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VIEWPOINT

Therapeutic validation and targeting of signalling networks that are dysregulated in intellectual disability

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Intellectual disability (ID) represents a major burden on healthcare systems in the developed world. However, there is a disconnect between our knowledge of genes that are mutated in ID and our understanding of the underpinning molecular mechanisms that cause these disorders. We argue that elucidating the signalling and transcriptional networks that are dysregulated in patients will afford new therapeutic opportunities.

Introduction

Intellectual disability (ID) is a series of neurodevelopmental disorders with overlapping clinical features, including impaired adaptive and cognitive function. ID represents a major burden for patients, families and healthcare systems [1]. These conditions are a critical unsolved biomedical problem that affects an estimated 1–3% of the world population, especially children [2–4]. Therefore, there is an urgent need to develop novel therapies for ID, as most have no known cure [5]. We attribute the dearth of therapeutic strategies for ID to lack of

knowledge as to the underlying molecular basis of these disorders.

Emerging sequencing, genomic and annotation tools in human genetics are effective in identifying genes which cause ID driven by inherited or de novo gene variation [6–9]. This is particularly true of X-linked ID (XLID), which affects mostly males [10,11]. XLID is estimated to account for 5–15% of all ID cases, and around 150 genes have been identified from studies of inheritance patterns in affected males and their families [10,12]. XLID variants have been identified in

Abbreviations

DUB, deubiquitinase; ID, intellectual disability; PKU, phenylketonuria; PROTAC, proteolysis targeting chimera; SRPK, SRSF protein kinase; TOKAS, Tonne–Kalscheuer syndrome; XLID, X-linked intellectual disability.

genes with diverse biological functions, including those encoding signalling enzymes and transcriptional regulators [12]. Understanding the signalling networks surrounding these key components could, in principle, provide insight into how cell communication and gene expression are disrupted in patients. This in turn affords potential opportunities to therapeutically re-establish regulatory logic and restore intellectual functioning [5,13] (Fig. 1).

Cell signalling and ID

Clinical genetics has identified an extensive panel of post-translational signalling components that are mutationally disrupted in ID patients. These include protein kinases, ubiquitylation enzymes including E3 ligases and deubiquitinases (DUBs) (reviewed in [14–16]) and other regulators of post-translational modifications such as acetylation and methylation [12]. Intriguingly, despite decades of research in this area, the products of ID genes remain largely unstudied. As a result, there is very little information on the molecular, cellular and neurodevelopmental functions of these enzymes.

Most of the functional information on XLID genes identified by clinical genetics comes from genetic disruption and phenotyping in whole organisms [17–19]. However, these models provide little or no information

on the upstream signals and mechanisms that regulate these enzymes, the key substrates whose function is modified by their catalytic activities and the downstream implications for transcription, cell biology and neurological development and functioning. This illustrates a major gap between clinical genetics and molecular research that must be bridged in order to develop effective therapies. Current research approaches are often oriented towards chemical screens to identify compounds that reverse phenotypes caused by ID gene disruption. However, we propose that rationally identifying mechanisms by which ID variants disrupt signalling components and defining how these integrate with wider cell signalling networks and processes will provide opportunities to specifically modulate the function of these enzymes and signalling networks to the benefit of patients. This approach will also delineate how these gene products control normal neurodevelopment and yield valuable information on protein function and may identify hypomorphic variants that may be missed by conventional functional screens.

Identifying specific molecular disruptions that cause ID

First, it is essential to understand the mechanisms by which ID-associated gene variants impact on encoded

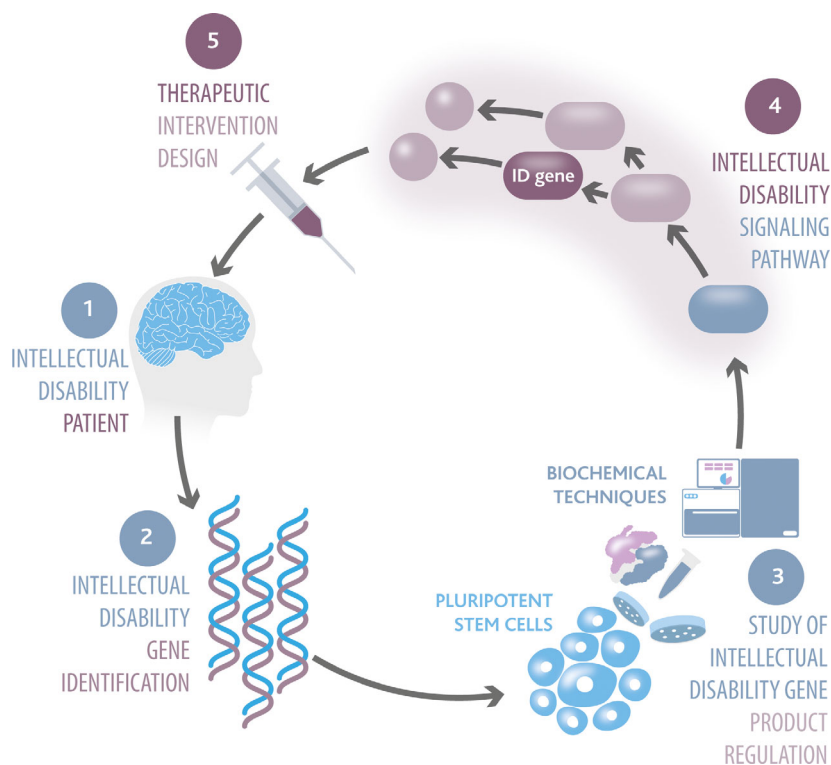


Fig. 1. Study of signalling networks towards development of therapies for ID. ID patients are diagnosed in the clinic (1) and genetic testing is carried out to identify potential ID genes (2). We propose to combine a variety of stem cell-based model systems with state-of-the-art techniques to functionally study biochemistry and regulation of ID gene products (3). This paradigm allows dissection of novel signalling pathways that are disrupted by gene variants that cause ID (4) which will be crucial to identify molecular targets for the design of therapies focused on restoring disrupted cell signalling (5). These therapies will then serve to treat ID patients and improve cognitive function.

signalling components. This involves initial investigation of whether the gene variant disrupts protein translation, folding or stability, which may be sufficient to classify ID variants as “likely pathogenic”. In other cases, ID-associated gene variants may alter the biochemical properties and/or molecular function of the encoded protein in more subtle ways. These can include disrupting subcellular localisation, interaction with key functional partners such as substrates and/or co-regulators or by directly disrupting biochemical or enzymatic activity. Investigating these functional alterations may require *a priori* knowledge of regulatory mechanisms, partner proteins and downstream substrates, which is often lacking in this field. Nevertheless, we and others have been able to exploit limited biochemical information to determine specific impacts of ID mutations on signalling proteins, thereby confirming variants as likely pathogenic.

If ID-causing variants impact on either protein levels or specific signalling functions such as catalytic enzymatic activities, it can be inferred that unravelling the downstream substrates, gene expression programmes and cellular functions will shed light on the molecular and cellular process processes that are disrupted to cause ID. In the case of core signalling components such as protein kinases or ubiquitylation enzymes, quantitative proteomic methods for profiling changes in the phosphorylation or ubiquitylation status of proteins can reveal the key downstream substrates [20,21]. Finally, transcriptomic profiling will identify the genetic programmes into which the emerging network feeds, enabling crucial functional insights into largely unstudied but critical neurodevelopmental signalling networks.

Research by our group and others illustrates the value of this approach. The E3 ubiquitin ligase RNF12/RLIM is mutated in patients with a syndromic form of XLID recently termed Tonne–Kalscheuer syndrome (TOKAS) [22,23]. RNF12/RLIM TOKAS variants lead to impaired E3 ubiquitin ligase activity, which disrupts substrate ubiquitylation and proteasomal degradation [24,25]. Previous evidence suggests that RNF12 ubiquitylates transcriptional regulators, most prominently the developmental transcriptional controller REX1/ZFP42 [26–28]. Our investigations find that REX1 may be a RNF12 substrate that is highly relevant for development of TOKAS [29]. In the presence of RNF12 TOKAS variants, REX1 ubiquitylation and degradation are impaired, leading to REX1 accumulation in cells [24]. This in turn leads to deregulation of an RNF12-dependent neurodevelopmental gene expression programme [29] and aberrant neural differentiation [24]. In principle, disruption of this signalling pathway for neurodevelopmental

regulation by RNF12/RLIM TOKAS variants could drive ID in TOKAS patients, providing detailed molecular insight into the mechanisms underpinning this ID syndrome.

This approach is further illustrated in elegant work by Werner and colleagues, who showed that the deubiquitylating enzyme OTUD5 is mutated in a form of ID known as LINKED syndrome [30]. This study identified a series of OTUD5 variants in ID patients, although the substrates of OTUD5 were not known. Quantitative proteomics pinpointed a cohort of key chromatin modifiers, including ARID1A/B, HDAC2, HCF1 and UBR5 that are direct substrates of OTUD5 which work in combination to regulate expression of neurodevelopmental genes [30]. This again provides an elegant molecular framework to explain the development of ID in LINKED syndrome patients with OTUD5 gene variants.

Importantly, these examples also illustrate how new molecular insights into signalling defects in ID can reveal potential therapeutic strategies. In the case of RNF12/RLIM signalling that is dysregulated in TOKAS, restoration of REX1 substrate degradation is predicted to normalise the expression of neurodevelopmental genes in TOKAS patients. In the case of OTUD5, restoring deubiquitylation activity, perhaps by destruction of the cognate E3 ubiquitin ligase(s) which ubiquitylate OTUD5 substrate proteins involved in chromatin organisation, could be employed to re-establish neurodevelopmental gene expression. In principle, each of these objectives could be achieved via targeted protein degradation approaches. These include proteolysis targeting chimeras (PROTACs) [31], which are heterobifunctional molecules that chemically juxtapose a target protein and a non-cognate E3 ubiquitin ligase, leading to neomorphic target ubiquitylation and destruction. Therefore, understanding the key molecular processes that are disrupted in pathways downstream of ID gene variants can enable personalised and/or targeted approaches to restore signalling and potentially normalise neurodevelopmental gene expression.

Comprehensively mapping signalling networks that are disrupted in ID

Initial focus is to address the key downstream molecular processes that are disrupted by ID gene variants. However, upstream regulation of signalling components that are mutated in ID will be highly relevant to gain a complete understanding of the molecular and cellular processes that are disrupted to cause ID. Furthermore, genes encoding these key upstream

regulatory components may themselves be mutated in ID patients with overlapping clinical features to introduce further ID-associated gene variants. We suggest that iteratively mapping ID signalling networks in this way will eventually provide a complete picture of the regulatory logic that is disrupted in various forms of ID and will in turn reveal further potential ID genes and signalling nodes that may be exploited in therapeutics (Fig. 1).

Our own research again supports this notion. In searching for upstream regulators of the E3 ubiquitin ligase RNF12/RLIM, which is mutated in TOKAS ID, we found using chemical inhibitor and recombinant kinase screens that the splicing factor kinase SRSF protein kinase (SRPK) directly phosphorylates RNF12, leading to activation and nuclear anchoring [29]. As a result, SRPK phosphorylation of RNF12 plays a key role in regulation of RNF12 substrate ubiquitylation and in regulation of neurodevelopmental genes [29]. Therefore, SRPK is directly linked to downstream processes that are relevant for ID. This prompted a search of databases reporting identification of gene variants associated with ID phenotypes to determine whether the SRPK gene family is also disrupted in these disorders. Indeed, *SRPK2*, which is highly expressed in the human brain, is deleted in ID patients, while the X-linked *SRPK3*, which is expressed in a very specific subset of human neurons, is frequently amplified, deleted and mutated in patients. Investigation of the biochemical impact of *SRPK3* ID-associated point mutations suggests that a subset display severely impaired kinase activity [29]. Taken together, these findings suggest that SRPK lies upstream of RNF12 in an emerging ID signalling pathway, of which multiple components are mutated in patients with overlapping clinical features. Based on this paradigm, future studies will involve high-throughput -omics approaches to map networks of post-translational modifications, comprehensively identifying key nodes and shedding light on the signalling networks that are disrupted in ID patients.

Understanding ID signalling in context

One of the major challenges in the ID field is contextual, that is, to effectively combine study of the relevant tissues in the nervous system with a tractable experimental system to unravel signalling networks in molecular detail. Standard laboratory cell culture systems are tractable but are far removed from the developing nervous system. Therefore, developmental components may not be expressed and/or regulation and function are not conserved. Animal models can

provide an important description of how relevant signalling systems are expressed and wired in the developing nervous system. However, whole organisms are not amenable to in-depth analysis and acute signalling perturbation, and neurological phenotypes are frequently marginal and/or difficult to detect.

In our experience, this gap is most effectively bridged using pluripotent stem cell models including mouse embryonic stem cells, but particularly human embryonic stem cells or patient-derived human induced pluripotent stem cells [32]. This enables researchers to perform detailed molecular and biochemical analysis in a tractable mammalian/human neuronal cell system while simultaneously garnering insights into developmental phenotypes that may be relevant for ID [33,34]. Furthermore, the capacity of pluripotent stem cells to differentiate into any cell type in the body including adult neural lineages provides a powerful tool to directly investigate how signalling networks engage the neurodevelopmental gene expression programmes that are exquisitely controlled to ensure correct neurological development and functioning [33]. Moreover, the advent of organoid-based technologies affords us future opportunities to investigate how signalling pathways control development of increasingly sophisticated and complete human “mini-brains” grown in the laboratory [35]. When combined with single-cell RNA-sequencing, organoids could provide unprecedented insight into the impact of ID gene variants on temporal and region-specific neurodevelopmental trajectories [36]. In short, pluripotent stem cells provide a surprisingly direct route by which to determine how complex molecular connections at the biochemical level impact on neurodevelopmental gene expression and therefore development of the human nervous system.

Therapeutic opportunities

What are the immediate therapeutic opportunities afforded by dissecting the signalling networks underpinning ID? A major benefit of research in this area is that signalling enzymes (and protein kinases in particular) have been exploited as therapeutic targets for decades, with scores of small molecule kinase inhibitors approved for clinical use. Furthermore, phosphatase inhibitors show growing promise as therapeutic targets that may reverse defects in kinase signalling caused by ID variants that disrupt kinase activity. In the ubiquitin field, PROTACs that are entering clinical trials induce or restore degradation of key therapeutic targets [37], and as discussed earlier could be exploited to normalise levels of protein substrates that accumulate in the presence of variants that disrupt activity of

the cognate E3 ubiquitin ligase. Finally, small molecule DUB inhibitors targeting distinct mechanistic aspects of enzymatic function [38] have shown recent clinical promise [39] and may enable modulation of ubiquitin-dependent signalling networks to modulate protein degradation in a similar manner. Excellent progress in the area of therapeutic tools and development pipelines to target the relevant signalling networks in ID suggests these will form important resources to ultimately benefit patients.

Future challenges

An ever-present challenge in this field is the ability to identify causal gene variants that strongly segregate with ID patients. In our view, this requires rigorous genetic analysis in combination with biochemical investigation to functionally characterise patient variants and assign causality. Although many ID genes are identified as inherited or *de novo* germline variants, somatic mutations during brain development must be considered as these can contribute to neurodevelopmental disorders [40]. ID gene identification will provide a robust starting point for mapping pathways and networks which are crucial for intellectual function. Although the primary objective will be dissection of discrete signalling pathways, understanding the links between distinct components and pathways that are mutated in related syndromes could require new systems biology and bioinformatic tools for network integration and annotation together with simulations/predictions of protein functional association and how these are disrupted by ID gene variants. Once the information flow within a signalling network is elucidated, it will then be possible to define nodes within that network that are appropriate for therapeutic intervention.

A key question that remains is whether the neurological manifestations of ID disorders are generally reversible in children and adult patients, or whether treatments or gene editing must be performed on embryos prior to embryonic neurological development. The latter may be true in many cases. However, in support of the former is the first example of mechanism-based therapy for ID caused by phenylketonuria (PKU) [41], where careful restriction of tyrosine uptake leads to marked improvement in intellectual functioning [42]. Recent investigations also address the reversibility of other genetic neurodevelopmental disorders by defining the relevance of ID genes for neurological function and determining whether phenotypes are reversed by restoring ID gene function. Thus far, adult neurological and behavioural

phenotypes have been shown to be at least partially reversible in mouse models of Rett syndrome caused by deletions in MeCP2 [43], SYNGAP1 haploinsufficiency [44], SHANK3-induced autism [45] and CDKL5 deficiency disorder [46]. Furthermore, clinical studies on Fragile-X syndrome, which is caused by inactivation of the mRNA binding protein and translational regulator FMR1, show promise in reversing the effects of FMR1 disruption [47,48]. This suggests a potential postnatal therapeutic window for reversal of neurological dysfunction associated with at least a sub-set of specific ID disorders. It is important to note that these interventions were developed based on a profound understanding of the molecular mechanisms underlying the disorder. If the same is true of other IDs caused by disrupted cell signalling, then signalling network mapping followed by target validation in the developing nervous system at the developmental stage of interest may enable mechanism-based targeted chemical interventions to normalise signalling and reverse neurological phenotypes.

This raises a final challenge of how to effectively model ID syndromes to provide pre-clinical validation of therapeutic strategies. In our view, it is essential to understand both molecular signalling disruptions and high-level neurological pathophysiology in order to develop effective therapies. Current experimental systems in the ID field each have major drawbacks. Human-derived pluripotent stem cells and neuronal differentiation systems suffer the obvious limitation of being an overly simplified *in vitro* system that is inappropriate for pre-clinical testing. In the longer term, this may be resolved using sophisticated stem cell-derived brain organoids, which may be sufficiently complex to reveal key physiological features of ID disorders. As discussed previously, some animal models of ID gene variants have shown utility for measuring ID phenotypes and therefore could provide pre-clinical validation. However, robust ID related phenotypes are not always obvious or even present making it very difficult to identify and measure impact of potential therapeutics on developmental, structural or behavioural defects of the nervous system. Therefore, the challenge remains to identify model systems that can provide tractable and quantifiable ID phenotypes, as this remains essential to enable pre-clinical testing of therapeutic interventions.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

GMF and FB conceived, drafted, edited and revised the manuscript.

Data availability statement

Data sharing is not applicable to this article as no new data were created in this study.

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