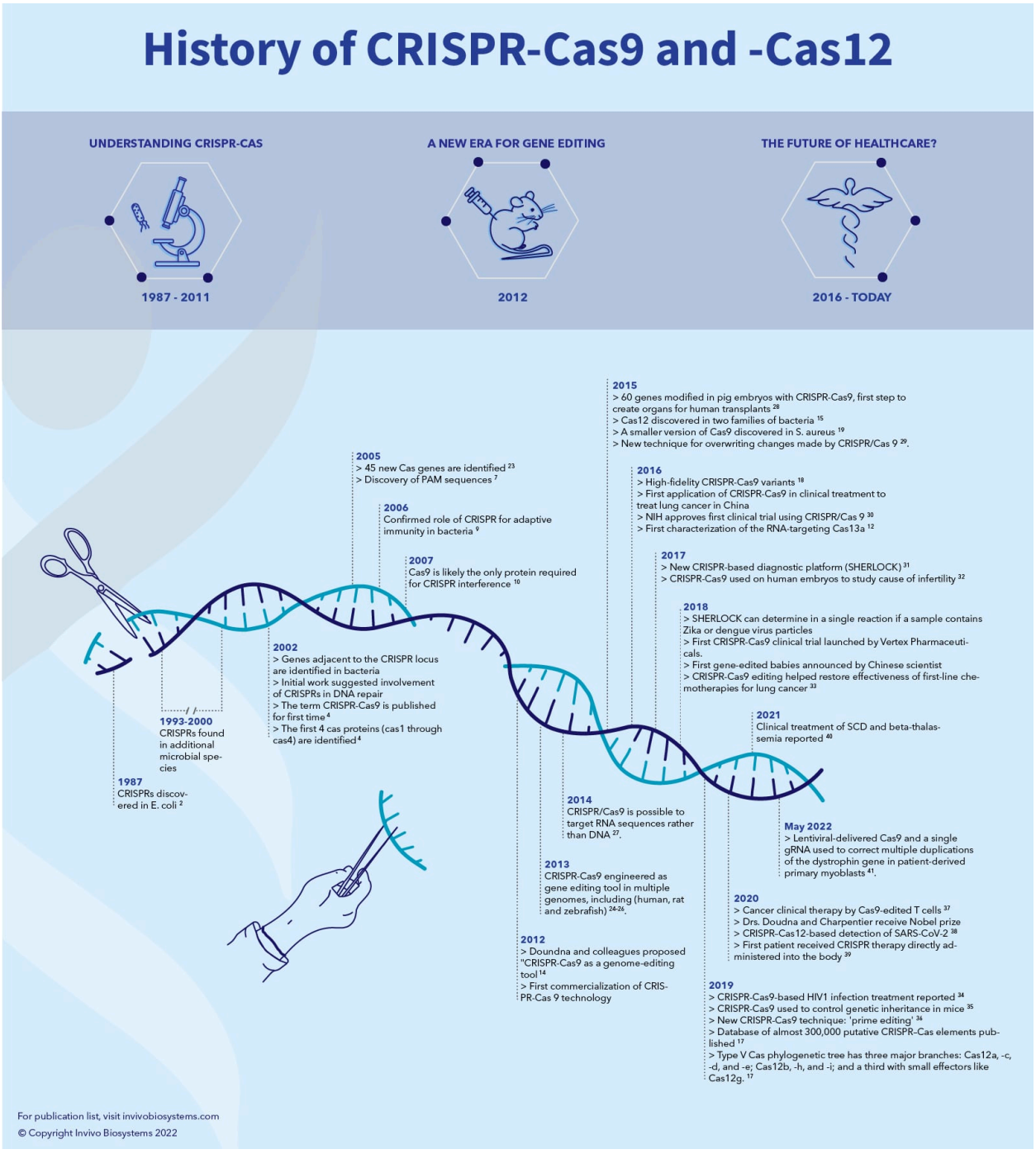
History of CRISPR: Cas9 and Cas12

Between the early 2000s and mid-2010s, artificial enzymes engineered to manipulate genomes – Zinc-finger nucleases (ZFNs) and transcription-activator–like effector nucleases (TALENs) – had garnered considerable interest. Starting in 2012, the CRISPR-Cas system was developed into a revolutionary molecular tool for DNA and RNA “surgery”. Clustered regularly interspaced short palindromic repeats (CRISPRs) are a family of nucleotide sequences present in multiple copies in the genome. Those sequences are called DNA direct repeats. Thanks to these patterns, CRISPRs can be used as a way to find a specific sequence of DNA inside a cell, or to shut one off by targeting and cutting a specific gene.

In the last decade, scientists have focused on the Cas endonucleases in an attempt to continuously improve their efficiency as genome editing tools. Despite their short history, CRISPR-Cas systems and Cas proteins have already been the subject of over thirty thousand publications [1]

In this blog post, we summarize the history of two of the most widely studied Cas proteins and how they are shaping the future of research and medicine.



Discovery of CRISPR

In 1987, while studying a gene responsible for the conversion of alkaline phosphatase, Dr. Yoshizumi Ishino and his team from the University of Osaka observed an "unusual

arrangement with repeated sequences" in the bacteria E. coli.[2] However, due to the lack of sufficient DNA sequence technology and data (the sequencing process had not yet been automated), the function of these arrays remained a mystery. It wasn't until 1993 that Dr. Francisco Mojica, then a doctoral student at the University of Alicante in Spain, characterized for the first time what is now called a CRISPR locus.[3] Almost a decade later, in the year 2000, Mojica reported that the different repeat sequences that he had observed shared a common set of features. In collaboration with Ruud Jansen from the University of Utrecht in the Netherlands, the term CRISPR was invented. Jansen was the first to publish these findings in 2002, as he identified the first 4 cas (CRISPR-associated) proteins (cas1 through cas4) [4]. He notes that those cas are present in prokaryotes (Archaea and Bacteria), but absent from eukaryotes or viruses. In 2005, Mojica reported that these sequences matched snippets from the genome of bacteriophage [5]. This finding led to the hypothesis that CRISPR is an adaptive immune system [5–7].

What is the CRISPR-cas System?

The role of CRISPR systems in adaptive immunity was pursued by various teams in the late 2000s [8,9] until Philippe Orvath and colleagues finally demonstrated it empirically in 2007 [10]. They showed that cas genes do not directly provide resistance but rather mitigate the insertion of additional fragments of foreign DNA (spacers), as well as repeats. The CRISPR spacers are used as immunological recordings to combat future infections.

The Cas protein sequences and the genomic organization of CRISPR-cas loci display a wide diversity and are rapidly evolving. Since the discovery of CRISPR, 2 classes, 6 major types and more than 33 subtypes of Cas proteins have been identified [11]. Thanks to the screening of constantly updated genomic and metagenomic databases, our knowledge of this diversity of Cas proteins is ever expanding. For example, we know that while some Cas proteins like Cas9 cleave DNA, others, like Cas13 cleave RNA [12,13].

Cas9: the Original CRISPR enzyme

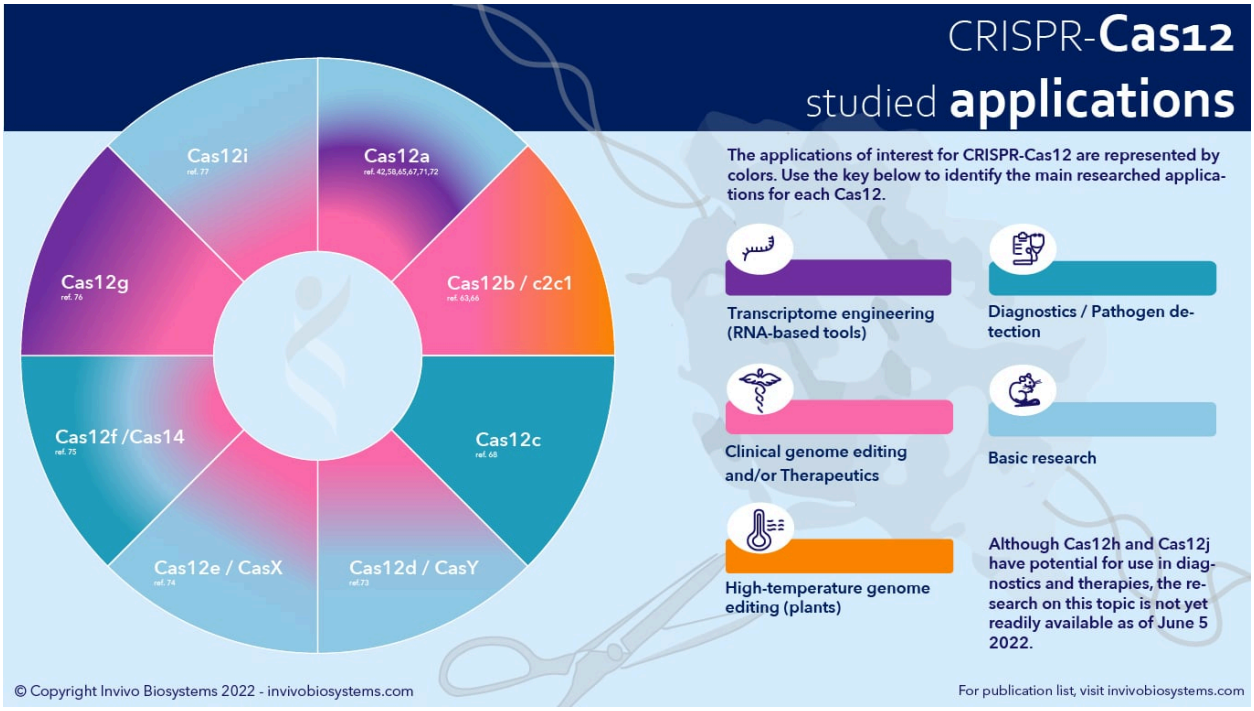
The best known Cas protein to date is SpCas9, the original gene editing enzyme. Cas9 is found in several strains of bacteria, where it originally evolved to cut invading foreign DNA. In 2005, Alexander Bolotin revealed an unusual CRISPR locus while studying the newly sequenced bacteria Streptococcus thermophilus [7]. Although the CRISPR array

(alternating conserved repeats and spacers) was similar to previously reported systems, it lacked some of the known cas genes and instead contained novel cas genes.

One of these new cas genes encoded a large protein predicted to have nuclease activity, Cas9. Bolotin also noted that the spacers, which had homology to viral genes, all shared a common sequence at one end. This piece of targeting DNA located next to the desired cleavage site is called the protospacer adjacent motif (PAM) and is required for target recognition. Bolotin's work on bacterial immunity ultimately led to the groundbreaking discovery by Jennifer Doudna and her team that Cas9 is indeed an RNA-guided endonuclease [14]. Based on this discovery, Doundna and colleagues proposed "an alternative methodology based on RNA-programmed Cas9 that could offer considerable potential for gene-targeting and genome-editing applications." [14].

Cas12: The Future of CRISPR-Dx?

Discovered in bacteria in 2015 [15], Cas12 is a more recent addition to the CRISPR family. Yet, in the last couple of years, Cas12 has gathered growing interest for its potential use in diagnostics [16]. Contrary to Cas9, which requires two RNAs to recognize and cut its target, and possesses no inherent RNAse activity, Cas12 processes its own guide RNAs (single RNA-guided endonuclease) and only requires crRNA for targeting.



David Scott and colleagues from Arbor Biotechnologies, in collaboration with Eugene Koonin, recently studied the entire Cas12 family to find effectors with new functionality [17]. Together, they built and searched a database of almost 300,000 putative CRISPR–Cas elements for type V systems. They then discovered that the type V phylogenetic tree has three major branches: one with Cas12a, c, d, and e; a second that includes Cas12b, h, and i; and a third with smaller effectors such as Cas12g. Interestingly, Cas12g can cleave both single-stranded DNA and RNA in trans in a non-specific manner. This ability could be exploited to detect viruses or the presence of certain mutations. Cas12i, on the other hand, does not need a trans-activating CRISPR RNA (tracrRNA) for single-strand DNA cleavage and is able to introduce a break only in the one strand of DNA that is not paired with the guide RNA. Because of this, Cas12i has the potential to become a highly specific platform for therapeutic genome editing.

	Cas9 (also known as SpCas9)	**Cas12 (also known as Cas12a or Cpf1)**	**Notes**
Discovered in	2012	2015	Cas 9 is the go-to option for genome editing thanks to its longer history and more publications.
number of publications as of May 2022	~ 20,100	~ 700	
patent rights	Broad Institute	Broad institute, MIT, and Harvard.	
Cas family type	Type II	Type V	
size (amino acids)	~ 1,000-1,600	~ 1,300	Cas12's smaller size allows it to be more easily delivered inside cells using common viral vectors like adeno-associated viruses (AAVs)
Nuclease activity	single	Dual (create double-stranded breaks in DNA)	The dual nuclease activity of Cas12a is essential for its ability to create double strand breaks in the DNA and is dependent on the RuvC domain [42,43]
RNA(s) needed	crRNA + tracrRNA (or single-guide RNA)	crRNA	Cas12a processes its own pre-crRNA into mature crRNAs, without the requirement of a tracrRNA, making it a unique effector protein with both endoribonuclease and endonuclease activities [44]. This dual nuclease activity is advantageous for

			multiplex gene editing, transcription, epigenetic modulations and base editing [45]
Nuclease site	2 nuclease domains: HNH and RuvC	Single nuclease site RuvC-Nuc	
non-specific ssDNA cleavage	no	yes	Once the complex Cas12/crRNA/target DNA is established, the non-specific cleavage of any collateral single-stranded DNA (ssDNA) is induced. This activity can be exploited to create biosensing systems that merge Cas12 effectors with amplification methods to enable rapid and specific detection of pathogen DNA samples [46,47].
type of cut in dsDNA	blunt cut	staggered 5' overhang (may facilitates site-directed integration with variable efficiency)	This feature facilitates site-directed integration, making Cas12a a useful tool similar to restriction enzymes for precise in vitro DNA assembly [48,49]. Cas9's blunt double-stranded may also induce more aberrations during repair.
trans cleavage	no	yes	The non-specific single-stranded DNA (ssDNA) cleavage activity in Cas12a is sufficient to completely degrade both linear and circular ssDNA molecules [50].
seed sequence	10 nt	5-6 nt	
PAM sequence recognised	3' G-rich 3-5 nt	5' T-rich	Most mammalian genes are GC-rich, so finding the requisite GG in mammalian cells for targeting Cas9 to a specific location is easier than for Cas12. Cas9's preference for GC-rich PAMs limits the targeting of AT-rich sequences, for example most of the non-coding genome in zebrafish is AT-rich [51].
specificity in vivo	+	++	[52]
mismatch tolerance *in vivo*	+*	++	Off-targeting effects can occur when the CRISPR nuclease tolerates some levels of imperfect complementarity between gRNA spacer sequences and protospacer sequences of the targeted genome. A recent comparison of Cas12a and Cas9 from various species in vitro revealed that both enzymes share similar types of specificities and tolerate similar

			mismatches [53]. However, in vivo reports show a lower off-target effect of Cas12a [54,55]. *Mismatches in the seed region have been reported to be more deleterious for binding and cleavage by Cas12a than by SpCas9 [24,27,46,56–58].
Knock-in accuracy in mammalian cells	+	++	Cas12a mediates efficient and precise endogenous gene tagging via MITI: microhomology-dependent targeted integrations (Pan Li 2020) [79]
Key application	mammalian gene editing	non-mammalian gene editing and diagnostics	CRISPR/Cas9 system with specific CRISPR-guided nucleases has evolved as prime DNA editing tool in number of gene editing studies in a variety of organisms, including mammalians and primates [24,59–63] The CRISPR-Cas9 system has been used in cancer therapeutic studies in vivo and ex-vivo [64]

The Future of Cas-9 and Cas-12: Beyond Genome Editing

Since the early 2000s, researchers have been trying to engineer new variants to improve the accuracy of CRISPR editing. Cas9-HF1 (or “HiFi”), for example, was designed by J. Keith Joung and team to reduce non-specific contacts [18], making this Cas9 variant particularly useful for research and commercial applications. In parallel, a smaller version of Cas9 was discovered in Staphylococcus aureus, allowing it to be packaged into a single adeno-associated virus (AAV) vector [19]. Its small size makes it a potentially viable candidate for therapeutic in-vivo gene transfer. As the first CRISPR effector nuclease, Cas9 has a much longer research history, publication record and greater investment than Cas12.

Interestingly, Cas9 and Cas12 recognize different PAM sequences. The GG sequence required for Cas9 is more abundant in mammalian cells than the canonical TT required by Cas12. For these reasons, many researchers find Cas9 more flexible for use in mammalian systems. In addition, due to its higher specificity and efficiency, CRISPR/Cas9 has been widely applied to many genetic ailments like X-linked diseases, and non-genetic disease such as acquired immunodeficiency syndrome (AIDS), cardiovascular diseases, and neurodegenerative diseases [20]. Furthermore, researchers

are studying the use of the CRISPR/Cas9 technique to cure or alleviate cancers through gene therapy and immunotherapy (ref).

On the other hand, Cas12's ability to cut single-stranded DNA in a non-specific manner can be leveraged to detect small amounts of DNA from sources such as viruses and cancer cells, making Cas12 a powerful tool for DNA diagnostics. Today, CRISPR tests like those being developed by Dr. Doudna and her team at Berkeley use the various properties of Cas proteins to target illnesses. For example, their diagnostic for HPV uses Cas12a. The test distinguishes between two types of HPV that studies have linked to cervical or anal cancer [21].

Conclusion

CRISPR-CasX is a simple system that enables the precise editing of any sequence in the genome of an organism. Today, CRISPR-Cas9 and CRISPR-Cas12 have generated tens of thousands of scientific publications and contribute to the development of novel products and medical advancement ranging from genetically modified crops and livestock to viral therapies. The CRISPR diagnostic technology leverages the different trans-cleavage activities of Cas9, Cas12a (and Cas13) and shows great potential in diagnostic sensitivity, specificity, convenience, and portability [22]. However, while Cas12 might hold potential for diagnostics applications, it is at a much earlier stage of development than Cas9 and may lack some of the accuracy needed for precision genome editing. Therefore, Cas9 remains the more appropriate option for commercial and research applications where accuracy and reliability are required, particularly in mammalian applications.

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