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The contribution of GTF2I haploinsufficiency to Williams syndrome

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Abstract

Williams syndrome (WS) is a neurodevelopmental disorder involving hemideletion of as many as 26–28 genes, resulting in a constellation of unique physical, cognitive and behavior phenotypes. The haploinsufficiency effect of each gene has been studied and correlated with phenotype(s) using several models including WS subjects, animal models, and peripheral cell lines. However, links for most of the genes to WS phenotypes remains unclear. Among those genes, general transcription factor 2I (*GTF2I*) is of particular interest as its haploinsufficiency is possibly associated with hypersociability in WS. Here, we describe studies of atypical WS cases as well as mouse models focusing on GTF2I that support a role for this protein in the neurocognitive and behavioral profiles of WS individuals. We also review collective studies on diverse molecular functions of GTF2I that may provide mechanistic explanation for phenotypes recently reported in our relevant cellular model, namely WS induced pluripotent stem cell (iPSC)-derived neurons. Finally, in light of the progress in gene-manipulating approaches, we suggest their uses in revealing the neural functions of GTF2I in the context of WS.

Keywords

GTF2I; Williams syndrome; hypersociability; TRPC3

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1.1 Williams syndrome

Williams syndrome is a rare multigenic neurodevelopmental disorder with the prevalence of 1 in 7,500 [1]. It is caused by hemizygous deletion of 26–28 genes on chromosome band 7q11.23 [2] (Fig. 1a) as a result of unequal non-allelic homologous recombination between low-copy repeats flanking the region of deletion [3, 4] during meiosis [2]. Due to haploinsufficiency of all these genes, classical (or typical) WS individuals, accounting for 98% of diagnosed cases [5], exhibit clinical symptoms including craniofacial (elfin) features, cardiovascular abnormalities especially supravalvular aorta stenosis (SVAS), hypercalcemia, and overall brain volume reduction [6–9]. In terms of cognitive profiles, typical WS subjects exhibit mild to moderate mental retardation, overuse of expressive language, and visuospatial deficits [10, 11]. Strikingly, they share a very unique behavioral profile, i.e. over-friendliness or hypersociability [12, 13], which is opposite the characteristics of autism spectrum disorders (ASD). Rarer atypical WS cases, especially the ones whose hemizygous deletion spans less than 26–28 genes, exhibit a partial spectrum of typical WS phenotypes [14–16]. These atypical cases provide vital clues for genotype–phenotype correlation studies, enabling the dissection of the role of each gene in WS etiology. Haploinsufficiency of *ELN*, for example, results in decreased elasticity of blood vessels and thereby leading to SVAS [14], the major cause of death in WS individuals [17]. In the past few decades, several attempts have been made to establish the links between other hemideleted genes and WS neurocognitive and behavioral phenotypes.

In addition to microdeletion, non-allelic homologous recombination during meiosis could result in duplication of this region, causing a disorder called 7q11.23 microduplication syndrome (7dup) [18]. 7dup individuals harbor three alleles of each of these 26–28 genes as opposed to one allele in WS patients. Interestingly, 7dup individuals are characterized by ASD-like phenotypes including developmental delay, language impairment, and poor social skills [18, 19], which are the opposite of WS characteristics, suggesting a gene-dosage effect. Mutations in *MECP2, FMR1, SHANK3, CDKL5, CACNA1C* and *TRPC6,* have been independently reported contributing to syndromic forms of ASD, i.e. Rett syndrome [20], fragile X syndrome [21], Phelan-McDermid syndrome [22], CDKL5-related disorder [23], Timothy syndrome [24], and TRPC6-related disorder [25], respectively. At first, proteins encoded by these genes may seem to act in different pathways, but their mutations ultimately result in common ASD phenotypes. These phenotypes are also shared by individuals with non-syndromic ASD, which make up the majority of ASD cases, yet the genetic bases for these phenotypes remain unknown. With relevant established models, studies of rare disorders with well-defined genetic mutations like WS and 7dup, which differ only in gene copy number, offer powerful insight into the functions of those genes in 7q11.23 as well as common mechanisms underlining neurocognitive profiles and social behaviors in ASD.

The use of neurons generated from patient-derived iPSCs has recently become a routine in neurological disease modeling [26–28] as genetic mutations in patients' somatic cells can be captured and passed on to neurons, which can then be manipulated and investigated for altered neuronal characteristics. We are the first group to report WS phenotypes in WS iPSC-derived neural progenitor cells (NPCs), specifically an increase in apoptosis as a result of frizzled9 haploinsufficiency, and in WS iPSC-derived neurons, including changes in

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morphology comparable to those observed in postmortem neurons as well as increases in calcium transient frequency when compared to NPCs and neurons derived from typical developing (TD) subjects' iPSCs, respectively [29]. In order to specifically look further into the role of each gene in the WS context and form appropriate hypotheses, information must be adequately gathered from previous studies using several different systems including atypical WS individuals as well as mouse and other cellular models. Among those 26–28 WS genes, *GTF2I* is of our interest as several genotype-phenotype correlation studies in WS individuals with partial deletions suggest its importance in regulating neurocognitive profiles and social behavior. Findings from those studies are, here, reviewed.

1.2 Atypical WS subjects and mouse models: Clues from genotype–phenotype correlation studies of *GTF2I*

GTF2I is one of the 26–28 hemizygously deleted genes in WS, being located at the telomeric end of the deleted region (Fig. 1a). It encodes general transcription factor 2I, which was first described in 1991 as a transcription initiation factor that binds to pyrimidine-rich initiator (Inr) elements at the transcription start site [30]. However, its role in WS etiology was implicated only when atypical WS cases with both alleles of *GTF2I* retained in the genome were reported for the first time in the early 2000s. Hirota *et al.* described three atypical WS subjects whose deletions were smaller than those with typical WS and did not include *GTF2I* and *GTF2IRD1* (Fig. 1b) [31]. Compared to typical WS subjects, these atypical WS individuals still have SVAS but do not exhibit elfin facies and visuospatial deficits [31]. Morris *et al.* reported five families, with members affected by SVAS, each having different deletions in 7q11.23 including *ELN* and some WS genes (Fig. 1c) [32]. In the genomes of all individuals analyzed, only *FKBP6* and *GTF2I* had two preserved alleles. Besides variation in WS cognitive profiles, none of the individuals had mental retardation [32]. However, social behaviors were not assessed in these two studies [31, 32]. Ferrero *et al.* reported atypical WS subject whose deletion was similar to one of those three patients described by Hirota *et al.* except that *FKBP6, FZD9* and partial *BAZB1* were retained in his genome (Fig. 1d) [33]. The patient exhibited partial craniofacial features, SVAS and particular cognitive deficits but no hypercalcemia and hypersociability [33]. Dai *et al.* described a very unique atypical WS subject whose deletions spanned almost all typical WS genes except *GTF2I* (Fig. 1e) [34]. The individual showed typical WS physical and developmental phenotypes as expected, but did not exhibit overly social behavior [34]. Antonell *et al.* reported another two families with different partial WS deletions preserving *GTF2I* (Fig. 1f) [35]. Despite variable cardiovascular abnormalities, members of both families exhibited normal visuospatial ability and, surprisingly, hypersociability [35]. Edelmann *et al.* described an individual with hemideletion of *GTF2IRD1* (partial)*, GTF2I* and another 14 genes outside the typical WS deletion region towards the telomeric end of chromosome 7 (Fig. 1g) [36]. While the subject met the criteria for ASD, including abnormalities in comprehension of simple language and conversation, she also exhibited WS cognitive and behavioral profiles, including visuospatial deficits, hypersociability, non-social anxiety, and language delay [36]. No heart defects, hypercalcemia, or typical craniofacial features were observed due to preservation of most typical WS genes [36]. Delgado *et al.* described another atypical case with deletion almost opposite to the individual in previous

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study, i.e. only partial *GTF2IRD1* and *GTF2I* were not hemizygously deleted (Fig. 1h). While the individual showed cardiovascular abnormalities and craniofacial features, her neuropsychological phenotypes, including hypersociability, visuospatial deficits and developmental delay, were, unfortunately, not assessed in this study [37].

Increasing studies of variation in single-nucleotide polymorphisms (SNPs) in *GTF2I* coupled with brain response using functional magnetic resonance imaging in different populations also suggest the gene's association with particular traits linked to certain brain regions. Jabbi *et al.* found that a minor allele of particular *GTF2I* SNP is associated with dorsolateral prefrontal cortex activity responding to aversive stimuli processing in the TD population [38]. Moreover, Malenfant *et al.* reported the association of another two *GTF2I* SNPs and their haplotype with social skill impairment and repetitive behaviors in ASD population [39]. Recently, Swartz *et al.* showed that one of these 2 alleles is also associated with reduction in amygdala activity relating to threat in female TD participants [40].

In addition to human studies, mouse models have also provided vital clues regarding the role of the murine homolog *Gtf2i* through genetic manipulation and whole animal characterization. Typical WS mice, with hemizygous deletion of their human gene counterparts in conserved syntenic region in reverse orientation on mouse chromosome band 5G2, were successfully generated [41]. This was achieved by breeding an atypical WS mouse with a proximal deletion (*Gtf2i* to *Limk1*) to another atypical WS mouse with a distal deletion (*Limk1* to *Fkbp6*) [41]. The typical WS progeny mice recapitulate phenotypes observed in typical WS individuals while atypical WS mice exhibit partial traits, allowing mapping of those features to a set of genes in either the distal or proximal deletion region. Using these mice, Li *et al.* found that the proximal genes were associated with elevated sociability and hypersensitivity to sounds, but not with cognitive defect and brain volume reduction [41]. Single-gene deficient mice also offer insight into the haploinsufficiency effect of specific gene of interest. Sakurai *et al.* reported that homozygous deletion of *Gtf2i* is embryonic lethal due to abnormal neural tube closure during embryonic development, while mice with heterozygous deletion of *Gtf2i* appeared to be physically normal accompanied by normal learning and memory and no increase in anxiety [42]. However, these animals exhibited hypersociability as indicated by significant increase in time exploring unfamiliar mouse and decreased habituation [42]. Lucena *et al.* generated mouse model expressing truncated form of GTF2I, i.e. only first 140 amino acids were deleted [43]. Instead of embryonic lethality, homozygous mutant mice showed compromised viability [43]. Interestingly, homozygous and, to a lesser extent, heterozygous mutants exhibited craniofacial phenotypes. Additional phenotypes reported for heterozygous mice include hypersensitivity to sound and high anxiety [43]. Another study by Mervis *et al.*, in which mice with varying copy numbers of *Gtf2i* were generated, demonstrated that a significant increase in separation anxiety was observed in mouse pups with more than two copies of *Gtf2i* relative to pups with one or two copies, both of which surprisingly exhibited comparable levels of separation anxiety [44]. Borralleras *et al.* characterized different mutant mice (intragenic deletion of *Gtf2i*, proximal and complete deletions of WS critical region) [45]. All mutants exhibited poor motor coordination, anxiety and hypersociability [45]. In addition, as GTF2I is phosphorylated by SRC tyrosine kinase and, consequently, translocated to nucleus [46], mutation in *Src* disrupting its kinase activity also negatively

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affects GTF2I function. Mice with homozygous mutation of *Src* showed WS phenotypes including visuospatial deficits, craniofacial features and hypersociability [47].

These genotype–phenotype correlation studies have suggested association of *GTF2I* in the manifestation of many WS phenotypes such as social behavior, anxiety, visuospatial deficits, hypersensitivity to sound, mental retardation as well as craniofacial features. Nevertheless, in order to establish unambiguous links between this gene and any of these potential phenotypes, further investigations of more rare atypical WS individuals, SNPs in different populations, and transgenic mice with a deletion or duplication of *GTF2I* are needed.

1.3 Function of GTF2I: Collective studies in different cellular models

GTF2I together with the GTF2I repeat domain-containing protein 1 (*GTF2IRD1*) and 2 (*GTF2IRD2*) genes are members of the transcription factor 2I family [48]. *GTF2IRD1*, located upstream of *GTF2I*, is also hemizygously deleted in typical WS individuals, unlike *GTF2IRD2* which is located downstream of *GTF2I* (Fig. 1a). GTF2I contains a leucine zipper at the N terminus for homodimerization, a nuclear localization signal, a basic region (DNA binding domain), and six repeated regions (I-repeats) with a helix-loop-helix motif in each repeat for protein-protein interactions [49]. Through alternative splicing, there are at least four human GTF2I isoforms (α, β, γ, and Δ) [50], with the γ isoform being highly expressed in neuronal cells [51]. These isoforms can interact with one another to form homomers and heteromers [50] in various combinations that may enable binding to different target promoters and, therefore, drive expression of different genes [50]. As suggested by its distinct domains for both DNA-protein and protein-protein interaction, GTF2I is multifunctional transcription factor that binds to both Inr core promoter element for basal transcription and to upstream elements for signal-induced transcription [52, 53]. *c-fos* promoter is described as first transcriptional target of GTF2I [54]. In mouse fibroblasts, it is demonstrated that, at resting state, the Δ isoform is found in the cytoplasm while the β isoform resides in the nucleus, controlling basal transcription of *c-fos*. Upon signaling-induced phosphorylation at tyrosine residues, the Δ isoform is translocated to the nucleus, recruits other activators and activates *c-fos* gene, while the β isoform is transported to the cytoplasm [55]. GTF2I has been shown to be involved in several cellular processes, including endoplasmic reticulum stress response, cell cycle, apoptosis or even virus transcription, etc. through its interaction with multiple proteins. Examples of binding partners of GTF2I and its corresponding transcriptional targets and cellular processes that have been reported are demonstrated in Table 1. Microarray analysis in mouse embryonic fibroblasts overexpressing GTF2I followed by qRT-PCR and chromatin immunoprecipitation of selected candidates confirmed that primary downstream targets of GTF2I were *Bax, Shrm, Cfl-1, Ezh2, Epc1, Ccnd3* and *Hdac1* [56]. Another microarray analysis comparing between mouse cortex of heterozygous mutants expressing truncated GTF2I, generated by Lucena *et al.* [43], and that of wild-type animals as well as between XS0353, embryonic stem cell line with heterozygous mutation for *Gtf2i,* and AB2.2, wild-type embryonic stem cell line, indicated that downstream target of GTF2I are genes in phosphatidylinositol 3-kinase signaling pathways, e.g. *Pik3r1*, *Cab39l*, and *Eif4b*, involved in dendritic spine formation during development and synaptic plasticity, as well as genes in transforming growth factor beta signaling pathways including *Shc1* and *Snw1* [57]. In terms of particular

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WS phenotypes, direct targets of GTF2I, namely *Cfdp1*, *Sec23a* and *Nsd1*, genes strongly associated with craniofacial development, have been identified [58].

One of the cytosolic functions of GTF2I relatable to WS etiology is its negative regulation of agonist-induced calcium entry (ACE), which may be associated with hypercalcemia described in WS individuals. Using rat neural cell lines, Caraveo *et al.* reported that, upon binding of agonists (uridine triphosphate and bradykinin) to G protein-coupled receptors on cell surface, phospholipase C-gamma (PLC-γ) becomes activated and its split pleckstrin homology (PH) domain binds to the PH-like domain of a calcium channel protein, namely transient receptor potential cation channel subfamily C member 3 (TRPC3), leading to an increase in surface expression of TRPC3, and, therefore, Ca^{2+} influx (Fig. 2a) [59]. As GTF2I also contains a PH like domain, activation by phosphorylation enables it to compete against TRPC3 for binding to PLC-γ at the split PH domain as well as to Src homology 2 (SH2) domain of PLC-γ, leading to decreased surface accumulation of TRPC3, and, hence, reduced Ca^{2+} influx (Fig. 2b) [59].

There are intriguing hints that these molecular data may have physiological relevanc. TRPC3 has been functionally implicated in synaptic transmission in murine cerebellar Purkinje neurons [60]. On the human side, TRPC3 dysregulation has been described in WS patient, where hypercalcemia has been associated with TRPC3 overexpression of TRPC3 in intestines and kidney tissues [61]. Taken together, one can speculate that haploinsufficiency of GTF2I, as seen in WS individuals causes an increase in surface expression of TRPC3, and, consequently, an increase in ACE in human Purkinje neurons. This hypothesis needs further investigation.

1.4 Uncovering the function of GTF2I in brain cells: the future path to understanding WS

Although multiple functions of GTF2I have been reported in several cellular models, the lack of human neurons and astrocytes has been a major obstacle hindering investigation of its cell lineage-specific roles, leaving mechanisms underlying WS etiology unclear. Recently, however, Adamo *et al.* investigated the GTF2I complexes in WS- and 7dup-derived iPSCs by mass spectrometry analysis and identified BEND4, a transcription factor highly expressed in brain and involved in neural processes, as one of direct targets of GTF2I [62]. As pluripotent stem cell technology, including iPSCs and embryonic stem cells (ESCs), together with established differentiation protocols has allowed researchers to generate not only 2D culture of desired brain cells, including neurons [63, 64], glia [65] and oligodendrocytes [66], but also 3D culture, i.e. mini brains or brain organoids [67, 68], both transcriptional targets and direct target proteins of GTF2I in, for example, cortical neurons, derived from iPSCs/ESCs can be identified. It also offers opportunities to determine whether candidate proteins, e.g. BEND4, described in non-neural cells actually interact with GTF2I in any brain cells. According to expression pattern of GTF2I in mouse, it is ubiquitously expressed in developing brain and becomes exclusively restricted to neurons with high levels of expression in cerebellar Purkinje neurons and hippocampal interneurons [69]. As iPSC-derived neurons in culture are considered temporally comparable to the ones in developing

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brain, we should be able to determine whether previously reported increase in calcium oscillation frequency observed in WS iPSC-derived cortical neurons [29] is caused by the increase in surface accumulation of TRPC3 as a result of reduction in GTF2I expression. Similar experiment should also be performed in human iPSC-derived Purkinje neurons as both GTF2I and TRPC3 expression was reported in mouse Purkinje neurons [69, 70]. Furthermore, single-gene deficient cellular model could be now established using genome-editing techniques such as CRISPR/Cas9 [71], facilitating the functional studies of specific gene of interest. By combining these 2 technologies, researchers could generate and characterize specific subtypes of neurons harboring one or three working alleles of *GTF2I* (Fig. 3). These studies would identify true targets of GTF2I in neural cells, reveal altered molecular mechanisms underlying WS etiology, and ultimately help establish the link between gene, brain, and behavior observed in WS individuals.

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Highlights

- Copy number variation of genes on 7q11.23 results in converse phenotypes
- Gene-phenotype correlation studies in atypical WS suggest role of GTF2I in WS
- GTF2I is a candidate for neurocognitive and behavioral profiles of WS
- GTF2I plays roles in both transcription and signal transduction
- GTF2I hemideletion is possibly responsible for calcium alteration in WS neurons

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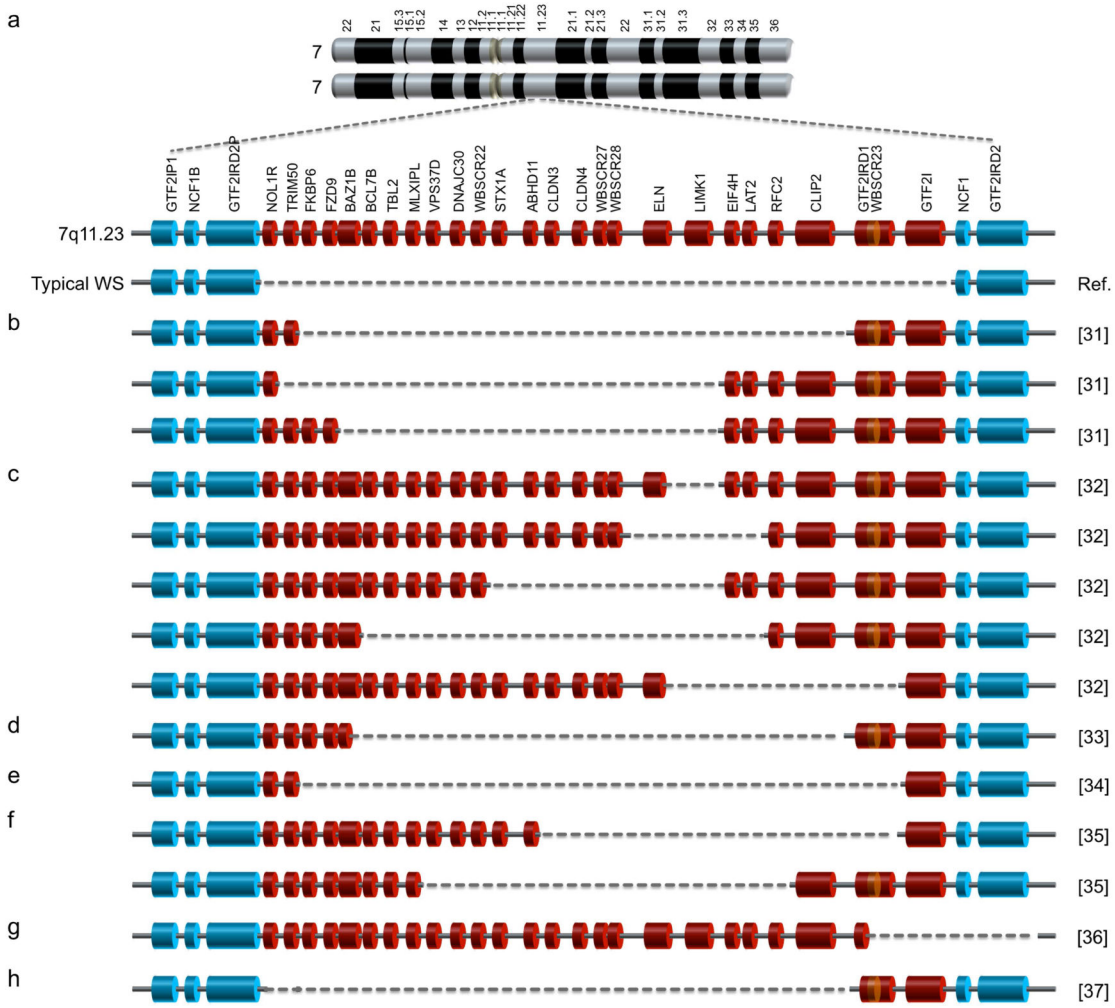

Figure 1. WS genetics and atypical WS cases in genotype–phenotype correlation studies

a, WS is caused by hemizygous deletion of multiple genes on chromosome band 7q11.23. A typical (classical) WS deletion encompasses every gene in red (*NOL1R* to *GTF2I*). **b**–**h**, Individuals with atypical partial WS deletions (**b**–**f, h**) [31–35, 37] and a subject with an extended distal WS deletion (**g**) [36] have been reported in genotype–phenotype correlation studies, suggesting a link between *GTF2I* and multiple WS phenotypes (see text for detail).

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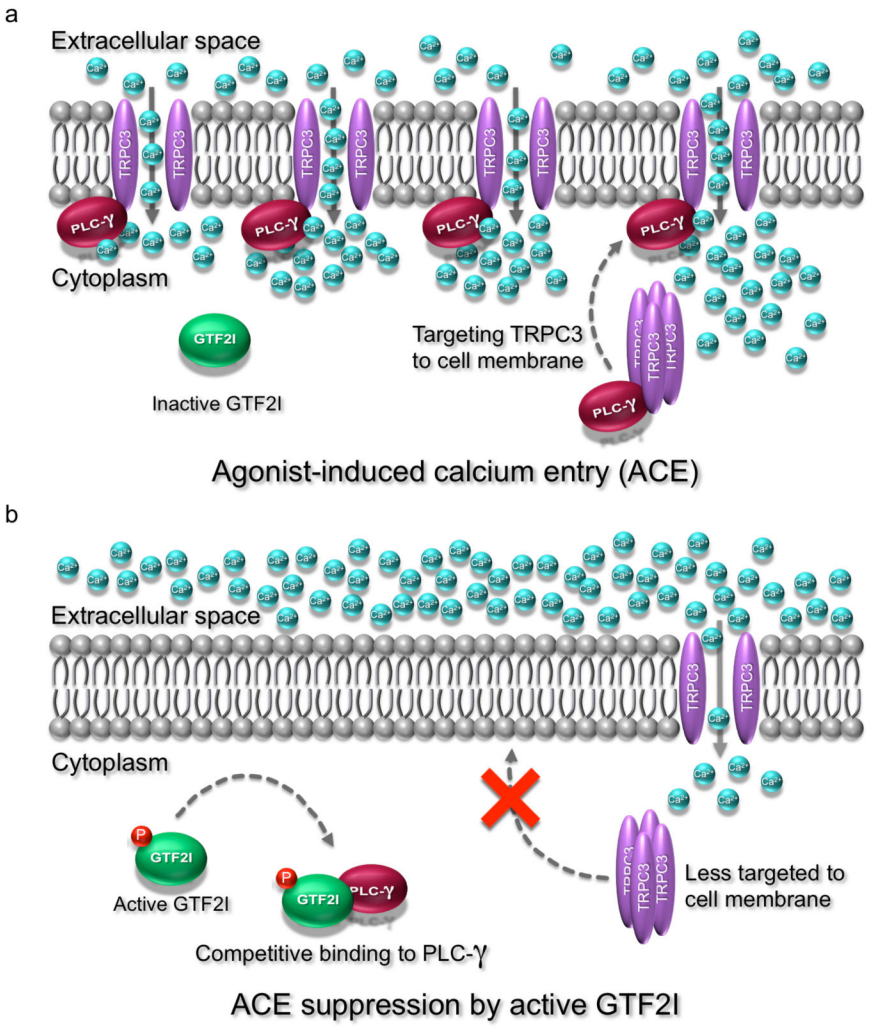

Figure 2. The potential role of GTF2I in agonist-induced calcium entry suppression in neurons

a, When GTF2I is inactive, activated PLC-γ binds to TRPC3, stimulating recruitment of more TRPC3 to the cell membrane. This results in an increase in calcium influx via agonist-induced calcium entry (ACE). **b,** When its tyrosine is phosphorylated, GTF2I becomes active and compete with TRPC3 for binding to activated PLC-γ, leading to a decrease in TRPC3 surface accumulation and, therefore, a reduction in ACE. GTF2I, general transcription factor 2I; TRPC3, transient receptor potential cation channel subfamily C member 3; PLC-γ, phospholipase C gamma; P, phosphorylation; Ca^{2+}, calcium ions.

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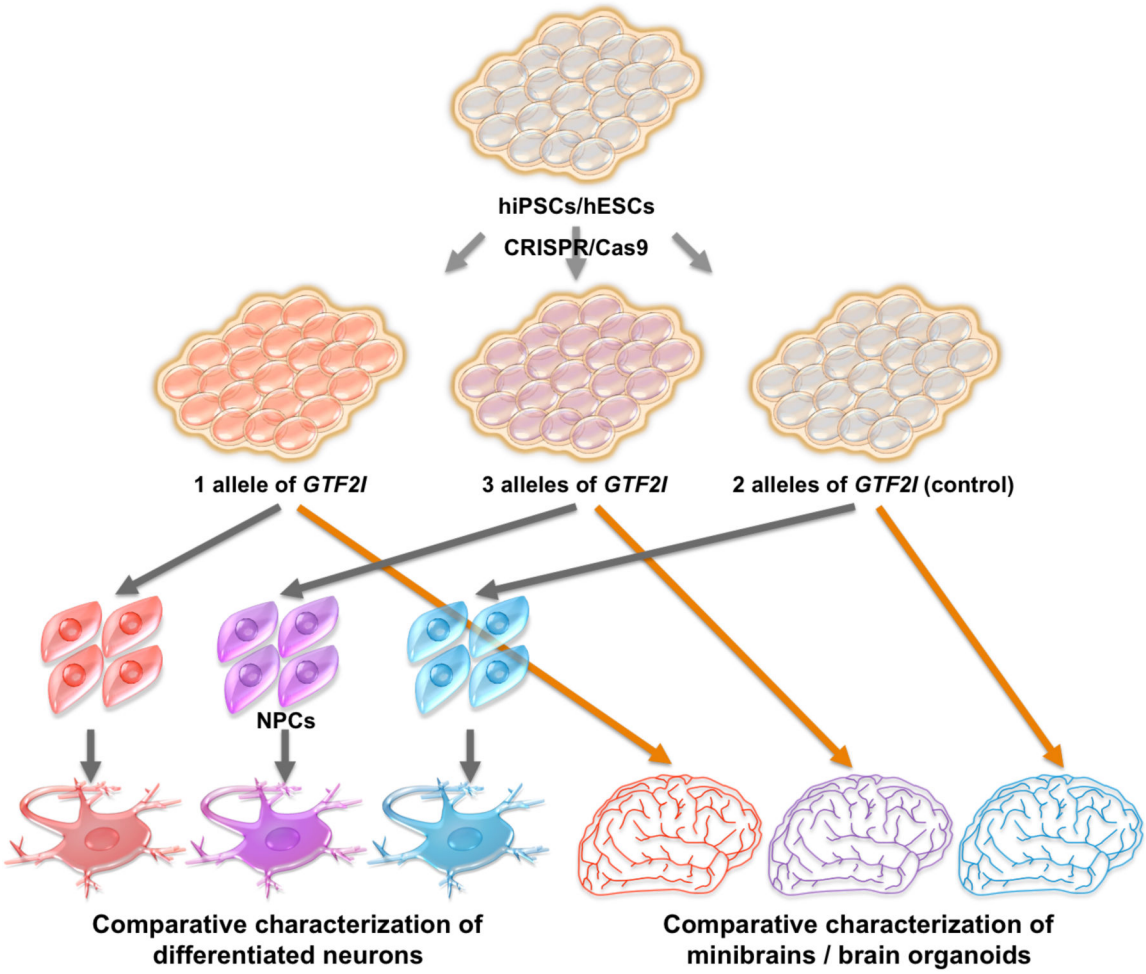

Figure 3. Pluripotent stem cell and CRISPR/Cas9 approaches in revealing the role of GTF2I in brain cells

In order to study the neural effects of *GTF2I* copy number, CRISPR/Cas9 genome-editing technique can be used to generate hiPSCs/hESCs with one (as observed in WS) and three (as in 7dup) *GTF2I* alleles. Generated cells can be differentiated into NPCs and neurons, for example, or minibrains (brain organoids). Comparative characterization of these cells can reveal cellular and molecular differences in neurons as a result of haploinsufficiency or duplication of *GTF2I*. hiPSC, human induced pluripotent stem cells; hESCs, human embryonic stem cells; NPCs, neural progenitor cells.

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Table 1

Roles of GTF2I in transcription and signal transduction through interaction with different binding partners

Binding partners	Inducing Signal	Action	Cell type	GTF2I target promoter /sequence	Processes	Ref.
BTK	BCR	Phosphorylates GTF2I	B cells	*c-fos*	B-cell development	[72, 73]
ITK	TCR	Phosphorylates GTF2I	T cells	*c-fos*	T-cell activation and function	[74]
CTCF	-	Form complex with GTF2I	WEHI-231	metabolic genes	Epigenetic processes	[75]
SMAD2	TGFβ/activ in signaling	Form complex with GTF2I	P19	*Gsc*	Development and patterning	[76]
ERK	Serum stimulation	Phosphorylates GTF2I	Mouse fibroblasts	*c-fos*	Cell growth	[77]
JAK2	Serum stimulation	Phosphorylates GTF2I	Mouse fibroblasts	*c-fos*	Cell growth and proliferation	[78]
c-Src	ER stress	Phosphorylates GTF2I	Mouse fibroblasts	*Grp78*	Cell survival and proliferation	[79, 80]
c-Src	α_2M	Phosphorylates GTF2I	1-LN	*Grp78*	Cell survival and proliferation	[81]
SRF,	Serum	Phosphorylates	HeLa cells	*c-fos*	Cell growth	[54]
Phox1	stimulation	GTF2I				
USF	Ras/MAPK	Phosphorylates GTF2I	T cells	HIV-1 LTR	Viral transcription	[82]
PLC-γ	RTK	Binds to phosphorylated GTF2I	PC12	N/A	Inhibition of agonist- induced calcium entry	[59]

Btk, Bruton's tyrosine kinase; BCR, B-cell receptor; ITK, inducible tyrosine kinase; TCR, T-cell receptor; CTCF, epigenetic regulatory protein; P19, mouse embryonic carcinoma cells; Gsc, Goosecoid; ERK, extracellular signal-regulated kinase; ER, endoplasmic reticulum; PC12, mouse neural cells; 1-LN, human prostate cancer cells; α_2M, alpha(2)-macroglobulin; SRF, serum response factor; USF, upstream stimulatory factor; MAPK, mitogen-activated protein kinase; RTK, receptor tyrosine kinase; N/A, not applicable