

Mouse with gene mutation (left) and healthy mouse. Bone is shown in red, cartilage in blue. The mutated animal has significantly shortened front and back legs and is much smaller than the healthy one.



# Genes

## That Come Close to the Bone

Nearly a quarter of all known illnesses are extremely rare and affect just a few thousand patients worldwide. **Stefan Mundlos**, a research group leader at the **Max Planck Institute for Molecular Genetics**, and his team specialize in the study of rare bone diseases. They are looking for the genes that trigger these disorders.



Photos: MPI for Molecular Genetics

TEXT **KLAUS WILHELM**

**T**he much vaunted “decoding” of the human genome is something Stefan Mundlos dismisses as a rumor. “Sequenced – yes,” he says. “But decoded? We are nowhere near understanding the jumble of text.” The “jumble of text” he refers to is the sequence of three billion chemical building blocks in DNA, the hereditary molecule and human genetic substance. In it are hidden approximately 30,000 genes that contain the instruction manual for our body’s proteins and that control every second of its growth and metabolic processes, usually via biochemical signaling pathways.

The doctor from the Max Planck Institute for Molecular Genetics and Head of the Institute for Medical Genetics and Human Genetics at the Charité Universitätsmedizin hospital in Berlin goes on to speak about the “enormous opportunity” finally being used by genetic researchers to understand what actually goes on in our genome. This could enable us to detect the existence of diseases that, unlike cancer and diabetes, are actually determined by just a single genetic defect.

#### **RARE INDIVIDUALLY BUT COMMON AS A GROUP**

Stefan Mundlos and his colleagues from the “Development and Disease” research group focus their scientific ambition on diseases that are completely unknown to the majority of people. They are extremely rare and yet, together, they affect an estimated four to six million people in Germany alone, often from birth.

According to the European Union definition, a disease is rare if it affects fewer than one in 2,000 people. The World Health Organization (WHO) assumes that, of the 30,000 known diseases, between 6,000 and 8,000 fall into

## DIAGNOSIS BY COMPUTER NETWORK

When children become ill due to a rare genetic defect – either shortly after birth or in the early years of life – it is difficult for their doctors to associate the often diffuse symptoms with a defined disease. No doctor can note the clinical symptoms of the thousands of diseases that affect ten or a hundred people throughout the world at a given time. Moreover, the symptoms associated with many rare diseases overlap and this also renders them difficult to diagnose.

Peter Robinson, a doctor and bioinformatician from Stefan Mundlos's research group, aims to resolve this problem by adopting a systematic approach. He feeds the ever-expanding information about rare diseases into a computer program. The program links different symptoms and then assigns them to similar cases that have been encountered

by other doctors in clinical practice. It can generate cross-references to all possible main and secondary symptoms. The program also stores the data about the underlying genetic mutations and molecular mechanisms.

"If such a system is available to researchers and doctors throughout the world and they can also enter data about their patients into it, a lot of time and effort can be saved in the diagnosis of rare diseases," says Stefan Mundlos. The program's "intelligence" increases with the volume of data and this, in turn, will enable doctors to make more precise diagnoses. This establishes a solid foundation for the diagnosis of rare diseases – one of the biggest problems facing doctors to date – that reflects the digital possibilities of the 21st century.

this category. According to research carried out by the Network for Rare Diseases, five new ones are described every week in the specialist medical literature.

Rare diseases take very different forms, but are usually caused by mutations in certain genes and can thus be passed on from one generation to the next. They have names like brachydactyly type A, cutis laxa type II, and craniosynostosis, Philadelphia type. Many of these diseases are chronic and can involve severe impairments that patients survive for only a few years.

Other rare diseases have little impact on life expectancy, but cause obvious physical deformities. Persons af-

ected by these conditions can expect little help from the pharmaceutical industry – the patient groups and corresponding market are simply too small. What is worse, however, is that the parents of affected children often consult numerous doctors in the hope of obtaining a diagnosis, and generally receive little more than a puzzled shrug of the shoulders in response.

## HELP FOR DESPERATE PARENTS

"Knowing the cause provides a degree of relief for the parents," says Stefan Mundlos. The doctor and his colleagues confirm this experience on an

almost daily basis at the clinic of the Institute for Medical Genetics and Human Genetics in Berlin's Charité hospital. They are also confronted with the gnawing question as to whether another child – or grandchildren – could also be affected and, if so, with what degree of likelihood.

"A correct diagnosis is crucial," explains the pediatrician. "Without a diagnosis there can be no prognosis or therapy development." The problem, however, is that, due to the recent nature of the research, diagnosis is not possible for many rare diseases because the corresponding genetic tests have not yet been developed.



- 1 Shortness of fingers through duplication of a regulatory region in the DNA: the intermediate phalanges of the index and little finger are missing or too short.
- 2 Mouse embryo with the same duplicated regulatory region in its DNA. The region controls genes in the emerging legs and fingers (blue). The duplication alters the activity of the BMP2 gene and gives rise to brachydactyly as a result.



Ideally, Mundlos's scientific projects feed directly into everyday clinical practice – when his team has managed to nail down the cause of a previously unexplained rare condition and it can then be demonstrated using a new genetic test. Every day, the doctors encounter rare diseases that share some features and symptoms but differ in others.

Blood samples and files from patients all over the world arrive at the institute every day. The scientists have now assembled a collection of diseases that they are systematically recording in a database. This enables them to identify certain “phenotypes,” as the geneticists call them, from all of the described disease patterns.

## DISEASES REVEAL THE FUNCTION OF GENES

To what extent can specific defects, as arise for instance in diseases of the skeleton, be predicted on the basis of genetic information? And, above all, how is the information from a single mutated gene used to provide a specific clinical picture?

“These questions can be extremely well researched in the case of rare genetic diseases,” says Stefan Mundlos. “This means we can really understand how the text of a gene determines a function and how the function, in turn, determines a disease.” In other words: a gene can literally be decoded. “The task for the future is to seek answers for all possible rare diseases using functional genomics.”

This process is driven by the technical progress achieved in molecular medicine over the past two decades – particularly in the area of DNA sequencing and another method known as array CGH analysis. This “gene chip diagnosis” enables the discovery of very small chromosome mutations that cannot be detected using conventional chromosome analysis.

Defined DNA fragments (arrays), which cover the entire human genome as evenly as possible, are placed on a special device. The matching sections of the DNA to be tested bind to these fragments. The analysis requires both



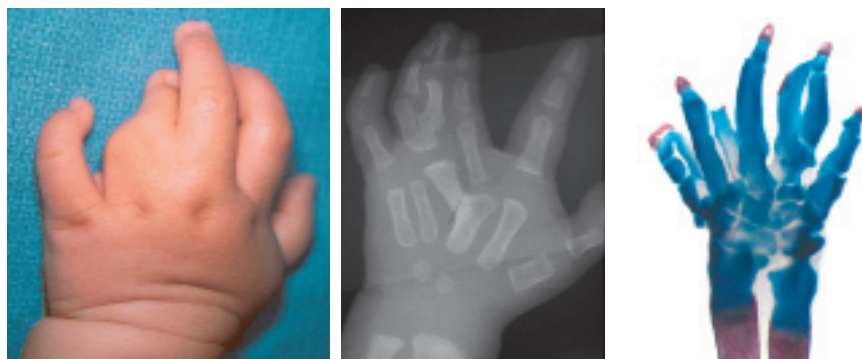
The human skeleton consists of more than 200 bones. Developmental defects are often visible as deformities. Stefan Mundlos looks for the genes behind such diseases of the skeleton, and analyzes their functions. This knowledge enables the development of new treatment options.

the patient DNA in which the genetic origin of the disease is being sought, and reference DNA. Both DNA samples bind to the array fragments, and are marked with different fluorescent dyes.

The fluorescent signals are then identified with the aid of a scanner. Depending on how strong a given fluorescent signal is, it is possible to establish whether both DNA samples have bound equally or whether one has bound more and the other less.

This enables the scientists to detect various minute DNA mutations. The individual signals are then assigned to certain gene regions using a computer program.

New technological developments also enable the fast and relatively cheap sequence analysis of complete genomes. The sequencing of an entire human genome used to take years; now a computer can, in just a few days, perform this task so accurately that



A mutation of the HOXD13 genes causes a complicated form of brachydactyly in which the fingers are fused together. The gene mutation causes a similar deformation in mice (right).

“high throughput sequencing” will soon become the standard method used in routine human genetic diagnostics. The quality, speed and now lower costs of these new technologies have long since revolutionized research into biological issues.

Equipped in this way, from the thousands of rare diseases, the Max Planck Researchers selected diseases of the skeleton as their research focus. There are 400 such diseases alone, along with a few hundred deformities of the extremities. Of these deformities of the hands and feet, at most a third have been characterized, ensuring that the researchers will have enough work to keep them busy for decades.

Stefan Mundlos’s team has been investigating the many forms of brachydactyly – literally “shortness of fingers and toes” – at the molecular level for more than ten years. This disease involves varying degrees of shortening of the fingers and, sometimes, metacarpals. Different forms of the disease with very similar effects arise in affected families. The average frequency of brachydactyly in the population is 1:200,000. The shortness in the digits is often symmetrical, and sometimes entire finger joints are missing. The likelihood of passing this disorder from parent to child is 50 percent.

In the embryo, the arms and hands arise from the extremity buds – a small mass consisting of two layers of tissue with stem cells that can develop into different types of cells. While these cells multiply and generate the form of

the extremity, the individual elements of the skeleton also differentiate – into the upper arm, lower arm and hand, and, finally, the fingers.

The skeleton parts are initially established as a basic cartilaginous frame in which the joints gradually form. However, the segmentation of the cartilaginous mass into the individual finger bones doesn’t function correctly in children with brachydactyly. “Different signaling pathways join forces to regulate the normal process,” says Stefan Mundlos. And various genes with the corresponding proteins play a role in these signaling pathways.

## SIGNALING PATHWAYS FOR BONE FORMATION

By carrying out tests on different forms of brachydactyly, the researchers discovered different mutations in these genes that give rise to malfunctioning proteins. The signaling pathway named after the bone morphogenetic proteins (BMPs) appears to play a particularly prominent role here. The signals from the BMPs cause the formation of cartilage structure. They also control segmentation with joint formation.

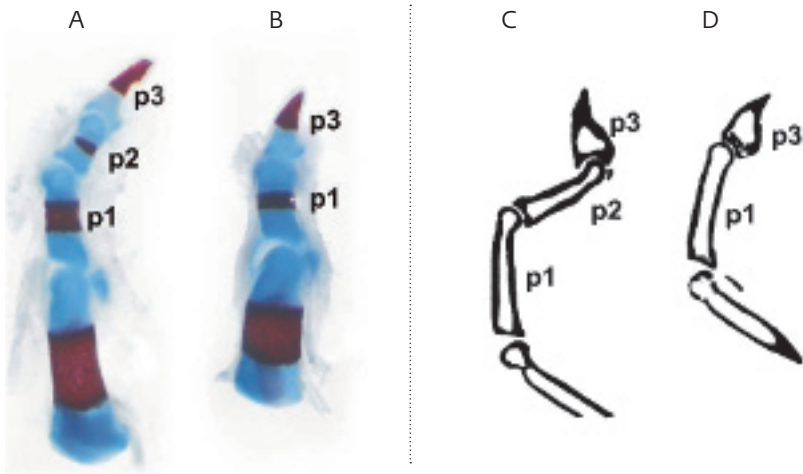
“This entire process requires extremely fine tuning,” says Max Planck scientist Mundlos. This involves various receptor molecules to which the BMPs bind and, in this way, transmit their effect, along with different inhibitors that block their activity. The mutations the researchers discovered in

these molecules also cause the same clinical manifestations in mice that they observed in humans.

In addition, there are molecules, such as ROR2, that interact with the BMP signaling pathways. A mutation in the ROR2 gene generates an “impressive phenotype” as Mundlos says. It looks like a truncated hand, as the top bones in the fingers are missing. ROR2 and the growth factor Wnt regulate the activity of a small mass of cells right at the top of the fingers that produces the cells for the development of the fingers. In the absence of an intact ROR2 gene, this small cell mass is not formed.

One thing is certain from the scientists’ discoveries: the genetic root of brachydactyly is heterogeneous. This means that different mutations can cause the same physical changes. Up to now, it appeared that traditional mutations in the genes that code for proteins were solely responsible for the impaired pattern formations in the fingers. However, the mutation of a particular regulatory DNA sequence can also trigger brachydactyly.

This duplication is located in a sequence of the genome that is highly conserved from an evolutionary point of view and, moreover, is almost identical in different species – including chickens and mice. Further, it is located in a section of the DNA that doesn’t code the instruction manual for a protein. This area, which was largely unknown up to now, contains regulators that must switch genes and proteins on



Phalanges (p1–p3) in a healthy (A) and mutant (B) mouse. The mutant mouse lacks the intermediate phalanges (p2). The bones in the skeleton preparation are shown in red and the cartilage in blue. A microcomputer tomography shows the longitudinal section of the bones of a healthy (C) and mutated (D) mouse.

and off at exactly the right time during the development of the embryo for complex structures such as the hands or the skull to form. “This is a veritable treasure trove for future research,” predicts Stefan Mundlos.

#### DIAGNOSIS: FAULTY GENE REGULATION

Within the duplication, there is an enhancer element that regulates the activation of a BMP gene. “Consequently, we have proven for the very first time that changes in the non-coded DNA areas can also cause diseases,” says Mundlos. “The duplication of the regulatory element that influences the detailed control of genes during em-

bryonic development disturbs the balance in the BMP signaling pathway considerably during the initial development of the fingers.”

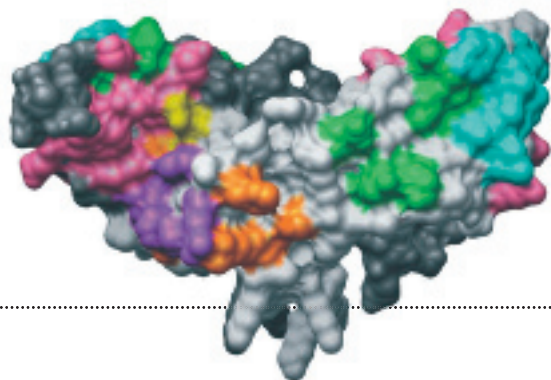
Once discovered, the researchers are now also finding the new type of mutation in other rare diseases, such as craniosynostosis, Philadelphia type, which also involves the duplication of this kind of enhancer element. With this disease, individual fingers and a child’s cranial sutures, in particular, fuse far too soon after birth and cause corresponding deformities. Mutations in the Hox genes, in contrast, cause phenotypes with, for example, more than five fingers. The Berlin-based researchers already discovered this many years ago.

Now they also understand, in molecular terms, how the genetic misinformation causes the additional fingers to grow. At the start of its development, the hand consists of a uniform plate. Out of this grow the individual elements – the fingers, which must be separated from each other. The cells in the spaces between the fingers receive a signal to not chondrify – that is, develop cartilage.

This “anti-cartilage signal,” as Stefan Mundlos calls it, is transmitted by one of the Hox genes. It, in turn, controls the activity of an enzyme that produces retinoic acid. Retinoic acid is a substance that is found at all possible stages of embryonic development. Mice with Hox mutations produce less

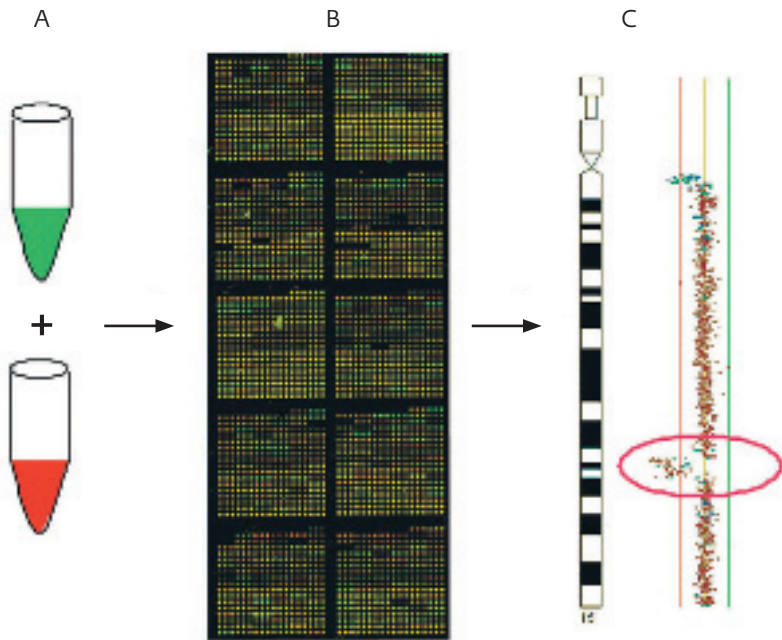
#### PROTEINS FOR CARTILAGE

In the course of their work on brachydactyly, the researchers from the Max Planck Institute for Genetics in Berlin produced proteins using methods from genetic engineering that trigger the production of cartilaginous mass. The artificial pro-



teins for cartilage are modified by mutations as compared with the natural bone morphogenetic proteins (BMP). Cell culture tests showed that two of the modified proteins (light gray, dark gray, mutation: yellow) sometimes trigger the formation of cartilage far more intensively than the natural proteins. One of the two proteins binds more strongly to certain areas (orange, red, green) than other proteins and is not adequately blocked by the body’s own inhibitor (pink) to hinder the effect of the BMPs. In both cases, however, the BMP signaling pathway is excessively active. “This could also be used for treatment,” says Stefan Mundlos. For example, cartilage is needed for the production of bone tissue in patients with complicated bone fractures. The new BMPs are currently being tested in animal experiments.





The principle of chip-based chromosome analysis: Dye-stained genetic samples of patient and control DNA are mixed (A) and applied to a gene chip. Patient and control DNA bind differently to short DNA segments and stain them accordingly (B). The color of the individual dots on the chip indicates changes in the chromosomes. The color of a dot here has shifted in the direction of red (circle), which means that the green sample binds more weakly and does not have the corresponding chromosome section.

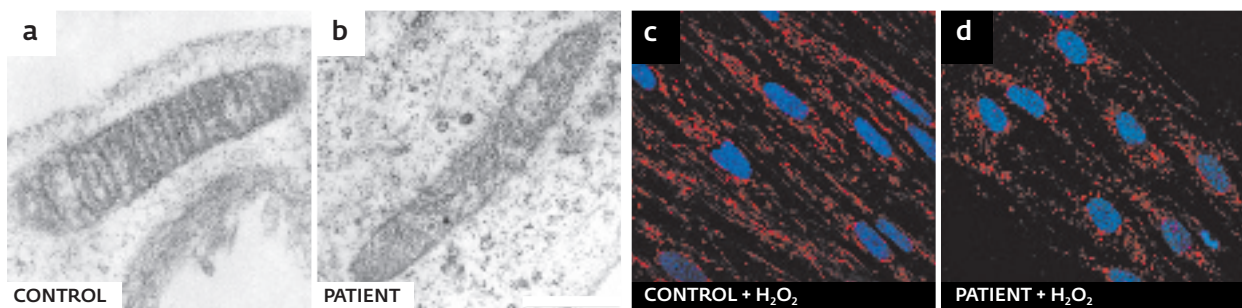
retinoic acid and thus form, in the intermediate spaces, cartilage that develops into additional finger digits.

If the mutated mice are given retinoic acid during embryonic development, the polydactyly disappears again. "However, retinoic acid is not a suitable drug for humans," stresses Mundlos. It would cause serious problems for other aspects of embryonic development. Moreover, in mice with certain Hox mutations, the long tubular bones are transformed into rounded ones. The Berlin team proved the existence of such homeotic transformations in the extremities of vertebrates for the first time. This shows that Hox genes influence the shape of bones.

## ANTI-POWER-PLANT RADICALS

Wrinkly skin and the loss of bone substance are typical features of aging. People with the rare genetic disease cutis laxa display such symptoms from childhood, however, and also suffer from intellectual disabilities. Five different forms of this disease are known. Together with an international research group, Stefan Mundlos's team has now discovered the genetic defects at the root of cutis laxa. It appears that the gene PYCR1 is mutated. The protein product of this gene is involved in the metabolism of the amino acid prolin in the cell's powerhouses, the mitochondria.

Due to the mutation, the patients' cells are more sensitive to free radicals. These are harmful oxygen molecules that are produced by certain metabolic processes. Free radicals have long been suspected of triggering aging processes through programmed cell death (apoptosis). When the mitochondria are stimulated by the radicals, their membranes open and the fate of the entire cell is sealed. PYCR1 probably protects people against the consequences of stress from free radicals.



The mitochondria are the powerhouses of the cell. They have an elongated shape and invaginations of the inner membrane. The differences between healthy mitochondria (a,c) and those of cutis laxa patients (b) are visible under the electron microscope. The mitochondria of cutis laxa patients are sensitive to free radicals (d): treatment with the radical builder  $H_2O_2$  destroys their network of thread-like proteins (red, nucleus blue).

Although it was already known that the Hox genes play a key role in the development of the extremity bud, the new tests have revealed that they also control delicate differentiation processes in subsequent phases of embryonic development – for example, the formation of connective tissue, cartilage and bones – around the eleventh or twelfth day of gestation in mice and the sixth to eighth week of pregnancy in humans.

### GENE ANALYSIS REVEALS NEW DISEASE

The Berlin researchers seldom apply their expertise in functional genomics to diseases outside of the skeleton. Some time ago, however, a patient with Mabry syndrome – a disease characterized by delayed intellectual development and raised blood levels of the enzyme alkaline phosphatase (ALP) – came to them for a consultation. High ALP values are usually indicative of bone diseases, but the patient in question did not have any such disorders and nobody could understand the origin of his high ALP value.

“We discovered a new gene defect in him and consequently defined a new disease,” says Stefan Mundlos. From a molecular perspective, the enzyme hangs on the outside of liver and bone cells from a kind of anchor. According to the findings of the Berlin-based scientists, a mutation renders

the anchor unusable. As a result, the enzyme can no longer adhere to the cells and large volumes of it swim around in the blood.

Exactly how the absence of the anchor ultimately causes the delay in brain development is still unknown. However, if a child with raised ALP levels and no indication of a bone disease presents somewhere in the world, now it will soon be able to undergo a genetic test for Mabry syndrome. This is how basic research is immediately incorporated into everyday clinical practice at the Charité’s Institute for Clinical Genetics.

At present, however, for most rare diseases, there is no treatment available that targets their molecular causes. “The research on these causes is a crucial prerequisite for the development of a treatment,” says Stefan Mundlos, and refers to Marfan syndrome – a disease from which the famous US President Abraham Lincoln suffered and that involves life-threatening complications of the aorta – as a positive example of such a disease.

Now, some 20 years after the discovery of the genetic defect that causes this disorder, a drug that is used in the treatment of high blood pressure and that addresses the cause of this condition is being tested. “It appears to work,” says the Max Planck researcher, and reports that one of his colleagues is now developing a specific therapeutic concept for the treatment of Marfan syndrome. ◀

### GLOSSARY

#### Enhancer element

An enhancer is a base sequence in the DNA that plays an important role in transcription from DNA to RNA. It strengthens the transcription activity of a gene by influencing the accumulation of RNA polymerase (enzyme complex) at a particular start sequence, the promoter. The enhancer and promoter sequence may be located several bases apart. If the enhancer moves closer to the promoter through the removal of sections of the DNA, the gene transcription continues to increase. In the case of tumor cells, for example, an oncogene can reproduce very quickly in this way.

#### Hox genes

Hox genes are regulatory genes that control the processes involved in the early development of organisms. Their most important task consists in segmenting the embryo along the body’s longitudinal axis. In humans, for example, these genes control, among other things, the shape and formation of the vertebrae and ribs. They also regulate the generation and degeneration of blood vessels during embryonic development, and control the formation of vessels during pathological processes, for example during the emergence of tumors.

#### Marfan syndrome

Marfan syndrome (MFS) or the Marfan phenotype is a disease of the connective tissue caused by a genetic mutation. The mutation arises in the gene for fibrillin – one of the most important components of the microfibrils, which play a key role in the development of connective tissue. Marfan patients present with a wide variety of symptoms; often, their cardiac, vascular and skeletal systems, eyes and internal organs are affected. Marfan syndrome affects an average of one to two people in 10,000. This connective tissue disorder is still incurable.

#### Duplication

Duplication refers to the doubling of a certain section within a chromosome. This happens, for example, through the unequal exchange of gene sections between sister chromatids. The chromosome becomes longer as a result. This kind of gene mutation usually can’t be repaired by the body’s own repair mechanisms, and often causes congenital defects.

### MAX PLANCK FOUNDATION

Through the Max Planck Foundation, a patron provided 250,000 euros in funding for a research project led by Stefan Mundlos on rare diseases in children. The non-profit Max Planck foundation was established in June 2006. It is funded by private patrons through a German national initiative. The Max Planck Foundation provides funding that enables the rapid and flexible support of cutting-edge research. The funding is used to provide support for special research projects, foster outstanding young scientists and recruit top scientists, thus guaranteeing the competitiveness of the Max Planck Society at the international level.

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