UBE3A reinstatement as a disease-modifying therapy for Angelman syndrome

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Half a century ago, Harry Angelman reported three patients with overlapping clinical features, now well known as Angelman syndrome. Angelman syndrome is caused by mutations affecting the maternally inherited *UBE3A* gene, which encodes an E3-ubiquitin ligase that is critical for typical postnatal brain development. Emerging evidence indicates that UBE3A plays a particularly important role in the nucleus. However, the critical substrates that are controlled by UBE3A remain elusive, which hinders the search for effective treatments. Moreover, given the multitude of signalling mechanisms that are derailed, it is unlikely that targeting a single pathway is going to be very effective. Therefore, expectations are very high for approaches that aim to restore UBE3A protein levels. A particular promising strategy is an antisense oligonucleotide approach, which activates the silenced paternal *UBE3A* gene. When successful, such treatments potentially offer a disease-modifying therapy for Angelman syndrome and several other neurodevelopmental disorders.

ANGELMAN SYNDROME: FROM THREE INDIVIDUALS TO A WELL-CHARACTERIZED SYNDROME

In 1965, the English paediatrician Harry Angelman reported three patients with severe neurodevelopmental delay and overlapping features, now well known as Angelman syndrome.[1] The estimated birth incidence of Angelman syndrome is approximately 1 in 20 000[2] and it is characterized by intellectual disability, impaired motor coordination, seizures, characteristic EEG abnormalities, sleep impairments, increased anxiety, lack of speech, and high comorbidity with autism spectrum disorder.[2] The first clinical manifestations usually present during the first year of life, when parents notice the lack of psychomotor activity and seizures.[3–5] Currently, only symptomatic treatments are available for Angelman syndrome, which aim to reduce seizures, improve sleep, or improve behavioural aspects.[6]

Angelman syndrome is caused by the loss of UBE3A protein.[7] The *UBE3A* gene lies in the imprinted Angelman syndrome/Prader–Willi syndrome 15q11.2-q13 locus, such that in neurons only the maternally inherited *UBE3A* gene is expressed. Four different genetic causes can lead to loss of functional UBE3A protein in neurons.[8] The most common cause (approximately 70%) represents a de novo deletion of maternal chromosomal region 15q11.2-q13 encompassing the *UBE3A* gene. The second frequent cause is a de novo or inherited mutation in the maternal *UBE3A* gene itself, leading to a loss of functional UBE3A protein. A less frequent cause is paternal uniparental disomy of chromosome 15, which results in decreased UBE3A protein levels owing to two imprinted chromosomes. Similarly, imprinting defects,

due to mutations affecting the Prader–Willi syndrome Imprinting Center, affect UBE3A protein expression through repression of the maternal *UBE3A* gene (Fig. 1).

Some patients with Angelman syndrome-like phenotypes have been reported who meet (most of) the clinical criteria for having Angelman syndrome but who do not fall into these aforementioned four genetic categories. These patients are typically affected by a mutation in a different gene, and hence represent a different syndrome.[8,9] For instance, patients with a *HERC2* mutation share many clinical features with those who have Angelman syndrome.[10] Interestingly, it has been shown that HERC2 is a positive regulator of UBE3A activity.[11] It needs to be determined whether the genes that are identified in patients with Angelman syndrome-like symptoms can also be directly linked to UBE3A function. Importantly, it should be noted that a deviation of the typical Angelman syndrome phenotype does not rule out a bona fide Angelman syndrome mutation. Recent reports have shown that certain *UBE3A* gene mutations lead to phenotypes that are milder than typical Angelman syndrome, and patients with these do not meet the clinical criteria for Angelman syndrome.[12,13] Given these recent findings, it would be useful within the field to discuss whether we should adopt genetic criteria rather than clinical criteria to define patients as having Angelman syndrome.

MOUSE MODELS PROVIDE INSIGHT INTO UBE3A FUNCTION

The identification of the *UBE3A* gene as the causal gene for Angelman syndrome[7] allowed for the

generation of mouse models of Angelman syndrome to study its function.[14] The *UBE3A* gene encodes the E3 ubiquitin ligase UBE3A (previously identified as E6-associated protein [E6-AP][15]) which is an enzyme that is responsible for linking ubiquitin molecules to their target proteins. This can either result in the degradation of these target proteins (poly-ubiquitination) or change the localization or function of target proteins (mono-ubiquitination).

In humans, the *UBE3A* gene encodes for three isoforms that are generated by alternative splicing, such that they all display unique amino (N) termini.[16,17] The two most abundant human UBE3A isoforms, 1 and 3, make up more than 95% of human UBE3A protein and are conserved in nearly all placental animals.[17] The human UBE3A isoform 2 evolved during early primate evolution and is not found in other mammals.[17] Although the significance and function of the different UBE3A isoforms has yet to be revealed, it is clear that the unique N terminus of the isoforms dictates the localization of UBE3A.[17,18] The predominant expression of the nuclear isoforms in both mice and humans explains the highly enriched UBE3A staining observed in these neurons.[16–20] Notably, the mouse UBE3A isoform 2 (homologous to human UBE3A isoform 3) that is cytosolic in mice (and given the sequence conservation, probably in most other mammals as well) acquired a mutation during late primate evolution. Hence, in humans and Old World monkeys, this isoform is primarily targeted to the nucleus.[17] The finding that most UBE3A protein is nuclear could indicate that the critical targets of UBE3A might be nuclear as well. In support of that, loss of the cytosolic UBE3A isoform does not result in a discernible phenotype in mice. In contrast, loss of the major nuclear isoform results in typical Angelman syndrome phenotypes in mice and humans, although in humans this is milder than in typical cases of Angelman syndrome.[12,18] Although several

What this paper adds
- Loss of UBE3A affects multiple signalling pathways in the brain.
- Emerging evidence suggests that UBE3A plays a critical role in the cell nucleus.
- Trials using antisense oligonucleotides to restore UBE3A levels are continuing.

nuclear targets have been identified[21] and UBE3A function has directly been linked to transcription regulation,[22–25] it is not clear which nuclear targets are really critical for Angelman syndrome pathophysiology. This greatly hinders the search for UBE3A-target-based treatments.

Apart from the nuclear targets that have been identified, several cytosolic targets have been reported. However, similar to the nuclear targets, it is unclear what their relevance is to Angelman syndrome pathophysiology.[14,21,26] It should be further noted that direct ubiquitination by UBE3A has not been demonstrated for most of the presumed UBE3A targets. Instead, increased levels of target protein in *Ube3a* mice are often used as an indication that the breakdown of a protein is UBE3A dependent. But if nuclear UBE3A plays a direct or indirect role in gene expression, increased protein levels could also be the result of transcriptional changes. This cautionary note is well illustrated by the observation that the most cited target of UBE3A, the synaptic protein ARC,[27] is no longer considered to be a direct substrate of UBE3A.[28,29] Instead, ARC is a target of the ubiquitin ligase TRIAD3A/RNF216[30] and UBE3A probably regulates ARC at a transcriptional level.[29]

Several research laboratories have used mouse models of Angelman syndrome (or sometimes induced pluripotent stem cells) to identify novel therapeutics to treat Angelman syndrome. Such therapeutics can be classified into two major categories: (1) targeted treatments aimed at correcting pathophysiological deficits associated with Angelman
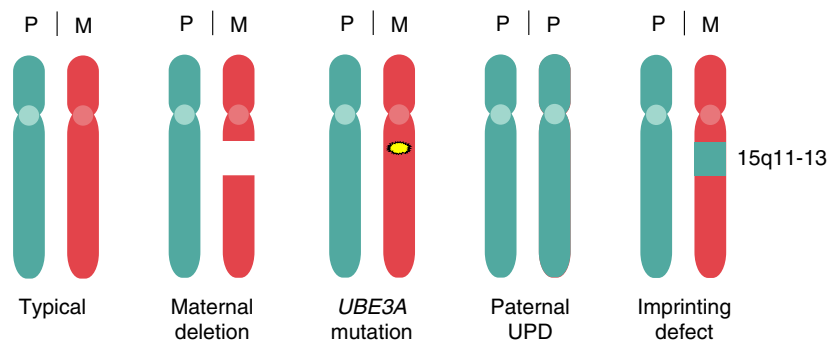

P | M   P | M   P | M   P | P   P | M

Typical   Maternal deletion   *UBE3A* mutation   Paternal UPD   Imprinting defect

15q11-13

Figure 1: Genetic causes of Angelman syndrome. Representation of the paternal (P) and maternal (M) chromosomes in individuals with Angelman syndrome compared with neurotypical individuals. Note that in all cases the maternally inherited *UBE3A* gene is affected, either by a deletion of the maternal 15q11-13 locus, an intragenic mutation in the *UBE3A* gene, the inheritance of two copies of the paternal chromosome (uniparental disomy [UPD]), or by imprinting defects that repress maternal *UBE3A* expression.

syndrome; and (2) treatments aimed at restoring the loss of UBE3A expression in the brain.

TARGETED TREATMENTS FOR PATHOPHYSIOLOGICAL DEFICITS ASSOCIATED WITH ANGELMAN SYNDROME

It has been consistently shown that mouse models of Angelman syndrome show marked changes in synaptic plasticity and excitatory/inhibitory balance.[14] Therefore, correcting these changes could provide a great target for treatment. However, over recent years, the view has emerged that these neuronal changes cannot be attributed to just a single mechanism. Several mechanisms have been identified that could underly the changes in neuronal excitability: for instance, changes in the alpha1 subunit of Na/K ATPase,[31] in small-conductance calcium-activated potassium channels (SK channels)[32] in calcium and voltage-dependent big potassium (BK) channels,[33] and in GABA (γ-aminobutyric acid) transporter (GAT1) levels.[34] Moreover, multiple changes have been observed in signalling cascades that are indirectly critical to neuronal function, such as CAMK2,[35,36] Ephexin5-EphB,[37] neuregulin-ErbB4,[38] TrkB-PSD-95,[39] ERK,[40] mTORC,[41] and dopamine signalling pathways.[42–45] These widespread changes are consistent with the suggestion that UBE3A probably plays an important global regulatory role, such as transcriptional regulation and/or by regulating (nuclear) proteasome activity to which it is strongly attached.[14,18,22–25]

In the face of these widespread changes, it is rather surprising that by specifically targeting a single mechanism, an electrophysiological or even a behavioural rescue could be observed in most of the aforementioned studies.[31–33,36,38,39,41] It is unclear how these highly specific treatments, which reversed the behavioural phenotypes in mouse models of Angelman syndrome, can be reconciled with the notion that so many mechanisms are affected. We believe that the observed rescue may in part be due to using underpowered studies in combination with behavioural readouts that are only weakly affected in mice with Angelman syndrome. The development of a standardized behavioural test battery of robust phenotypes, and the concomitant power analysis for each test, may allow better drug selection to then be moved to clinical trials.[46] For example, minocycline treatment was successful in a low-powered study that focused on a limited number of readouts.[47] However, when tested in the standardized behavioural test battery with a sufficiently high number of mice with Angelman syndrome, there was no improvement with minocycline on any of the readouts,[46] nor in clinical trials of patients with Angelman syndrome.[48] Similarly, levodopa treatment did not show an improvement in the Angelman syndrome mouse behavioural test battery nor in a clinical trial.[46,49]

Given that so many molecular mechanisms seem to be affected, a treatment that acts at a more global level may ultimately be more successful in a clinical trial. Several such treatments were successful in Ube3a mice. For some of these treatments, the precise mechanism targeted in Angelman syndrome is unclear, and it is conceivable that they act more generally as cognitive enhancers (e.g. treatment with reelin,[50] ampakines,[51] a neurogenesis stimulator,[52] or IGF-2 treatment[53]). Other drugs target the excitatory/inhibitory imbalance by the GABA modulators, such as ganaxolone and gabaxadol.[34,54] The latter drug was able to partly correct motor impairments and anxiety phenotypes in mice with Angelman syndrome.[34] But recent data of a randomized, double-blind, placebo-controlled, phase 3 study (Neptune trial, NCT04106557), which enrolled 97 patients treated with gabaxadol (OV101) or placebo, failed to show a change in the primary endpoint (overall score on the Clinical Global Impression-Improvement-Angelman syndrome scale). In addition, secondary outcome measures were not significantly changed. These disappointing results indicate that even identifying more broadly acting treatments is still very difficult.

As a cautionary note of the translational failures, it should be pointed out that all drug testing in mice to date has been performed on mouse models of Angelman syndrome that specifically lack Ube3a, whereas most trials have predominantly included patients with 15q11-13Del Angelman syndrome lacking the entire (maternally inherited) 15q11-13 gene cluster. The deletion of the additional genes in this locus may make it more difficult to obtain a clinical effect of the tested therapies. Hence, some of the translational failures might be caused by using an inappropriate mouse model.[14] Developing additional mouse models of Angelman syndrome that lack the equivalent genes of the 15q11-13 gene cluster, or using 15q11-13Del Angelman syndrome patient-derived induced pluripotent stem cells,[33,55] might circumvent such issues.

TREATMENTS AIMED AT RESTORING UBE3A EXPRESSION IN THE BRAIN

Undoubtedly, the most promising therapeutic approach for treating Angelman syndrome aims at restoring UBE3A expression. Such a therapy would potentially be disease-modifying and get as close to a cure as possible. There are two ways to achieve this: either by gene therapy that introduces UBE3A protein into the brain through viral vectors, or through activation of the imprinted (silenced) paternal UBE3A allele.

Viral-mediated delivery of UBE3A in the brain was shown to be partly effective in mice with Angelman syndrome.[56] However, a major translational challenge will be to create safe viral vectors with a good biodistribution that allow UBE3A expression throughout the human brain. Moreover, getting the correct amount of UBE3A per cell is very important, as too much could potentially be detrimental and may result in autism.[57] Last but not least, such a viral vector would preferably express both dominant UBE3A isoforms (or possibly all three isoforms), to ensure that all UBE3A functions are restored.[18]

To circumvent these issues, a very elegant approach to re-express neuronal UBE3A is by activating the dormant *UBE3A* gene on the paternally inherited chromosome (commonly referred to as 'unsilencing'). In both humans and mice, the paternally inherited *UBE3A* allele is silenced owing to the paternal expression of the long *UBE3A-ATS* transcript (>460 kilobases in humans, approximately 1000 kilobases in mice) that runs antisense to the *UBE3A* locus.[58] Elegant experiments in mice have demonstrated that this interferes with the expression of the paternally inherited *Ube3a* allele[59,60] (Fig. 2). Interfering with the synthesis of the UBE3A-ATS by topoisomerase inhibitors[61] or by Cas9-mediated targeting[62] induces paternal *UBE3A* gene expression, without the risk of inducing too much UBE3A expression.

Alternatively, the *UBE3A-ATS* can be targeted by antisense oligonucleotide (ASO) treatment. With this approach, binding of the ASO to the UBE3A-ATS leads to RNase-H-mediated cleavage of the ASO-RNA heteroduplex, thereby unsilencing the paternal *UBE3A* gene[63]

(Fig. 2). ASO treatment would solve most of the limitations associated with viral injection, especially with respect to controlling the level and the type (isoform) of UBE3A expression as well as its biodistribution. ASOs are readily absorbed by neurons, and given the great success of ASO treatment for spinal muscular atrophy (a motor neuron disorder), such a therapy holds great promise for Angelman syndrome and many other (genetic) neurodevelopmental disorders.

Despite its great therapeutic promise, ASO treatment of mice with Angelman syndrome resulted in a rather disappointing lack of improvement of behavioural phenotypes, despite significant upregulation of UBE3A protein levels in most areas of the brain.[63] These disappointing results may have resulted from treating the mice when they were adult. Subsequent studies showed that the critical period for the full recovery of well-established Angelman syndrome mouse phenotypes by *Ube3a* gene reinstatement lies around birth.[64,65] This suggests that UBE3A function is critical for typical brain development, which is further highlighted
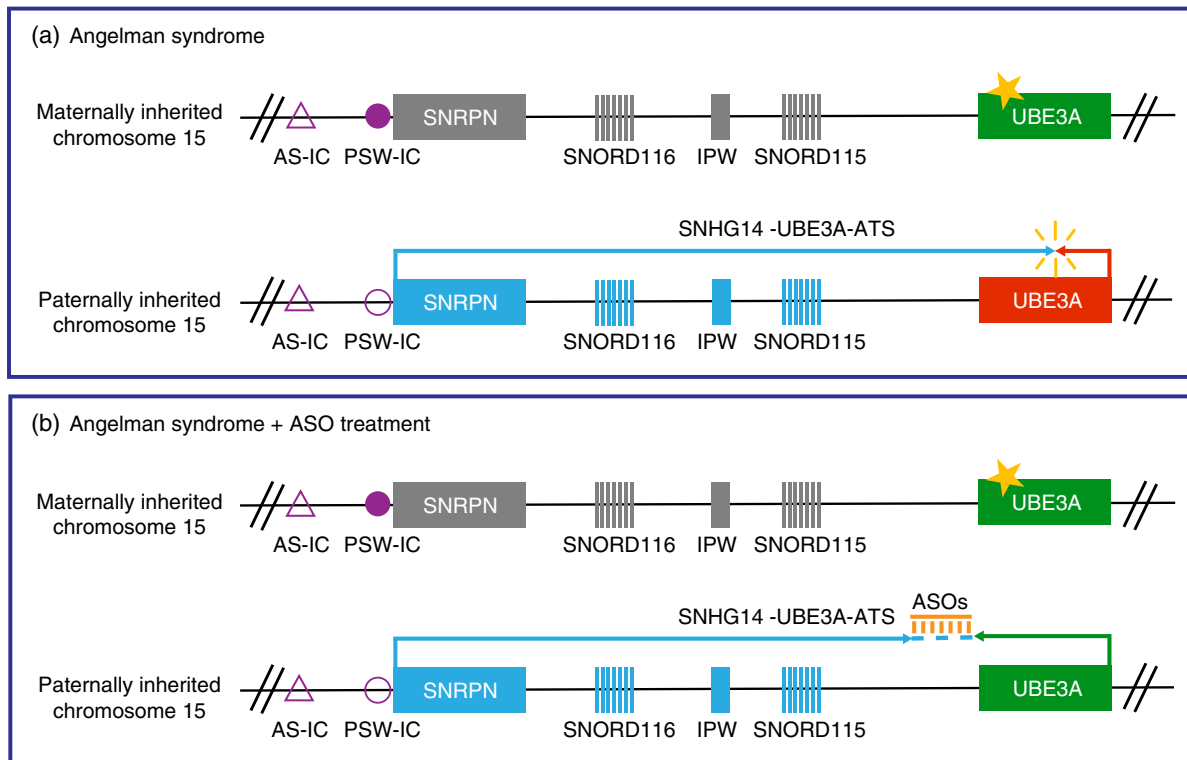

Figure 2: Mechanism of neuronal UBE3A imprinting and antisense oligonucleotide (ASO)-mediated unsilencing of paternal *UBE3A* gene expression. (a) Overview of the *UBE3A* locus in neurons of an individual with Angelman syndrome with a mutation in the *UBE3A* gene (indicated with a star). Note that most patients with Angelman syndrome carry a maternally inherited deletion of the depicted region extending far beyond the Angelman syndrome Imprinting Center (AS-IC) and the *UBE3A* gene. (b) Overview of paternal *UBE3A* expression in the Angelman syndrome condition upon ASO treatment (orange). Maternalli imprinted genes are depicted in grey, the AS-IC is indicated as an empty purple triangle. The lack of a methylated Prader–Willi syndrome Imprinting Center (PWS-IC; indicated as an empty purple circle) allows for the transcription of the long non-coding *SNHG14* gene, also known as *UBE3A-ATS*, which is responsible for suppressing paternal *UBE3A* transcription (red rectangle). The administration of ASOs leads to cleavage of the *UBE3A-ATS* transcript, resulting in the unsilencing of the paternal *UBE3A* gene (depicted by a green rectangle), allowing restoration of synthesis of the UBE3A protein.

by the observation that deletion of the *Ube3a* gene in adult mice has little effect.[66] How this critical period in mice with Angelman syndrome relates precisely to the optimal treatment of patients with Angelman syndrome is unknown, but it is noteworthy that there is no critical period for restoring hippocampal plasticity.[64] Since hippocampal plasticity is often used as a cellular proxy for learning and memory formation, this would suggest that gene reinstatement will improve cognitive function, at least to some extent, at any treatment age.[65,66] Another important question is whether UBE3A reinstatement is equally beneficial in patients who carry the 15q11-13 deletion as in those carrying the *UBE3A* mutation. Owing to the lack of a mouse model of 15q11-13 deletion,[14] it is difficult to predict to what extent the effect of losing the additional genes in this locus is masked by the severe consequence of losing UBE3A. However, restoring UBE3A in these patients will probably still significantly improve symptoms.

Since the sequence of the *UBE3A-ATS* is poorly conserved between mice and humans, the use of patient-derived induced pluripotent stem cells[67] has been instrumental in identifying efficient ASOs that can be used in the clinic. Currently, two active clinical trials are taking advantage of the ASO-mediated *UBE3A* gene reinstatement approach. One trial is sponsored by Hoffmann-La Roche (molecule RO7248824; NCT04428281) and the other is sponsored by GeneTX Biotherapeutics (molecule GTX-102; NCT04259281). A third trial (sponsored by Ionis/Biogen) is expected to start soon as well. Since ASOs do not cross the blood–brain barrier, they will need to be administered through intrathecal injections. It is important to note that all current studies are phase I trials, primarily aimed at investigating the safety and tolerability of ASOs, as well as looking into their pharmacokinetic and pharmacodynamic properties. The safety and tolerability aspects are particularly important as these ASOs cannot be tested in unaffected population norm volunteers, owing to the risk of inducing levels of UBE3A that are too high. Notably, all trials make use of different target sequences, as well as different phosphodiester linkages and sugar modifications that stabilize the ASO.[68] Hence, both in terms of efficacy (ASO stability and absorption, *UBE3A-ATS* binding and breakdown) and (dose-related) side effects, these trials may have different outcomes. Needless to say, the results of these ASO trials are eagerly awaited by the families and the medical and scientific communities alike. Success of these trials would not only transform the lives of patients with Angelman syndrome and their families, but could also offer great opportunities for treating many other genetic neurodevelopmental disorders.

Data Availability Statement
Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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