of *Trp53* mutated cells correlated with the early stage upregulation of *Cdkn1a* transcription, as well as the off-target activity level of the sgRNAs used.

In summary, our study showed that CRISPR could enrich for *Trp53* mutated cells, and that the enrichment effect correlated with the severity of DDR induced by CRISPR. In addition, the early stage upregulation of *Cdkn1a* could be a parameter for low off-target sgRNA selection and also to predict how the cells may be affected by the CRISPR-induced DDR in the long term.

## 4.2 Identification of a core CRISPR-p53 interactome using CRISPR-based screen and Depmap portal. *[Paper I]*

To expand our understanding of CRISPR-induced DDR and identify more p53 related non-redundant genes in this pathway, we performed a CRISPR-based screen. We designed a custom library targeting DNA damage-related genes and performed the screen in both Hox and B16-F10 cells. In the initial screen, DDR was induced by lentiviral delivery of sgRNA into the cells and the KO of the target gene. We discovered that *Trp53* sgRNA was significantly enriched, and *Mdm2* sgRNA was significantly depleted.

We further performed another screen using the same custom library, but cultured the cells for 14 days after the lentiviral delivery and then performed another controlled CRISPR event, by electroporating with a *GFP* targeting sgRNA. Within this screen design, we could separate the CRISPR-induced DDR and the lentiviral delivery induced DDR, and also make it possible to include the control stimulation such as Etoposide and AMG232. We identified that sgRNAs targeting a set of tumor suppressor genes were enriched after CRISPR or pharmacology activation of p53 (**Figure 6**).

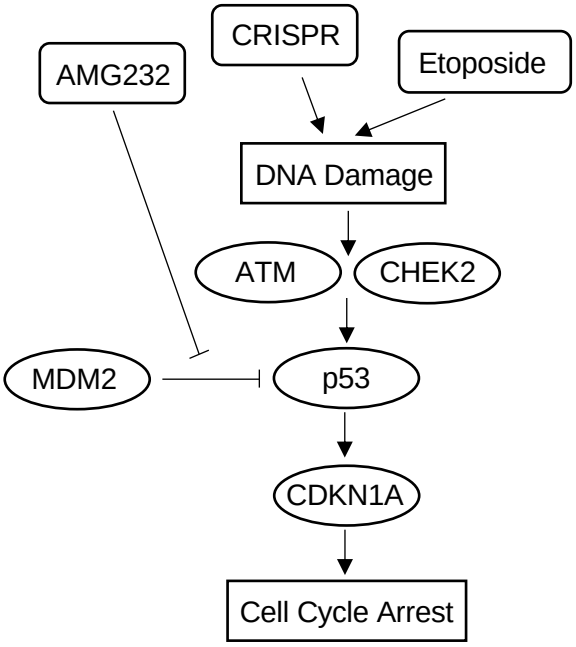


**Figure 6.** Model indicating genes playing a non-redundant role in the DNA damage response, discovered by CRISPR-based screen.

Since the enrichment of mutated tumor suppressor genes challenges the use of CRISPR for clinical gene therapy, we tested a set of inhibitors targeting p53 or non-redundant proteins in the p53 pathway. Although most of the inhibitors did not show any effects, we identified that a collection of *Trp53* siRNAs could efficiently inhibit p53 activity after CRISPR stimulation without negatively impacting the CRISPR KO efficiency. Additionally, the delayed cell growth was normalized after *Trp53* siRNA treatment during CRISPR stimulation. Most importantly, the enrichment of sgRNAs targeting tumor suppressor genes was eliminated when we used *Trp53* siRNA-treated cells or *Trp53* KO cells in the CRISPR-based screen.

The Depmap portal is a database containing full genome CRISPR screen, baseline gene expression, mutation status as well as drug sensitivity data of >800 human cell lines. Based on the analysis using the data from the Depmap portal, we observed that the *TP53* sgRNA enrichment was significantly higher in *TP53* WT cells. In addition, the *TP53* sgRNA enrichment was correlated with the sensitivity to a set of p53 activation drugs, as well as the depletion of *MDM2* sgRNA. These suggested that the correlation of *TP53* sgRNA enrichment can identify factors related to the CRISPR-p53 pathway. A list of genes was thus generated using this method, and most importantly, all the genes identified in our CRISPR screen experiment were included in this list. We continued to use a similar method to identify another list of genes correlated with the *TP53* mutations status, instead of the *TP53* sgRNA enrichments. Not surprisingly, there was a large overlap between those two lists. Cells with mutations in the positively correlated overlap genes or overexpression in the negatively correlated overlap genes can be enriched by CRISPR stimulation. Based on this, we highlight TP53 as well as TP53BP1, CDKN1A, USP28, CHEK2, ATM, XPO7, UBE2K as a core CRISPR-p53 tumor suppressor interactome. (**Figure 7**).

| Positively Correlated Genes (+) | Negatively Correlated Genes (-) |
|---|---|
| *TP53BP1 #* | ***MDM2 #*** |
| ***CDKN1A #*** | *PPM1D #* |
| *USP28 #* | *MDM4 #* |
| ***CHEK2 #*** | *PPM1G #* |
| ***ATM #*** | *WDR89 #* |
| *XPO7 #* | *USP7 #* |
| *UBE2K #* | *DDX31 #* |
| *RPL22* | *FERMT2* |
| *FAM193A* | *TERF1 #* |
| *ZNF326* | *USP38* |

**Figure 7.** Top 10 genes with the strongest positive (+) and negative (-) correlation with *TP53* sgRNA enrichment from full genome CRISPR screens of 808 cell lines (Depmap portal). Bold indicates genes identified experimentally in the CRISPR-based screen. # indicates genes that overlap with the list correlating with *TP53* mutation status.

Finally, we further analyzed the baseline gene expression pattern in cells for which CRISPR screen data also was available. We found that cells that enriched for *TP53* sgRNA generally had a strong baseline *CDKN1A* expression. This suggested that baseline *CDKN1A* expression is an important parameter to estimate if the CRISPR-p53 pathway is active in a cell and *TP53* sgRNA can be enriched by CRISPR. In comparison, *TP53* expression itself was not correlating with enrichment of *TP53* sgRNA.

In summary, we identified non-redundant genes in the DDR induced by CRISPR, and described a core CRISPR-p53 interactome. Cells with mutations in these genes can potentially be enriched by CRISPR. Most of them belong to tumor suppressor genes, and mutations of

these genes can lead to cancer. Therefore, those genes should be monitored during the clinical CRISPR use and are potential drug targets related to the CRISPR-p53 response.

Our data related to transient inhibition of p53 was in line with previous findings, showing that it can increase the KO efficiency (*189, 190*) while not interrupting the normal cell function (*191*). Importantly, we also identified that transient inhibition of p53 suppressed the enrichment of mutations in a set of tumor suppressor genes. This can partially solve the safety concern relate to the CRISPR clinical use.

## 4.3 CRISPR-based drug target discovery in the hematopoietic system: Rapid CRISPR Competitive (RCC) assay. *[Paper II]*

When CRISPR/Cas9 is used to inactive a gene in a cell population, a uniform genotype is not achieved. Instead, various genotypes, including different forms of InDels, are generally generated. Such genetic heterogeneity makes the analysis of the role of the target gene difficult. One solution to this is to generate clonal cell lines with uniform genotypes. However, the generation of clones may lead to bias to the cell lines, and it is not always possible to generate clonal cell lines for all cell types, such as primary cells. It is also time-consuming. An alternative is to accept the genetic heterogeneity generated by CRISPR and quantify the heterogeneity using next-generation sequencing (NGS) platforms, fragment length analysis (FLA), or standard Sanger sequencing. The latter one is faster and cheaper than the other approaches, and several analysis tools have been developed to quantify the genetic heterogeneity in sanger sequenced samples, such as ICE (Inference of CRISPR Edits) and TIDE (Tracking of Indels by DEcomposition). Benefiting from these software, with a focus on ICE, we developed a simple discovery approach analyzing enrichment or depletion of CRISPR generated genotypes. We call this approach the Rapid CRISPR Competitive (RCC) assay, and used it in study II to discover genes affecting the hematopoietic system (**Figure 8**).

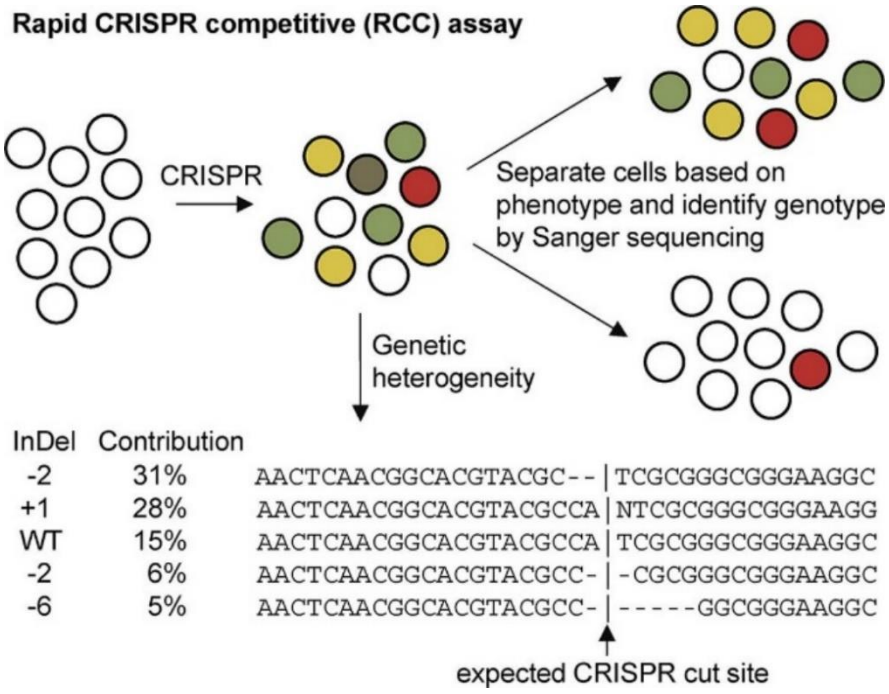


**Figure 8.** Model describing the Rapid CRISPR Competitive (RCC) assay.

We optimized delivering a *GFP* targeting sgRNA into Lineage negative (Lin-) BM cells from Cas9+ GFP+ mice and quantified the generated mutations using flow cytometry (GFP) and Sanger sequencing. We also developed the co-targeting method to enhance the target gene KO efficiency. We co-targeted *GFP* and the studied gene, then sorted the cells with successfully inactive GFP, to confirm that the KO efficiency for the gene of interest was higher in the *GFP* KO cells compared to GFP+ cells. Such a method with readouts based on Sanger sequencing showed a high correlation with the flow cytometry readouts, although the sensitivity was lower at low mutation frequencies. This could be because of the minor peaks of the low-frequency mutations in the Sanger sequencing.

Next, we generated "immuno-CRISPR" (iCR) mice by transplantation of CRISPR-treated Lin- BM cells to irradiated recipients. We targeted *Zap70* as a proof-of-concept in the Lin- BM cells, and the data showed that the mutation rate of *Zap70* was high in B cells, but low in T cells. This suggested that *Zap70* is essential for T cells but redundant for B cells, which was in line with the previous literature (*192*). This proved that iCR mice can be used to study a gene of interest, such as *Zap70 in vivo*. Additionally, we developed secondary transplantation methods to increase the KO cell percentage when the KO percentage was low in the CRISPR-treated Lin- BM cells. Comparing the complexity and the expensive cost to generate gene-modified mice, the iCR mice models and RCC analysis are simple to generate and cost-efficient. As such, we think there is immense potential in using iCR mice to study the role of genes in immune cells *in vivo*.

As an alternative discovery approach, we *in vitro* differentiated Lin- BM cells, electroporated with a *GFP* sgRNA into macrophages and dendritic cells and found that KO *GFP* did not affect the differentiation. This proved that the differentiation of CRISPR treated Lin- BM cells was not affected and that this model system could be used to study the behavior of differentiated cells such as phagocytosis *in vitro*. We also used RCC to study the activation of T cells. We targeted the gene *LCP2* and used either anti-CD3/28 or PMA to stimulate cells. The data was in line with the previous literature showing that *LCP2* is essential for the TCR signaling pathway (*42*). This expanded the application of RCC in researching genes related to the activation of T cells.

Finally, we assessed RCC in studying the role of genes related to malignancies in the hematopoietic system. We generated Hox cells in which the activity of the proto-oncogene *Hoxb8* can be induced. The activity of HOXB8 makes the BM cells proliferate and keep them at a behavioral and phenotypic state overlapping with acute leukemia cells. Turning off the HOXB8 activity makes the cells differentiate into Lin+ cells. When we optimized the settings, we delivered sgRNA targeting *Hoxb8* into the cells, resulting in half of the cells acquiring the Lin+ phenotype. As expected, the sequencing showed 100% mutation frequency of *Hoxb8* in the Lin+ population. Interestingly, although almost half of the Lin- cells also showed mutations in *Hoxb8*, a more detailed analysis of the genotypes in the Lin- population showed InDels with a multiplier of three nucleotides, resulting in deletion of amino acids (AAs), which may not interrupt the function of the protein. In contrast, InDels of the multiplier of one or two

nucleotides, as found in the Lin+ population, led to a frameshift and finally resulted in premature stop codons and nonsense-mediated decay (*193, 194*). Although the observed phenotype was likely dependent on the specific site where the gene was targeted, comparing InDels that cause frameshifts to such that only cause insertions or deletions of AAs could be a strategy for identifying functionally redundant amino acids in proteins.

After that, we used geneMANIA to discover a set of proteins physically interacting with HOXB8, and used the RCC assay to confirm that *Hoxb8* and *Meis1* targeting sgRNAs were able to differentiate the cells into Lin+ phenotype, while *Pbx1* targeting sgRNA somewhat surprisingly could not. This could be due to the redundancy of PBX proteins, where PBX1 is only one of the family (*195*). Taken together, this suggested that PBX1 may not be a good drug target related to HOXB8 related transformation but that MEIS1 could be a drug target candidate. Additionally, this showed that the RCC approach could be used to study genes involved in the malignant transformation of cells.

## 4.4 CRISPR-based drug target discovery: Identification of *Gcn1l1* regulated amino acid deprivation response behind IL-4 suppressing tumor growth. *[Paper III]*

The important role of IL-4 in the protective response to helminth infections is well established (*196*). IL-4 has also been shown to suppress autoantibody-mediated joint inflammation in several animal models for arthritis (*47, 197*). However, the role of IL-4 and IL-4Rα has not been fully established in the cancer setting, and both positive and negative roles of IL-4 have been observed on tumor growth (*198, 199*).

Based on our previous studies, where we discovered that IL-4 can suppress autoantibody-mediated effector functions (*47, 197, 200*), we tested if IL-4 could suppress the therapeutic immune activities in the cancer setting. To our surprise, we found that IL-4 alone could suppress the B16-F10 tumor growth. We further confirmed the suppression was dependent on IL-4Rα in the host, instead of IL-4Rα directly in the B16-F10 tumor cells, by using IL-4Rα KO mice and IL-4Rα KO B16-F10 cells. Importantly, the IL-4 mediated suppression was seen in both prophylactic and therapeutic protocols.

Next, we found that IL-4 injections activated CD8+ T cells in lymph nodes and we hypothesized that T cells may be involved in the IL-4 mediated protection. Several previous studies have suggested that T cells could be involved in the IL-4 mediated protection in cancer models (*199, 201*). However, we found that IL-4 injection did not show any synergistic activity combined with anti-PD-1 treatment. Additionally, depletion of CD4+ or CD8+ cells did not eliminate the suppression effect of IL-4, and IL-4 still showed a suppression effect in T cell-deficient mice. These results clearly rejected our initial hypothesis, and we concluded that T cells were activated by IL-4 administration but that this was not involved in the therapeutic activity of IL-4 in the used model. Instead, we found macrophages were involved in this phenotype by chlodronate liposome-induced macrophage depletion.

To further investigate the IL-4 induced phenotype, we performed an RNAseq of tumors from mice injected with IL-4 or control and discovered an enrichment of the genes linked to amino acid deprivation in IL-4 injected mice. A mass spectrometry-based analysis showed that essentially no arginine was detected in the tumor bed after IL-4 injection. This was in line with RNAseq data showing that the arginine depleting enzyme arginase 1 (*Arg1*) was upregulated after IL-4 injection. Additionally, B16-F10 cells were observed to be sensitive to ARG1-mediated depletion of arginine *in vitro*. This suggested that IL-4 inducing upregulation of *Arg1* and the complete depletion of arginine could result in, the suppression of tumor growth.

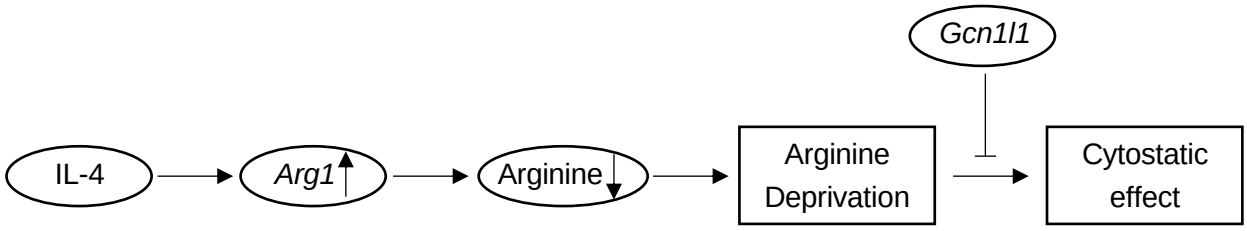


**Figure 9.** Model describing IL-4 suppressing tumor growth by inducing a *Gcn1l1* regulated amino acid deprivation response.

To identify potential synergistic drug targets related to the IL-4 induced amino acid deprivation, we designed a custom CRISPR screen library targeting genes involved in the amino acid deprivation response and also genes identified to be significantly changed in the RNAseq experiment. The library was introduced into Cas9 expressing B16-F10 cells, to generate a genetically heterogeneous cell population, and the cells were injected into groups of mice subsequently injected +/- IL-4. Analyzing the *in vivo* screen data using MAGeCK (*172*), we discovered that sgRNAs targeting *Gcn1l1* (also called *Gcn1*) and *Eif2ak4* (also called *Gcn2*) were depleted in the IL-4 injected mice. We further analyzed the Depmap portal database and found the depletion of *GCN1* sgRNA was significantly correlated with the depletion of *GCN2* sgRNA. Additionally, *GCN2* was the top gene correlating to the depletion of *GCN1* sgRNAs, and vice versa. This was in line with previous literature showing that *GCN1L1* (*GCN1*) and *EIF2AK4* (*GCN2*) are two interaction partners and play an early essential role in response to amino acid deprivation (*202*). For validation of the screen data, we generated a *Gcn1l1* KO version of B16-F10 cells and confirmed that the suppression activity of IL-4 was significantly enhanced in the *Gcn1l1* KO cells. This indicated that those two genes could protect the cells from IL-4 induced tumor suppression and could be candidate co-targets to enhance the IL-4 mediated protection effect. Also, mutations of these two genes in the tumor may decrease the suppression ability of IL-4 administration (**Figure 9**).

# 5 CONCLUSIONS

In Paper I, we discovered that CRISPR-mediated DNA damage enriches cells with mutations in *p53* and a core CRISPR-p53 interactome. Importantly, this enrichment can be inhibited by transient p53 inhibition. In addition, we found several parameters affecting the enrichment.

In Paper II, we presented a rapid and universal experimental approach, RCC, to discover potential drug targets in the hematopoietic system *in vitro* and *in vivo*, leveraging the genetic heterogeneity induced by CRISPR for discovery.

In Paper III, we identified that IL-4 could suppress the growth of B16-F10 melanoma tumors and further used an *in vivo* CRISPR-based screen to discover that *Gcn1l1* regulates the amino acid deprivation response in this phenotype.

In summary, we have developed novel CRISPR-based drug discovery approaches and investigated DNA damage-related safety concerns of potential relevance for CRISPR-based therapies.

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