Neurodevelopmental models of transcription factor 4 deficiency converge on a common ion channel as a potential therapeutic target for Pitt Hopkins syndrome

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Introduction

Pitt Hopkins syndrome (PTHS) is a rare, pervasive developmental disorder resulting from autosomal dominant mutations on chromosome 18 at the TCF4 (ITF2, SEF2, E2-2) locus that lead to TCF4 haploinsufficiency and/or a dominant-negative mechanism.1-4 PTHS shares neurodevelopmental characteristics with the 18q deletion syndrome including cognitive and motor learning deficits, a lack of language acquisition, stereotypical autistic behaviors, epilepsy, and breathing abnormalities.5-8 In addition, common variation within and around the TCF4 gene is genome-wide significant for association with risk for schizophrenia,9,10 as well as for cognitive endophenotypes linked to schizophrenia.11,12 Therefore understanding the impact of TCF4 dysfunction on brain development and function will be important for delineating the molecular mechanisms that drive the underlying pathophysiology in PTHS.

Here we summarize our novel approach to identifying these molecular mechanisms that link genetic dysfunction of TCF4 to functional deficits in neurodevelopment. Using both an in utero gene transfer model to identify cell autonomous effects of TCF4 dysfunction and a PTHS mouse model, we were able to determine specific electrophysiological phenotypes that consist of deficits in neuronal excitability and action potential (AP) output.13 Furthermore, our approach utilized recently developed molecular profiling techniques that involve immunoprecipitating...
mRNA that is actively being translated by ribosomal complexes, thus giving us access to the translatome of a given target cell population. The combined set of experimental techniques we describe is well suited for phenotypic discovery and for the identification of molecular mechanisms that can ultimately be targeted for potential therapeutic benefit. We believe this is an attractive experimental strategy that can be applied to other disorders of neurodevelopment and rare diseases affecting the brain.

**In vivo cell autonomous model of PTHS**

In order to gain insight into the prominent neurodevelopmental characteristics of PTHS, we sought to model the disease in vivo. This was accomplished by developing an in vivo cell-autonomous model of the disease through TCF4 gene expression knockdown in the medial prefrontal cortex (mPFC) of the rat. Two shRNA constructs targeting Tcf4 (shTCF4) were separately transfected by in utero electroporation (IUE) to knockdown expression in layer 2/3 pyramidal neurons of developing embryos. Each shRNA, one targeting a common 3' exon in all Tcf4 isoforms and the other targeting a common 3' UTR, resulted in a significant reduction of TCF4 protein. We observed that expression of shTCF4 produced no gross morphological deficits, but resulted in a clear reduction in spike frequency output compared to a control shRNA (shCon). A rescue of AP output was achieved by overexpression of the human TCF4B isoform along with an shRNA, providing evidence that intrinsic excitability deficits represent a sequence-dependent effect. Although this phenotype was replicated across both shRNA experiments, we further validated the observation by delivering a CRISPR-Cas9 construct to target Tcf4, thus controlling for sequence-independent off-target effects. The crTCF4 also resulted in a significantly reduced neuronal spike frequency, showing the disruption of TCF4-dependent signaling directly contributes to a deficit in intrinsic neuronal excitability.

**Targeted molecular profiling**

Translating ribosome affinity purification (TRAP) is a targeted molecular profiling method that has previously been used to identify cell type-specific mRNA expression that gives rise to the unique molecular profiles of different populations of neurons in the CNS. We adapted TRAP to our IUE model in a novel approach we term iTRAP. Our adapted protocol includes co-expression of an EGFP-fused L10a ribosomal protein (L10a-EGFP) along with either shRNA or shCon constructs during IUE. Transfected mPFC tissue from P21 animals was affinity purified to isolate mRNA associated with active translation at the ribosome at the time of sample capture. This method provides multiple advantages in answering our specific questions about the underlying mechanism behind the phenotype generated in our IUE model of PTHS. IUE has several distinctive advantages as a model, including the high fidelity of co-transfection of multiple plasmids, as well as temporal and spatial specificity of cell-types that are transfected. In addition, the relatively sparse expression pattern that results is ideal for identifying cell-autonomous phenotypes that result from the specific genetic manipulation employed. However, the mosaic expression pattern prevents the use of bulk tissue to assay protein or mRNA levels since the majority of cells are untransfected. Indeed, we found that mRNA measured from bulk shRNA or control homogenates was insufficient to produce a measurable differential expression of ion channels responsible for intrinsic excitability deficits. However, the enrichment of mRNA from transfected cells by iTRAP allowed us to identify significant upregulation in the translation of target ion channels that were shown to ultimately be responsible for the observed excitability deficit.

**Reduced TCF4 causes ectopic expression of ion channels**

The differential expression between shTCF4 and shCon neurons indicated that two ion channels, Kcnq1 and Scn10a, were at least partially responsible for the excitability phenotype due to TCF4 haploinsufficiency. Translation of these two ion channels were found to be significantly upregulated in the shTCF4 condition when profiled using a prefabricated PCR plate containing primers for all known rat ion channel genes. Additionally, genomic DNA close to each of these two ion channels was confirmed to be directly bound by TCF4 through the analysis of multiple independent TCF4 ChIP-seq data sets. The specificity of these signals was confirmed by multiple pharmacological and molecular experiments in order to validate the biological relevance of these ion channels as downstream effectors of TCF4 initiated...
pathophysiology. Antagonists to both KCNQ1 (UCL2077 and JNJ303) and SCN10a (A-803467) were capable of rescuing spike frequency output in shTCF4 neurons while having no effect on shCon cells. Overexpression of a recombinant Scn10a resulted in a phenocopy of excitability deficits of TCF4 knockdown. Lastly, co-expression of CRISPR-Cas9 constructs targeting both Kcnq1 and Scn10a along with the shTCF4 resulted in a complete rescue of AP output. Thus, molecular profiling by iTRAP resulted in a remarkable advance in identifying biologically relevant mechanisms underpinning the TCF4 haploinsufficiency phenotype, a result unlikely to be achieved using traditional pharmacological methods in a comparable timeframe.

**Mouse model of PTHS**

Having found convincing evidence of ion channel regulation by TCF4, we sought to confirm these observations in an additional rodent model of PTHS. Using a mouse model initially generated to produce haploinsufficiency of TCF4 through constitutive germline deletion of one Tcf4 gene allele, we repeated measurements of intrinsic neuronal excitability through whole-cell recordings of layer 2/3 cortical neurons and observed a similar reduction in spike output compared to control littermates. Surprisingly, through western blot analysis of our PTHS mouse model, we determined that instead of the targeted constitutive Tcf4 deletion, a truncated TCF4 protein was expressed and this indicated the possibility that this transgenic mouse was instead a dominant-negative model. Therefore, we originally speculated that expression of a truncated TCF4 protein could be one explanation for the ion channel expression differences we observed between our in utero TCF4 knockdown models and this PTHS mouse model.

**Transcriptional control of TCF4**

In the PTHS patient population, both large deletions of the entire gene and single point mutations within the bHLH are observed, and both haploinsufficiency and dominant-negative models have been posited. Therefore, this PTHS mouse model is relevant regardless of whether it is a haploinsufficiency or dominant-negative model. However, to understand underlying molecular mechanisms downstream of TCF4 dysfunction it is critical to determine which model is being employed. To determine if the truncated TCF4 protein expressed in our PTHS mouse model could result in a dominant-negative effect, we tested its ability to bind to full-length TCF4. We PCR cloned both the full-length and truncated cDNA from our PTHS mouse model and added different N-terminal tags to each of the recombinant genes, where they would be predicted to not interfere with the bHLH domain of the protein. We co-transfected these constructs into HEK293T cells and performed co-immunoprecipitation experiments. As shown in Figure 1, HA-tagged full-length TCF4 (HA-mTCF4fl, red) can pull down Myc-tagged truncated TCF4 protein (Myc-mTCF4tr, green). This protein pulldown experiment, in conjunction with previously reported evidence of dominant negative effects, indicates that a dominant-negative effect of the truncated TCF4 protein expressed in our PTHS mouse model is a probable explanation for the mRNA expression differences between our two rodent models. However, we still cannot completely rule out other possible explanations such as non-cell autonomous effects that are specific to the mouse model or that the truncated TCF4 protein has other unknown in vivo functions beyond being a dominant-negative.

This evidence of a dominant-negative pathological mechanism in the mouse model now further confirms that while the cellular and molecular mechanisms involved in TCF4 regulation may be context specific, the replicated deficit in intrinsic excitability and AP spiking output is likely to be a true pathophysiological consequence of PTHS that is consistent across individuals with either haploinsufficient or dominant-negative versions of the disorder. Although Kcnq1 expression diverged, Scn10a expression was consistent between our models and the SCN10A antagonist
A-803467 was effective at acutely rescuing AP output in the TCF4\(^{+/+}\) mouse neurons, just as it did in the in utero shTCF4 rat model. Our results clearly position Scn10a as a high priority target with therapeutic potential in PTHS, as multiple independent experimental methods all suggest that its ectopic expression is linked to the intrinsic excitability deficit resulting from TCF4 dysfunction.

**Future directions**

The cellular, molecular, and behavioral analysis of TCF4 in relation to PTHS is in relatively early stages of study. TCF4 is expressed in a variety of cell types throughout the body and it is estimated that it regulates hundreds to thousands of different genes. Therefore, it is very likely that a variety of phenotypes, beyond just the intrinsic excitability deficits we have observed so far, will be discovered in the near future. This is the case for other models of autism spectrum disorders, where deficits in cell proliferation, differentiation, synapse formation, and plasticity are observed within and across different model systems.\(^{21-23}\) Therefore, it will be important to further characterize models of PTHS during all stages of development and with a variety of cellular, molecular and behavioral techniques. In addition, it will be important for the field to perform unbiased whole genome RNA sequencing from PTHS models to aid in the identification of genes that are both regulated by TCF4 and whose expression is disrupted in PTHS, as these regulated genes could represent therapeutic targets. However, given the context-dependence of TCF4, it may be critical to use cell-type specific RNA purification approaches, such as iTRAP, to obtain better mechanistic insight into newly observed phenotypes.

**Conclusions**

Determining the underlying mechanisms of pathophysiology for rare diseases, as well as for more common disorders, is a formidable challenge. Here, and in our recent article,\(^{13}\) we have described a system for phenotype discovery in a rare disease model that utilizes a novel molecular profiling method we term iTRAP that is capable of identifying candidate molecular mechanisms underlying pathophysiological phenotypes.

By comparing a cell autonomous TCF4 haploinsufficiency rat model to a PTHS mouse model, we identified a common electrophysiological phenotype that is at least partially caused by inappropriate expression of Scn10a, a voltage-gated sodium channel. We now consider SCN10A to be a potential therapeutic target for PTHS. Further experiments are required to determine if these phenotypes and molecular mechanisms observed at the cellular level can be successfully translated to the level of mouse behavior, to human biology, and ultimately to the development of therapeutic agents.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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