GENETICS

The hexanucleotide hex

For years, researchers missed the most common genetic cause of ALS. Now they're on an accelerated track to treat it.

BY ELIE DOLGIN

Mark Price's family had a long history of neurological disease. His sister and uncle had died from amyotrophic lateral sclerosis (ALS), and his mother and aunt were living with dementia. But it was not until Price himself started to slur his words in 2010, shortly after his daughter Sharon's wedding, that it dawned on him that there might be a genetic basis to his family's tragic medical past.

Within a year, Price was diagnosed with ALS, and Sharon wondered if she — or her future children — would be next. "I stopped everything and said, 'I can't have a kid until we figure this all out,'" recalls Sharon, then aged 26. At first, Price's doctors couldn't pinpoint any defects in the ALS-associated genes that were known at the time. Then came reports in September 2011 that two teams of scientists had found a new gene linked to ALS, one that could explain up to 40% of familial cases of the disease and 10% of what are known as sporadic cases. What's more, this gene accounted for an estimated 30% of hereditary cases of a condition known as frontotemporal dementia (FTD), providing a long-sought genetic rationale for why that neurodegenerative disorder often struck members of families affected by the motor neuron disease ALS — families such as Price's.

The genetic culprit is called *C9ORF72*— from its location on chromosome 9 in a region known as open reading frame (ORF) 72. And it has an unusual nucleotide sequence pattern. In some people with ALS or FTD, a short stretch of DNA in a non-coding portion of *C9ORF72* is repeated hundreds or even thousands of times; in healthy individuals, the same sequence — GGGGCC — is repeated fewer than two dozen times.

In early 2012, Price was tested for the *C9ORF72* repeat expansion. The test came back positive, and he died a year later. And while Sharon and her two sisters grieved for their father, they also had to grapple with the fact that each of them had a 50:50 chance of carrying the genetic defect. Now, they had to decide — would they get tested?

HOUSE HUNTING

The story of *C9ORF72* starts with the German psychiatrist Anton von Braumühl, who in 1932 first made the link between ALS and FTD[1]. But it was not until the mid-2000s, when the genetics of large multi-generational families affected by both disorders were studied, that researchers began to zoom in on the short arm of chromosome 9 as harbouring the gene of interest. By 2010, they had narrowed the search down to a stretch of 232,000 nucleotides[2] — tiny by genomic standards. But none of the four genes in that region contained any protein-altering mutations that could explain the disease connection.

"It's like we knew the street, but we didn't know the exact house," says Ammar Al-Chalabi, a neurologist and clinical geneticist at King's College London.

The race was on to find the gene responsible. At least five research teams from across Europe and North America dedicated themselves to solving the problem. Many thought it would be straightforward. But *C9ORF72* proved to be "very sneaky", says Ekaterina Rogaeva, a molecular geneticist at the University of Toronto in Canada. "This region is not user-friendly."

A group led by Rosa Rademakers, a neurogeneticist at the Mayo Clinic in Jacksonville, Florida, focused on a three-generation family in which ten individuals had ALS, FTD or both. Not knowing what to search for in these patients' genomes, "we looked for anything that might be suspicious", Rademakers says. That included the

Mark Price, here in a family snapshot with his daughters in 1988, was diagnosed with ALS in 2011 and found to carry a faulty *C9ORF72* gene.

GGGGCC-rich section of *C9ORF72*.

She and her colleagues set up polymerase chain reactions (PCRs) to amplify that region and saw an unusual inheritance pattern: for everyone in the family who had a neurodegenerative disease, the PCR test showed them having two identical copies of *C9ORF72* when they should have had different variants.

It was a head-scratcher for Rademakers until it dawned on her that the genetic defect was larger than the upper size limit that the PCR could read. She and her collaborators turned to a more sensitive technique called repeat-primed PCR and observed a large repeat expansion — but only in affected family members. None of their unaffected kin had it. Nor did some 1,000 healthy controls.

The researchers tested another 696 people with ALS or FTD to make sure that this repeat was not unique to the family they had studied. Sure enough, they found the *C9ORF72* mutation in another 59 unrelated individuals, including 22 who had no known family history of neurodegenerative disease. Further experiments showed that the GGGGCC stretch repeated itself at least 700 times.

"Wow," Rademakers remembers thinking. "This is something that's going to have consequences." At the same time, an international consortium led by Bryan Traynor, a neurologist and geneticist at the US National Institute on Aging in Bethesda, Maryland, was making the same discovery. Traynor was clued in to the repeat expansion by the technical shortcomings of a different DNA analysis method — next-generation sequencing. "It was an amazing moment sitting in front of that computer and knowing what was truly going on there," he says.

The two teams published their results back-to-back in September 2011 in *Neuron*[3,4], beating other groups that were still on the hunt for it. "We were scooped," says Al-Chalabi. "But in a sense, we were pleased to be scooped."

EXCITING TIMES

The discovery had an immediate impact. The frequency of the *C9ORF72* defect in patients "made everyone who's seriously interested in ALS feel like they should work on it", says Pamela Shaw, a neurologist at the University of Sheffield, UK.

Brian Dickie, director of research development at the Motor Neurone Disease Association in Northampton, UK, recalls flying from London to a meeting in the United States that September. It was five days after Rademakers' and Traynor's papers were published. Several ALS researchers and clinicians were on board and someone had printed copies of the manuscripts. "They were being passed around the aircraft as we were flying over," Dickie says. "It was clearly an exciting time."

Several drugmakers jumped on the finding. "It was difficult to ignore something like the *C9ORF72* discovery," says Brian Zambrowicz, head of functional genomics at Regeneron Pharmaceuticals, a company in Tarrytown, New York, that was founded to tackle neurodegenerative diseases, but broadened its strategy 20 years ago after its first drug candidate failed to help people with ALS. According to Zambrowicz, the discovery of *C9ORF72* prompted the company to focus again on ALS therapies, starting with the creation of a *C9orf72* mouse model[5].

Ionis Pharmaceuticals, which specializes in antisense RNA-based therapies that can switch off disease-causing genes, also moved rapidly. "We put a plan together the day the papers came out," recalls Frank Bennett, senior vice-president of research at Ionis, based in Carlsbad, California. Within two years, Bennett and his academic collaborators had demonstrated that an antisense drug could reduce aberrant *C9ORF72* mRNA levels in cell cultures. They had proof-of-concept data in mouse models a little more than two years later[6]. A lead drug candidate from Ionis is now undergoing preclinical toxicology studies, and human trials could begin early next year.

That speed, says Lucie Bruijn, chief scientist at the ALS Association in Washington DC, was enabled in part by the influx of investigators driven to deduce the mechanism by which the *C9ORF72* defect causes disease. The repeat expansion recalled those found in other neurodegenerative disorders, including Huntington's disease, myotonic dystrophy and spinocerebellar ataxia. In addition, it overlapped genetically with FTD. Researchers who study these brain diseases had historically worked in isolation. After the *C9ORF72* discovery, they came together with a common purpose.

"We suddenly had a large number of clinicians and scientists interested in ALS," Bruijn says. "That gave the field an enormous boost." The first idea about why the GGGGCC mutations might cause ALS or FTD had less to do with the repeat expansion and more to do with the normal C9ORF72 protein. Rademakers noticed that levels of the normal protein were reduced in people with the gene defect. Although the protein's role is still poorly understood, it is thought to be involved in the transport of molecules within cells. Rademakers' observation led to the suggestion that lower levels of

SARAH KEAYES/THE PHOTO PITCH

***In vitro* fertilization has enabled Price's daughters Sharon Stone (left) and Jodie Price to avoid passing on the faulty gene to their children.**

normal C9ORF72 could be driving pathological brain responses.

Initial studies seemed to refute this hypothesis. Mice with little or no expression of the C9orf72 protein in their neurons displayed no behaviours indicative of a neurodegenerative disease, and nor did their brains have the molecular hallmarks of ALS or FTD. More recently, however, several teams have noticed immune defects in mice that lack C9orf72 in all tissues. Together, these findings indicate that the lower levels of working C9orf72 do not themselves cause neuron degradation, although the altered immune responses could add to the severity or progression of the disease. "It may contribute," says neuroscientist Jeroen Pasterkamp at the University Medical Center Utrecht in the Netherlands, "but in conjunction with other mechanisms."

NO GAIN, NO PAIN

The most obvious alternative mechanism is RNA toxicity. Other diseases caused by non-coding repeat expansions are explained by aggregations of aberrant RNA in the nucleus that bind and sequester housekeeping proteins that are otherwise needed for proper cell function. Pursuing this hypothesis, molecular neuroscientist Adrian Isaacs and his colleagues at University College London created transgenic fruit flies to test whether these aggregates caused disease. They were in for a surprise.

Flies with more than 100 GGGGCC repeats did indeed show signs of C9ORF72-mediated neurodegeneration — but only when the repeat-containing RNA could be translated into a protein, and not when the RNA was interspersed with translation stop signals[7]. RNA aggregates, in other words, were not enough to cause disease. Rogue proteins seemed to be the real drivers. "I was convinced the flies would tell us it was an RNA toxicity," Isaacs says, "but when we saw the data it was clear that that was not the case."

The proteins that emanate from the GGGGCC expansion are created through an unusual process that does not require a start signal and can occur even with repeat sequences located in non-coding gene regions. Laura Ranum, a neurogeneticist at the University of Florida College of Medicine in Gainesville, first described this phenomenon in 2010, in tissues from people with spinocerebellar ataxia and myotonic dystrophy, and in mouse models of these diseases[8].

According to Ranum, the research community initially largely ignored her findings. Many doubted that the mechanism was real. Then came the RNA-binding Proteins in Neurological Disease symposium in November 2011 in Arlington, Virginia, where Rademakers and Traynor discussed *C9ORF72* and Ranum spoke about the unusual form of protein translation. Scientists quickly connected the dots.

Dieter Edbauer recalls sitting in the audience, listening to Ranum's talk, and pulling out his laptop to see what kinds of protein the *C9ORF72* expansion might make. Because the repeat is six nucleotides long — and protein synthesis relies on a triplet code — Edbauer realized that *C9ORF72* might yield a handful of different proteins, each containing two amino acids repeated over and over again. He typed out each of these potential dipeptide repeat proteins. "I looked left and right to see if somebody saw what I did," recalls Edbauer, a molecular neuroscientist at the German Center for Neurodegenerative Diseases in Munich. "I thought that everybody must have had the same idea, but apparently not."

Fifteen months later, in February 2013, Edbauer and colleagues reported that these proteins accumulate throughout the brains of *C9ORF72*-affected people[9]. Within days, Rademakers and her Mayo Clinic colleagues, led by molecular neuroscientist Leonard Petrucelli, published similar findings[10], as did Ranum herself before the year was out[11].

> *"The evidence is pretty overwhelming that it's the protein that's toxic in these simple model systems."*

Since then, evidence has mounted that at least some of these repeating proteins are "uniformly wicked toxic", says Paul Taylor, a molecular geneticist at the St Jude Children's Research Hospital in Memphis, Tennessee. These proteins seem to cause neurodegeneration by snarling up the trafficking of molecular cargo between the nucleus and cytoplasm in brain cells. "The core defect in C9ORF72 is really that nuclear transport," says Jeffrey Rothstein, a neurologist at the Johns Hopkins University School of Medicine in Baltimore, Maryland.

CALL TO ACCOUNT

Some researchers are now willing to pin the blame for *C9ORF72*-mediated disease entirely on these problematic proteins. "I won't mince words here: the toxic poly-dipeptides do not contribute to the disease, they account for the disease," says Steven McKnight, a biochemist at the University of Texas Southwestern Medical Center in Dallas. McKnight describes RNA aggregates and decreased normal C9ORF72 protein levels as "sideshows".

But most researchers are more equivocal. "The evidence is pretty overwhelming that it's the protein that's toxic in these simple model systems," says Aaron Gitler, a molecular neuroscientist at Stanford University School of Medicine in California. However, he adds, "in the context of human disease it could be some combination of factors, and I have to keep an open mind."

The debate over disease mechanism is not purely academic: it guides drug development. Some companies, including Neurimmune of Zurich, Switzerland, and Voyager Therapeutics of Cambridge, Massachusetts, focus just on blocking the repetitive proteins or preventing their formation, whereas others, such as Karyopharm Therapeutics of Newton, Massachusetts, hope to mitigate defects in nuclear transport without targeting any *C9ORF72* gene products directly.

But some therapeutic strategies, such as antisense, do not depend on what the mechanism actually is. Because antisense drugs can shut off the production of both RNA and proteins, it does not matter which one is the causative agent in brain cells, says Paul Bolno, chief executive of Wave Life Sciences in Cambridge, Massachusetts, which is on track to start testing a C9ORF72-targeted antisense therapy in patients next year. And because you can track levels of the repeat proteins in the spinal fluid, it is straightforward to assess whether the drug is working. "You do have a measurable biomarker," Bolno says.

Given how far researchers and drug companies have come in such a short time, it's entirely possible that an effective therapy for *C9ORF72*-mediated disease will be available if more of Mark Price's relatives start to develop symptoms of neurodegeneration. Haley, his youngest daughter, finds that prospect encouraging. "Hats off to the scientific community," she says. But she worries that policymakers aren't doing enough to support preventive health measures available today, to help avoid *C9ORF72*-related disease in the first place.

For family-planning purposes, Haley and her sisters all opted to find out their *C9ORF72* status soon after their father tested positive. "Unfortunately," says Jodie, the oldest, "it was bad news for everybody." Each sister has since gone through multiple rounds of *in vitro* fertilization with the added step of checking that the embryos were free of the *C9ORF72* defect ahead of implantation. It was emotionally, physically and financially taxing on everybody, costing at least Aus$150,000 (US$120,000), they estimate. Ultimately, however, "it was a confirmation that the science worked, and we could get rid of the family curse", says Haley.

Sharon's son Jack recently celebrated his third birthday, Jodie is expecting a daughter in mid-November, and Haley has two frozen embryos, ready to use after her wedding on 9 December. ■

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