**Background information on the award of the
2023 Paul Ehrlich and Ludwig Darmstaedter Prize
to Professor Frederick W. Alt and Professor David G. Schatz**

Protected by a tamed parasite

How is it possible that we vertebrates can defend ourselves against all kinds of bacteria and viruses? How does our immune system succeed – with limited resources – in creating enough antibodies to defy an armada of attackers that form chemical structures of almost endless diversity? How can we, with our 20,000 genes, produce sufficient proteins to defend against billions of different attackers? This great mystery of immunology was solved almost 50 years ago – at least in principle: by randomly cutting out different ones again and again from a few hundred gene segments and assembling them together each time in a new combination to form functional genes that code for variable parts of antibodies. This variable part at the two upper ends of a Y-shaped antibody determines which antigen it can claw. However, this did not yet answer the question of how this "somatic recombination" works or how the individual gene segments are recognized and pieced together again. That we are today largely able to answer it is essentially thanks to the achievements of the two prize winners.

When Frederick Alt began his work as a postdoctoral researcher in David Baltimore's laboratory at the Massachusetts Institute of Technology (MIT) in 1977, the first experimental proof that antibodies assemble genes for their variable regions by recombining certain DNA sequences had only just been presented. Susumu Tonegawa had achieved this at the Basel Institute for Immunology and published it in 1976. With the help of genetic engineering, invented in 1973, Tonegawa and others were able to further develop this initial finding over the course of the following five years into the following sketch: maturing B cells assemble the variable regions of antibodies by randomly combining the gene segments V (for variable), D (for diversity) and J (for joining). These few hundred segments are scattered on chromosome 14 for the heavy antibody chain and on chromosomes 2 and 22 for the two light ones. Each of these segments borders onto a recombination signal sequence (RSS). Such an RSS consists of two pieces of DNA seven and nine letters long, in the middle of which sits a placeholder either 12 or 23 DNA letters long. The difference between 12 and 23 letters or bases corresponds to about one twist of the double helix. Next to the RSSs, the segments to be newly combined are spliced from the respective V, D and J libraries. According to the 12/23 rule, two gene segments can only then be joined together if their neighboring RSS have placeholders of different lengths. In contrast to the heavy antibody chain, the light

ones join only V and J segments together, which is why we speak of V(D)J recombination.

Center of immunological research

The heavy antibody chain is recombined before the light ones. In the process, DJ units are always created first, which are then joined together with a V segment. These findings were published in research papers in the first half of the 1980s, in which Tonegawa was by then only partially involved. Their first author was Frederick Alt.[1] Tonegawa had meanwhile moved to MIT but had increasingly turned his attention to neuroscientific questions. In 1987, he was awarded the Nobel Prize in Physiology or Medicine for his discovery of "the genetic principle for generation of antibody diversity".

Frederick Alt and other postdoctoral researchers supervised by David Baltimore had meanwhile expanded his laboratory into a leading center for immunological research. Baltimore had discovered the enzyme reverse transcriptase, which transcribes RNA into DNA, and been awarded a Nobel Prize in Physiology or Medicine for this achievement in 1975 at the age of just 37. Inspired by the perspectives opened up by Tonegawa and by the availability of genetic engineering methods, he decided the following year to focus on immunology in his future work.[2] Like few other researchers of his generation, Baltimore not only knew how to rally brilliant young scientists around him but also to give them free rein to put their own ideas into practice in an atmosphere of open and vigorous exchange. Thus, while still under Baltimore's aegis, Alt discovered that the enzyme TdT can insert short non-coding DNA sequences at the interfaces of the gene segments to be combined, like a random generator. Mixing in these N-nucleotides multiplies the diversity of the antibodies produced in the V(D)J lottery.[3]

One new insight after the other

In his own laboratory at New York's Columbia University – whose first postdoc, incidentally, was Michael Reth, the winner of the 2014 Paul Ehrlich and Ludwig Darmstaedter Prize – Alt elucidated numerous features of V(D)J recombination in the years that followed. He showed that transcription processes are necessary in order to make V segments accessible for genomic rearrangement in the first place. He described the special way in which the ends of the spliced gene segments are joined together in the course of V(D)J recombination. And he proved that the genes for variable parts of antibodies and T cell receptors, which had only been discovered in 1983, are assembled by the same recombinase[4]. That was in 1986, three years before David Schatz discovered this recombinase, which he succeeded in doing in his doctoral thesis in David Baltimore's laboratory[5].

Discovery of key genes

First, Schatz isolated the recombination-activating gene *RAG-1* – an achievement which was so significant that the *New York Times* devoted an extensive article to it, on 22 December 1989, entitled *Key Gene in Immune Defenses Is Believed Found*. Soon, however, it transpired that the activity of this gene alone was not sufficient to conduct recombination processes in maturing B and T cells. At a distance of a few kilobases, which is tiny on a genomic scale, Schatz and colleagues then discovered the gene *RAG-2*. Only

the joint product of both genes, the heterodimeric enzyme RAG1/2, is the motor for V(D)J recombination. Without it, no functional B and T cells, no effective adaptive immune defense can develop. Many cases of severe immunodeficiency are caused by mutations of the *RAG* genes, and some lymphomas and types of leukemia are associated with malfunctions of these genes.
RAG1/2 is primarily an endonuclease that cuts the double DNA strand solely in maturing B and T lymphocytes at those sites pre-marked by RSSs. The enzyme binds one RSS and bends the double strand towards itself so that it can capture a second RSS lying somewhere on the strand that satisfies the 12/23 rule. It then makes a double-strand break between each RSS and the gene segment flanking it. The result is two asymmetrical pieces at each of the interfaces: on the gene segment sides, the ends of the single strands are fused together like hairpins, and on the signal sequence sides, only phosphorylated stumps remain. Any double-strand break in the genome is potentially very dangerous. RAG1/2 therefore secondarily enlists the help of an internuclear repair workshop straight away, whose enzymes are specialized in repairing double-strand breaks. The specific non-homologous end-joining (NHEJ) pathway in which these repair enzymes work together to connect the ends generated by RAG 1/2 endonuclease was discovered by Frederick Alt[6]. The repair enzymes open the two hairpins and join the two gene segments together. Because the opening occurs two base letters next to the tip of the pin, the joint is imprecise, which is advantageous because it increases the diversity of the resulting antibody genes. In addition, the N-nucleotides already mentioned above are inserted in the course of the further repair process. The signal ends, on the other hand, are mostly joined precisely together via their sides facing away from the interface and subsequently lost to the genome in many cases.[7]

Loop through the center of recombination

The RAG enzymes are not stationary molecules. They do not, however, wander aimlessly through the cell nucleus of immature lymphocytes either, but instead bind themselves temporarily again and again to dynamic structures of the chromatin filaments. Chromatin is the structure in which our DNA, which is two meters long, is coiled up together with spherical proteins, like on a string of pearls and in such a space-saving way that it fits into the nucleus of every cell. Through the binding of RAG1/2 to chromatin, V(D)J recombination centers are created in which chromatin scanning takes place. In the process, a chromatin loop, which can be more than one million base pairs in length, passes through the recombination center. The two needle eyes of this loop are rings of cohesin, and the nodes that stop it from threading through are CTCF proteins. If J segments are already situated on the one side of the loop that passes through the active centre of the enzyme, only its other side, on which the D segments are located, continues to thread through – until both CTCF nodes abut the cohesin rings from the outside. The D and J segments, which are now juxtaposed in the enzyme, are best suited for joining in this round of scanning because loop extrusion comes to a halt and there is enough time to cut and join the segments safely. This loop extrusion mechanism of V(D)J recombination, discovered by Frederick Alt in recent years, also applies for subsequent joining with a V segment.[8]

The second multiplication step

Admittedly, V(D)J recombination is not enough to produce sufficient antibodies to defend against antigens. A second diversification stage is therefore sparked solely in maturing B cells, which is known as somatic hypermutation. It is characterized by the fact that the normal rate of point mutations, which affect only one base letter, is increased by a factor of one million at the point where V segments for light and heavy chains occur on chromosomes 2, 14 and 22. In a small fraction of cases, this arrangement leads to the formation of antibodies with a higher affinity for antigens. More frequently, antibodies with the same or lower affinity can result. The mutator that triggers these million-fold changes is the enzyme AID discovered by Tasuku Honjo at the turn of the millennium, a deaminase that converts the DNA building block cytosine into the DNA building block uracil. Frederick Alt helped to shed light on the paths this mutator takes to reach its molecular targets in the V-segment regions. He did similar explanatory work on the involvement of AID in the class switching of antibodies, which are synonymously referred to as immunoglobulins (Igs). Initially, B cells only produce immunoglobins of the classes M and D, which during B cell maturation must partially switch to the classes A, E or G. In class switching reactions, as Alt could prove, both the NHEJ pathway and the loop extrusion mechanism [9]play an important role, too.

Integration of an intruder

The most astounding feature of RAG1/2 is its origin. It most likely descends from a DNA parasite which, like a kind of scrounger, implanted itself in the genome of one of our very early ancestors millions of years ago, but was tamed in the further course of evolution in such an effective way that it was able to become the most important control room of the adaptive immune system. David Schatz tracked down this genealogy when he discovered in test tube experiments that RAG1/2 could excise a fragment of DNA and then reinsert it at another location in the genome. From this it was clear that the enzyme exhibits transposase activity *in vitro*. Transposases are enzymes with whose help transposons jump from one place in the genome to another. Transposons are mobile DNA segments. They make up almost half our genome. Mostly they are harmless, selfish DNA parasites (selfish genes), but they are sometimes also elements that cause diseases. RAG1/2 must therefore under no circumstances appear as an active transposon *in vivo*, otherwise it would run wild through our genome. To prevent this, evolution has indeed taken care to defuse the enzyme's potentially explosive power with the help of a double intervention: at position 848 of RAG1, it has replaced the hydrophobic amino acid methionine with a hydrophilic counterpart arginine and inserted a negatively charged coil into RAG2. Under *in vivo* conditions and according to David Schatz's findings, this fixes the former transposon in the genome and prevents its further transposition.[10]

Revolution in evolution

Although its transposase activity in the test tube made it plausible that RAG1/2 was originally a transposon, no evidence for this hypothesis could be produced. This changed when Schatz and his group analyzed a molecule with properties similar to RAG in the invertebrate lancelet *Amphioxus*, evidently a prototype of the transposon which at some point in time had jumped into the genome of vertebrates. Such RAG-like transposons have meanwhile been found many times in invertebrates, among others in the genome of the cotton bollworm *Helicoverpa zea*. Schatz has recently illustrated, step by step and in almost atomic resolution, how its transposase carries out transposition. Like a

butterfly, the enzyme opens and closes its "wings" in two successive beats, as if wanting to symbolize the beauty of natural development.[11] With these images, Schatz is giving science a fascinating look back at a revolution happening in the course of our evolution: the start of an adaptive immune system developing in jawed vertebrates in addition to the innate immunity that already existed in more primitive organisms. Building on this view from basic research, translational research will be able to open up new therapeutic perspectives for diseases in which our immune system plays a crucial role.

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[1] Cf. Alt, F. *et al.* Organization and reorganization of immunoglobulin genes in A-MuLV-transformed cells: rearrangement of heavy but not light chain genes. *Cell* 27, 381–390 (1981); Alt, F. W., G. D. Yancopoulos, T. K. Blackwell, C. Wood, E. Thomas, M. Boss, R. Coffman, N. Rosenberg, S. Tonegawa, and D. Baltimore. 1984. Ordered rearrangement of immunoglobulin heavy chain variable region segments. EMBO J. 3: 1209–1219.

[2] "Luckily a few of my postdocs, notably Fred Alt, Enzo Enea, and Al Bothwell, were as intrigued as I was by the puzzles of immunity, and they got the lab into experimental immunology (…) I note above that my postdocs got the laboratory into immunology. I phrased it that way because about this time I stopped doing experiments myself, and all the work of the lab was done by trainees." In: Baltimore, D. Sixty years of discovery. Annu. Rev. Immunol. 2019. 31: 1-17, 7.

[3] Alt, F. W. and Baltimore, D., Joining of immunoglobulin heavy chain gene segments: Implications from a chromosome with evidence of three D-JH fusions. Proc. Natl. Acad. Sci. USA 1982. 79: 4118–412.

[4] Alt, F.W. From gene amplification to V(D)J recombination and back: A personal account of my early years in B cell biology. Eur. J. Immunol. 2007. 37: S138-147.

[5] Schatz, D. G. and Baltimore, D., Stable expression of immunoglobulin gene V(D)J recombinase activity by gene transfer into 3T3 fibroblasts. Cell 1988. 53: 107–115; Schatz, D. G., Oettinger, M. A. and Baltimore, D., The V(D)J recombination activating gene, RAG-1. Cell 1989. 59: 1035–1048.

[6] Taccioli, G.E., Rathbun, G., Oltz, E., Stamato, T., Jeggo, P.A. and Alt, F.W. (1993) Impairment of V(D)J recombination in double-strand break repair mutants. Science, 260(5105), 207-210.

[7] Schatz, D.G. and Swanson Patrick C. V(D)J Recombination: Mechanisms of Initiation. Annu. Rev. Genet. 2011. 45:167-202.

[8] Zhang, Y. et al. The role of chromatin loop extrusion in antibody diversification. Nat. Rev. Immunology 2022

[9] ibid.

[10] Zhang, Y., Cheng, T.C., Huang, G., Lu, Q., Surleac, M.D., Mandell, J.D., Pontarotti, P., Petrescu, A.J., Xu, A., Xiong, Y., and Schatz, D.G. (2019). Transposon molecular domestication and the evolution of the RAG recombinase. Nature 569, 79-84. PMID 30971819

[11] Liu, C., Yang, Y., and Schatz, D.G. (2019). Structures of a RAG-like transposase during cut-and-paste transposition. Nature 575, 540-544.