Chapter 6
Induced Pluripotent Stem Cells Reveal Common Neurodevelopmental Genome Deprograming in Schizophrenia

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Abstract Schizophrenia is a neurodevelopmental disorder characterized by complex aberrations in the structure, wiring, and chemistry of multiple neuronal systems. The abnormal developmental trajectory of the brain is established during gestation, long before clinical manifestation of the disease. Over 200 genes and even greater numbers of single nucleotide polymorphisms and copy number variations have been linked with schizophrenia. *How does altered function of such a variety of genes lead to schizophrenia?* We propose that the protein products of these altered genes converge on a common neurodevelopmental pathway responsible for the development of brain neural circuit and neurotransmitter systems. The results of a

We dedicate this chapter to Patrick W. Lee whose courage and comradery inspired our work.

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multichanneled investigation using induced pluripotent stem cell (iPSCs)- and embryonic stem cell (ESCs)-derived neuronal committed cells (NCCs) indicate an early (preneuronal) developmental-genomic etiology of schizophrenia and that the dysregulated developmental gene networks are common to genetically unrelated cases of schizophrenia. The results support a "watershed" mechanism in which mutations within diverse signaling pathways affect the common pan-ontogenic mechanism, integrative nuclear (n)FGFR1 signaling (INFS). Dysregulation of INFS in schizophrenia NCCs deconstructs coordinated gene networks and leads to formation of new networks by the dysregulated genes. This genome deprograming affects critical gene programs and pathways for neural development and functions. Studies show that the genomic deprograming reflect an altered nFGFR1—genome interactions and deregulation of miRNA genes by nFGFR1. In addition, changes in chromatin topology imposed by nFGFR1 may play a role in coordinate gene dysregulation in schizophrenia.

Abbreviations

3C	Chromatin conformation capture
CBP	CREB-binding protein
ChIPseq	Chromatin immunoprecipitation sequencing
CNVs	Copy number variations
ESCs	Embryonic stem cells
FGFR1(SP-/NLS)	Constitutively nuclear active variant of FGFR1
FGFR1(SP-/NLS)(TK-)	Dominant negative nuclear active variant of FGFR1
GO	Gene ontology
INFS	Integrative nuclear FGFR1 signaling
IPA	Ingenuity pathway analysis
iPSCs	Induced pluripotent stem cells
NCCs	Neuronal committed cells (integrative nuclear (n)FGFR1 signaling—INFS)
nFGFR1	Nuclear fibroblast growth factor receptor-1
RNAseq	Global RNA sequencing
SNPs	Single nucleotide polymorphisms
TAD	Chromatin topologically associated domains

6.1 Schizophrenia: A Disorder of Brain Development

Schizophrenia is one of the most debilitating mental illnesses worldwide (Hanzawa et al. 2013), with a lifetime prevalence of about 1.5–2% (Saha et al. 2005). There are currently no treatments that are completely effective or treat all the symptoms of schizophrenia (Blanchard et al. 2011; Rummel-Kluge et al. 2012). Schizophrenia is classified as a neurodevelopmental disorder, even though symptoms of the disease do not appear until puberty/young adulthood (Fatemi and Folsom 2009; Rehn and

Rees 2005). In males the peak onset of symptoms is between 10 and 25 years old, while in females it is between 25 and 35 years old (Rajji et al. 2009). A less frequent but particularly severe form of schizophrenia is accompanied by motor dysfunction that occurs during the prepubertal stage (Erlenmeyer-Kimling 2000). Another group, who do not show schizophrenia-like symptoms until after the age of 60, is defined as having a very late-onset schizophrenia-like psychosis (Howard et al. 2000; Keshavan 1999; Keshavan and Hogarty 1999). Together it is proposed that schizophrenia will occur by a two-hit model etiology in which early brain maldevelopment is followed by additional changes occurring adolescence (Keshavan 1999; Keshavan and Hogarty 1999). In this chapter, we will focus on changes thought to occur during the early brain development. Alterations in the schizophrenia brain are thought to occur during the first and early second trimester of development (Kneeland and Fatemi 2013) leading to improper clustering of neurons in layers II, III, and V of the cortex (Arnold et al. 1997), alteration in the number of nonpyramidal neurons in CA2, alteration in the shape of the hippocampus (Benes et al. 1998), hypoplastic development of dopamine neurons, and cerebellar atrophy (Akbarian et al. 1993; Bogerts et al. 1983; Connor et al. 2004; Schiller et al. 2006). These alterations in neuronal numbers and clustering are not due to neurodegeneration, as no neurodegenerative markers are observed in schizophrenia. In addition to neuronal alteration, changes in white matter structure have been observed (Davis et al. 2003), suggesting that even oligodendrocytes are effected. This widespread alteration of brain structure is thought to underlie the complexity of the clinical symptoms observed: positive symptoms (delusions and hallucinations), negative symptoms (affective flattening, amotivation, and anhedonia) (Blanchard et al. 2011; Foussias et al. 2011), and cognitive symptoms (disorganized speech and cognitive deficits) (DSM 4th edition). In addition, minor physical anomalies are associated with schizophrenia; these anomalies are consistent with abnormal development during the first trimester (Lloyd et al. 2008).

6.2 Schizophrenia: An Integrated Perspective on the Disease of Hundreds of Genes

While the symptoms of schizophrenia have been characterized well, the underlying causes have been difficult to pin down. Schizophrenia is a heritable familial disorder with a complex mode of inheritance and expression (Sullivan et al. 2003). Even in identical twins, the likelihood of both having schizophrenia is only up to 50%. This suggests that the disease could be a resultant of an interplay between genetic and environmental factors. Factors listed as acting during pregnancy which increases the frequency of the disease include infections (mother's immune attack hypothesis), episodes of hypoxia, and nicotinism. Possible environmental factors include being raised in a city, cannabis use during adolescence, certain infections, parental age, and poor nutrition during pregnancy.

While schizophrenia has been shown to be inheritable, its polygenetic nature and complexity make it difficult to dissect out the underlying genetic mechanisms. Several linkage studies have been carried out to better understand the schizophrenia genetics; however a lack of highly significant and consistently reproducible results has generally characterized those studies (Need et al. 2009). Next-generation sequencing technology has enabled researchers to look at the hundreds of thousands of single nucleotide polymorphisms (SNPs) simultaneously. However, similar to linkage studies, even though over 500 SNPs have been found to be significantly associated with schizophrenia, the overall results have been inconsistent (Welter et al. 2014). In addition to SNPs, copy number variations (CNVs) have also been associated with schizophrenia. A common theme is the enrichment of rare (<1% minor allele frequency) and large (>100 kb) CNVs (International Schizophrenia Consortium 2008; Malhotra et al. 2011; Walsh et al. 2008), and those that occur de novo (Kirov et al. 2012; Malhotra et al. 2011; Xu et al. 2008).

Each year brings reports of new candidate "schizophrenia genes" further complicating the picture of this polygenetic disease. Even though many genetic alterations have been associated with schizophrenia, no single alteration has been found to make up more then 1–2% of the schizophrenia population (International Schizophrenia Consortium 2008; Stefansson et al. 2008; Xu et al. 2008). Hence, the genetic causes of schizophrenia appear to be a multiplicity of rare risk alleles, and schizophrenia has been defined as a common, rare variant disease.

How do various mutations lead to a common disorder? One possible answer, proposed by Cannon and Keller, is the watershed hypothesis (Cannon and Keller 2006). According to this hypothesis, individual mutations dysregulate distinct biological pathways that in turn converge on a common ontogenic pathway(s) (Fig. 6.1). The common affected pathway should integrate signals from various pathways in which the individual schizophrenia gene mutations have been found and command the early stages of the brain development. The dysregulation of these common pathway would lead to brain malformations which increase the risk of the disease. However, the nature of such a central pathway and its organization has been yet unknown. This chapter will discuss evidence that the recently discovered integrative nuclear FGFR1 signaling (INFS) pathway could serve as candidate common pathway in schizophrenia.

6.3 IPSCs Model Cellular Developmental Abnormalities in Schizophrenia

One key proposition of the watershed hypothesis is that there is a common dysregulation of the developmental genome in schizophrenia. Identification of an early developmental gene dysregulation in adult brain tissue specimens may not be possible once the disease has progressed to a late-stage form. Additionally, tissue samples from schizophrenia patients in past studies have historically been limited to

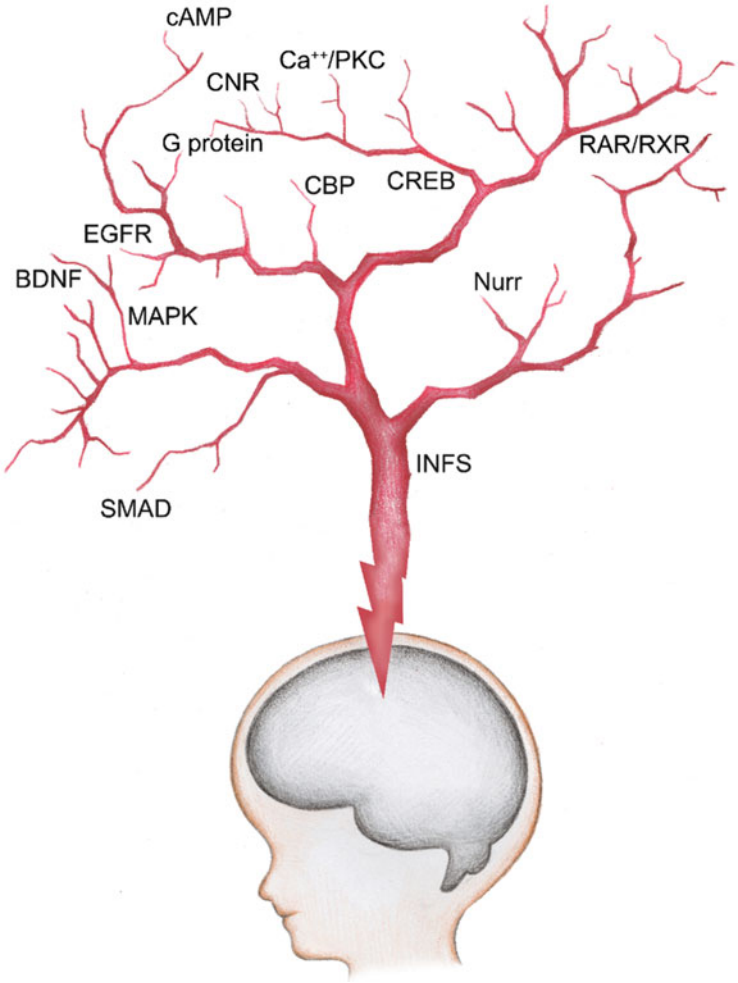

Fig. 6.1 Modified watershed hypothesis (Cannon and Keller 2006) of schizophrenia. In schizophrenia mutations are found in >200 genes of multiple signaling pathways which feed to a common pan-ontogenic mechanism the "Integrative Nuclear Fibroblast Growth Factor Receptor 1 Signaling" (INFS) pathway (for review see Stachowiak et al. 2011b, 2015; Stachowiak and Stachowiak 2016). In INFS, FGFR1 and its ligand, FGF-2, translocate into the nuclear interior, and through direct interaction with transcription gating factor CREB binding protein (CBP), nuclear (n) form of receptor, (n)FGFR1, directly controls the activation/inhibition of thousands of genes and epigenetic changes integral in ontogeny and brain development. INFS links downstream developmental gene programs to multiple upstream pathways such as cAMP, PKC, neurotrophins and MAPK, diverse growth factors, and nuclear retinoid and orphan Nur receptor-mediated pathways. Figure drawn by Sun Young Kang

postmortem individuals, whose samples were complicated by various factors such as substance abuse, drug treatment, postmortem interval, abnormal brain pH influenced by hypoxia, and nutritional deficiency (Deep-Soboslay et al. 2011). In recent years human-induced pluripotent stem cells (iPSCs) have emerged as new potential tools in testing the watershed hypothesis. In 2011, two laboratories reported successful development of iPSCs from schizophrenic patients (Brennand et al. 2011; Chiang et al. 2011). Brennand et al. had developed iPSCs from four patients diagnosed with schizophrenia or its schizoaffective variant and four from control non-schizophrenic subjects (Brennand et al. 2011). Schizophrenia hiPSC-derived NPCs have aberrant migration (Brennand et al. 2014b) and cellular polarity (Yoon et al. 2014), perturbed WNT signaling (Srikanth et al. 2015; Topol et al. 2015), increased oxidative stress (Brennand et al. 2014b; Paulsen et al. 2011; Robicsek et al. 2013), and altered responses to environmental stressors (Hashimoto-Torii et al. 2014), while schizophrenia hiPSC-derived neurons exhibit decreased neurite number (Brennand et al. 2011), reduced synaptic maturation (Brennand et al. 2011; Robicsek et al. 2013; Wen et al. 2014; Yu et al. 2014) and synaptic activity (Wen et al. 2014; Yu et al. 2014), and blunted activity-dependent response (Roussos et al. 2016).

6.4 Schizophrenia Patients' NCCs Share a Common Coordinate Pattern of Gene Dysregulation

The recent investigation into iPSC's neural progeny tested the watershed hypothesis of schizophrenia by examining whether patients with diverse genetic backgrounds and schizophrenia-linked copy number variants may show also common dysregulations of the genome. Such a possibility was suggested by broad transcription analysis using a microchip analysis method on mature neurons differentiated from different iPSC lines. A common set of 596 dysregulated genes in 4 patients was found (Brennand et al. 2011). Many of the changes in gene expression observed in mature neurons could reflect differences in the types of neurons that were generated from the patient and control IPSCs (Brennand et al. 2011, 2014a, 2015; Brennand and Gage 2011). Thus, to identify the genomic mechanism that leads to altered neuronal and brain development and therefore underlies the etiology of schizophrenia, we have focused on studying early neural development, i.e., the transition from iPSC-differentiated neural progenitor cells (NPCs) to neuron-committed cells (NCCs) induced by 2-day treatment with BDNF, GDNF, and cAMP.

Global RNA sequencing including small RNA has revealed a common set of 1349 dysregulated genes (FC $> \pm 1.5$ and q value > 0.05) in all 4 patients with diverse genetic backgrounds and different schizophrenia-linked copy number variants (Narla et al. 2017) (Fig. 6.2).

To determine whether this dysregulation of 1349 genes in schizophrenia NCCs represented a random or a correlated event, a pairwise correlation network analysis of all dysregulated genes was carried out (Fig. 6.3a1), for 909,226 potential

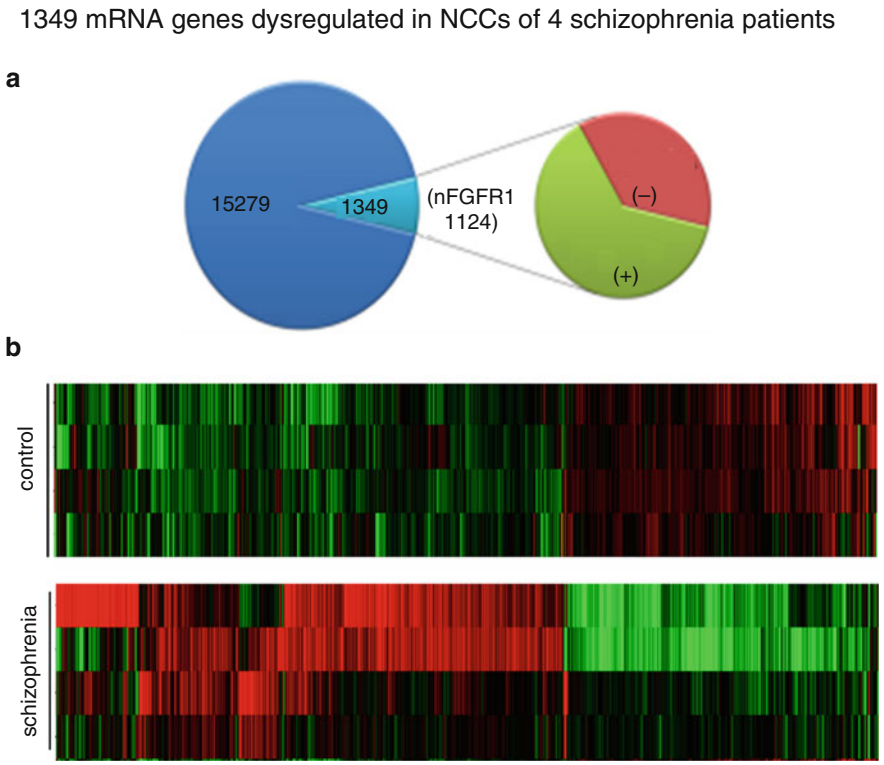

Fig. 6.2 RNAseq of control and schizophrenia neuronal committed cells (NCCs) derived from iPSCs of four schizophrenia patients and four control individuals (Narla et al. 2017). (**a**) Distribution of gene expression across eight samples: four control and four schizophrenia NCC lines. 15,279 expressed genes (mRNAs) were detected in all 8 samples, of which 1349 genes were dysregulated in all 4 schizophrenia NCC samples (FC $> \pm 1.5$ and q value >0.05). Among these the majority of genes, 63%, were upregulated. Nearly 84% (1124) of the dysregulated genes were targeted by nFGFR1 (ChIPseq analysis) (Narla et al. 2017). (**b**) Heatmap of 1349 genes that were dysregulated in all 4 schizophrenia NCC samples (FC $> \pm 1.5$ and q value > 0.05). Raw expression data were log transformed and then centered to the median of all eight samples. Red indicates higher value than median; green indicates lower value than median. This figure is based on the results from Narla et al. (2017)

relationships. Compared to control cells ($n = 4$), in which the distribution of correlations was flat (likelihood of genes having high or low correlation was similar), in patients' cells the numbers of positive correlations (genes changing in the same direction) and negative correlations (genes changing in opposite direction) were markedly increased.

The analysis of the highly interconnected nodes (genes which are highly correlated with a greater number of other genes) revealed that the networks formed by genes that were highly correlated in control cells were no longer found in the schizophrenia cells (Fig. 6.3b1) and that a new network of the highly correlated genes formed in schizophrenia cells (Fig. 6.3b2). Thus the control networks became

disrupted in the patient NCCs, and a new network of connected genes formed in their place. The ontological gene categories represented by the control network, disrupted in schizophrenia, included genes involved with extracellular matrix, synapse formation, neuronal projection, and nervous system development. This network change suggests an enhanced push toward a neuronal phenotype in patient samples compared to controls. The control networks included both up- and downregulated genes. In the schizophrenia networks, however, the up- and downregulated genes distinctly segregated into separate networks. These findings indicated further the concerted gene dysregulation by singular factors.

To further characterize the observed gene dysregulation, we performed separate gene ontology (GO) analyses of all genes that were upregulated and all genes that were downregulated in schizophrenia NCCs (Fig. 6.1a). Genes involved in glial differentiation and axon ensheathment were present only in the downregulated category, while neuronal ontologies such as axonogenesis, neurotransmitter transport, and learning were overrepresented in the upregulated group (Narla et al. 2017). Importantly, genes involved in positive regulation of cell proliferation (GO:0008284), positive regulation of cell migration (GO:0030335), positive regulation of cell motility (GO:2000147), positive regulation of cell morphogenesis involved in differentiation (GO:0010770), and positive regulation of neuron differentiation (GO:0045666) were all in the upregulated category. These findings have established a genomic mechanism for the increased NPC proliferation, migration, and premature neuronal differentiation found recently in schizophrenia iPSC brain organoids (Stachowiak et al. 2017).

6.5 Dysregulation of miRNA and mRNA Interactive Networks

One category of factors that could elicit a concerted dysregulation of transcriptome in schizophrenia is miRNAs, which are known to influence overlapping gene sets in a coordinated fashion. miRNAs influence mRNA levels by promoting mRNA degradation, inhibiting mRNA translation, and acting at the transcription level (Bartel 2009; Younger and Corey 2011). The NCCs from 3 schizophrenia patients examined displayed a concerted dysregulation of 16 miRNAs, all of which were overexpressed, albeit to different extents. Within this group, mir-132 (Miller et al. 2012), mir-134 (Moreau et al. 2011; Santarelli et al. 2011), mir-218 (Perkins et al. 2007), and mir-17 (Shi et al. 2012) have previously been implicated in schizophrenia (Miller et al. 2012; Santarelli et al. 2011). TargetScan and MirTarBase analyses predicted that the overexpressed miRNAs may interact with >400 dysregulated mRNAs, in a largely overlapping manner as illustrated on Fig. 6.3c. In control NCCs these 16 miRNAs displayed a high degree of positive correlation consistent with the model in which different miRNAs controlled shared mRNA targets. In schizophrenia NCCs, all 16 miRNAs were upregulated, but to different degrees (1.5-

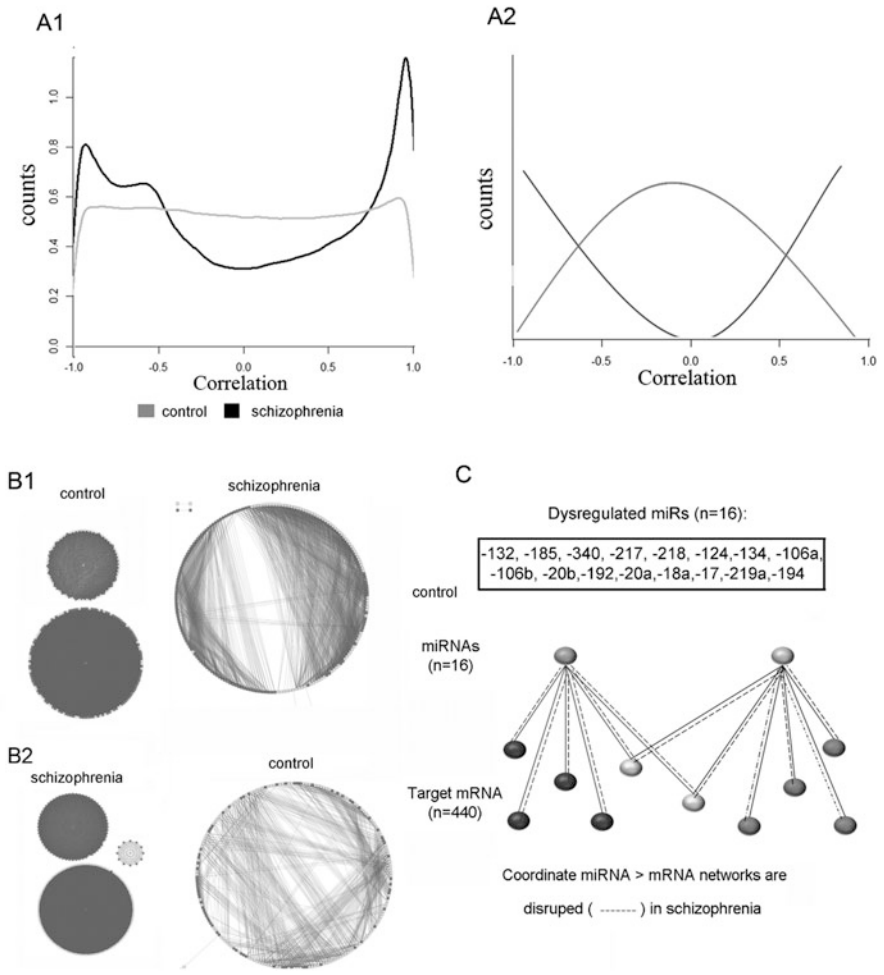

Fig. 6.3 Correlate gene networks in control NCCs are disrupted and replaced by new networks in schizophrenia [based on the results from Narla et al. (2017)]. (**a**) Analysis pairwise correlations among schizophrenia dysregulated 1349 mRNA genes. (**a1**) Correlation was performed using four control and four patient NCC samples. A flat distribution of correlation is observed in controls, while in patients an increase in the number of positively and negatively correlated genes was observed. (**a2**) Predicted forms of gene dysregulation: random-dyscoordinate (convex) and nonrandom-coordinate (concave). The dysregulation in schizophrenia (**a1**) follows the nonrandom-coordinate model. (**b**) Top 200 nodes (genes whose expression is highly positively correlated with that of multiple other genes) in control and in patient NCCs were identified (marked on the perimeters). (**b1**) Gray lines link pairs of genes whose correlation is >0.9. (**b1**) In the control set, two separate networks were observed, and each contained both upregulated and downregulated genes. These correlations were disrupted in schizophrenia. (**b2**) In the patient set, the upregulated and downregulated genes formed three separate networks which did not exist in control NCCs. (**c**) Dysregulation of miRNA in schizophrenia NCCs. NCCs from three control subjects and three patients were analyzed. In all 3 patients 16 miRNAs were dysregulated (all upregulated). Those dysregulated miRNAs target 440 mRNAs. The observed miRNA–mRNA correlations in control cells were eliminated in schizophrenia cells (indicated by interrupted lines) indicating a disassociation of the miRNA > mRNA networks (Narla et al. 2017). Results and (**b**) are from Narla et al. (2017)

to >70-fold), and the correlations among those 16 miRNAs found in control networks were lost. Analysis of the combined networks of miRNAs and mRNAs together demonstrated the loss of cooperation between the miRNAs and their target mRNAs in schizophrenia NCCs (Fig. 6.3c). This suggests that the normally tight miRNA to mRNA coordination is being overridden by a separate, possibly global mechanism, which is resulting in alterations in control of mRNA genes, and causing disruptions of correlated expression levels of normally interdependent miRNA.

One candidate pathway that could be involved is the pan-ontogenic integrative nuclear FGFR1 signaling (INFS) (Stachowiak et al. 2007, 2011b, 2015), which integrates signals from diverse pathways in which the schizophrenia-linked mutations have been found and which controls genes involved in neural development. A disruption of FGFR1 function in dopaminergic neurons of transgenic mice led to developmental brain malformation and behavioral changes that mimic the positive, negative, and cognitive deficits observed in humans (Stachowiak et al. 2013).

6.6 Pan-Ontogenic INFS in Brain Development

At the center of the INFS module are proteins that bear the historic name of fibroblast growth factors (FGF) and the high-affinity FGF receptors (FGFR). Neither FGFs nor FGFRs exist in single-cell organisms but are common to eumetazoans and are essential for the generation of tissues with specialized cells (Stachowiak et al. 2011b). Mutations of the single FGFR1 gene disrupt gastrulation and development of the central and peripheral nervous systems, mesodermal somites, muscles and bones, and the endoderm. These effects are accompanied by changes in the expression of genes (Ciruna and Rossant 2001; Ciruna et al. 1997; Dequeant and Pourquie 2008; Partanen et al. 1998) and microRNAs (Bobbs et al. 2012; Stuhlmiller and Garcia-Castro 2012) that control development. These findings firmly placed FGFR1 at the top of the developmental hierarchy; however, how could a single gene perform such a global ontogenic function was unknown.

FGFs emerged during early metazoan evolution equipped with nuclear localization signals (NLS), and their biological effects depend on nuclear accumulation (Popovici et al. 2006). In addition, NLS-lacking FGFs have evolved which act as extracellular secreted proteins. In the mammalian FGF family, NLS-containing FGFs act in the nucleus to promote cell speciation, whereas secreted FGFs act on the cell surface receptors as mitogens (Claus et al. 2003; Sherman et al. 1993; Stachowiak et al. 2007, 2011a). Individual FGF receptors (in mammals, FGFR1-4) likewise have adaptations directing them to different cellular compartments (Myers et al. 2003).

There are two separate pathways which have been characterized for FGFR1 processing. The newly synthesized FGFR1 can enter the constitutive membrane pathway (MP) in which receptor is processed and glycosylated in Golgi and accumulates in the plasma membrane. In the nuclear pathway, an atypical transmembrane domain in FGFR1 allows newly translated immobile receptor to be released from the

pre-Golgi into the cytosol in a process that engages proteasome, FGF-2 ligand, and ribosomal S6 kinase activities. Nuclear transport of FGFR1 is mediated by importin-β (Stachowiak et al. 2007) and stimulated by a variety of developmental signals, including EGF, NGF, BDNF, BMP, retinoids, hormones and neurotransmitters, calcium, cyclic AMP, and PKC, and is inhibited by cell contact receptors. This is the reason that this pathway has been referred to as an integrative signaling (Stachowiak et al. 2007, 2011b).

The INFS mechanism is involved primarily in developmental transitions, most commonly the switches to differentiation and postmitotic development (Stachowiak et al. 2007, 2011b). In proliferating neural stem/progenitor cells (NS/PC) of the brain ventricles, FGFR1 is present in the cytoplasm, while in differentiating brain cortical cells or midbrain dopamine neurons, FGFR1 is located within the cell nucleus (Fang et al. 2005; Stachowiak et al. 2009a, b). As this development is completed, FGFR1 localization becomes again predominantly cytoplasmic. Nuclear accumulation of nFGFR1 occurs during differentiation of diverse stem cells and growth and differentiation of glial, neuronal, endothelial, and mesodermal cells as well as cancer cells.

In loss- and gain-of-function experiments, nFGFR1 was found to be essential for the control of the pluripotent state, necessary for neuronal programing by retinoic acid (RA), NGF, BDNF, or cAMP, and was sufficient to induce neuronal differentiation in the absence of additional stimulation (Lee et al. 2012). How can a single nuclear protein program the development of ESCs—a process that involves the coordinated regulation of thousands of genes that are located on different chromosomes and contain diverse regulatory elements?

nFGFR1, which lacks a DNA-binding domain, engages indirectly in gene regulation by binding to the domain of the CREB-binding protein (CBP). CBP is a common transcription co-regulator and histone-acetylating protein that interacts with multiple transcription factors (Fang et al. 2005; Hu et al. 2004; Kasper et al. 2006; Vo and Goodman 2001). The interaction with CBP allows FGFR1 to target a wide variety of genes. Next-generation sequencing has delineated global and direct gene programing by nFGFR1 and its partner CBP, which guide pluripotent embryonic stem cells (ESCs) toward development into multipotent neural progenitor cells (NPCs) and toward further differentiation (Stachowiak and Stachowiak 2016; Terranova et al. 2015). nFGFR1 cooperates with a multitude of transcription factors (TFs), including RXR, RAR, and orphan nuclear receptors, and targets thousands of genes (both mRNA and miRNA) across the entire genome in a nonrandom manner. Additionally nFGFR1's binding on the genome is increased during the transition into neuronal lineage underscoring its importance for neuronal development (Stachowiak and Stachowiak 2016; Terranova et al. 2015). nFGFR1 binds genes involved in pluripotency leading to their inactivation during the transition into neuronal stem cells (Terranova et al. 2015). In addition nFGFR1 binds to and activates Hox genes, which regulate spatial development of organs and tissues (Stachowiak and Stachowiak 2016; Terranova et al. 2015).

In addition to these genes, nFGFR1 has been found to both regulate the expression of a multitude of transcription factors and work with said transcription factors in order to control expression of various genes. Due to the global role nFGFR1 plays

during development, INFS is a strong candidate for one of the downstream pathways theorized by Cannon and Keller's watershed hypothesis.

6.7 FGFR1 in Dysregulation of Schizophrenia Dysregulation of NCC Transcriptome

nFGFR1 is bound in a nonrandom fashion across all chromosomes in both control and schizophrenia NCC genomes. nFGFR1 binding across each chromosome was related to gene distribution. nFGFR1 binding was highly enriched in 5' UTR regions (>fivefold) and in promoters. Both control and schizophrenia NCCs and nFGFR1 almost exclusively associated with the promoters that were actively expressed. FGFR1 was found to be bound to promoters of >90% of 1378 genes dysregulated in schizophrenia but only to 55% of all genes (Fig. 6.4b). The majority of dysregulated genes had promoters targeted by nFGFR1, and the number targeted was higher in schizophrenia than in control NCCs. In addition, nFGFR1 was bound to more locations in schizophrenia compared to controls, and a large portion of the new binding sites were found in introns and distal intergenic regions. Potentially, such distal binding could be related to regulation of the 3D chromatin structure.

MACS2 analysis of the nFGFR1 binding score (the score reflects the abundance of nFGFR1 at a particular genomic locus in a cell population) showed that out of the 915 genes that bound nFGFR1 in both control and schizophrenia cells, the majority, 828 genes, showed stronger nFGFR1 binding in patient cells. Due to the broad genome binding alterations of nFGFR1 signaling in schizophrenia cell lines, nFGFR1 could be one possible factor in causing changes that are observed in schizophrenia.

Categories of dysregulated genes targeted by nFGFR1 were identified using gene ontology (GO), ingenuity pathway analysis (IPA), and reactome. As found for all 1349 dysregulated genes, the nFGFR1-targeted dysregulated gene (90%) NCCs overrepresented the pathways involved in axon guidance, neurotransmitter release, and glial cell differentiation (Table 6.1).

Many neuronal GO categories were overrepresented (Table 6.1) including genes involved in neural crest development, synaptic plasticity, learning, memory, and synapse organization. Several GO groups related to glial development were also overrepresented, including those involved in the processes of myelination, axon ensheathment, glial cell differentiation, and oligodendrocyte differentiation.

Transfection of the recombinant, constitutively nuclear variant of FGFR1 [FGFR1(SP-/NLS)], in which the cleavable SP is replaced with the NLS of FGF-2, and of dominant-negative variant FGFR1(SP-/NLS)(TK-), which lacks the tyrosine kinase (TK) domain, showed that nFGFR1 is sufficient and necessary for neuronal differentiation, both in the mouse brain (Bharali et al. 2005; Stachowiak et al. 2009a, b) and in cultured ESC or NPC cells treated with RA, NGF, BDNF, BMP, or cAMP (Fang et al. 2005; Horbinski et al. 2002; Lee et al. 2013; Stachowiak

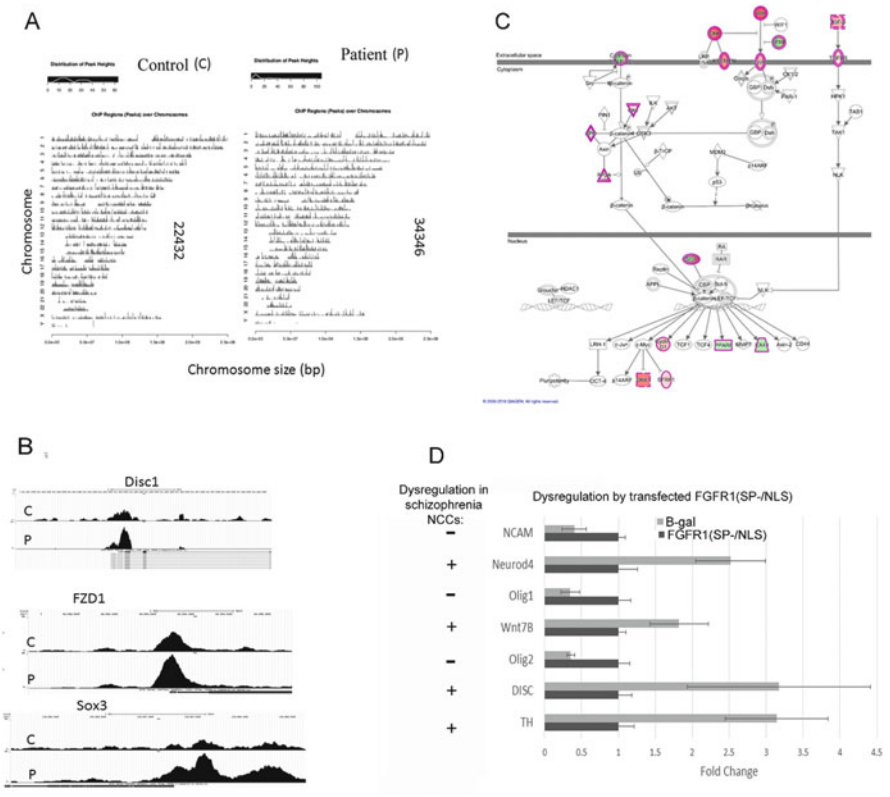

Fig. 6.4 (**a**) ChIPseq distribution of nFGFR1 peaks throughout the genomes of NCCs from control and schizophrenia iPSCs [based on the results from Narla et al. (2017)]. nFGFR1 binding sites (peaks) were enriched in the promoters but not in the intergenic regions. In patients, an increased nFGFR1 binding was observed in gene promoters, distal promoters ,and distal intergenic regions. (**b**) UCSC genome browser views of nFGFR1 binding for Disc1, FZD1, and Sox3 genes. Tag distribution of nFGFR1—increased binding is observed in schizophrenia compared to control NCCs. (**c**) WNT signaling is dysregulated in schizophrenia patients. IPA pathway for Wnt signaling—pink outline represents genes that are dysregulated in schizophrenia. Green fill represents genes that are downregulated, and red fill represents genes that are upregulated. WNT, cadherins, Frizzled, and Sox3 are some of the genes dysregulated in this pathway.(**d**) Examples of nFGFR1-targeted genes (TH, Wnt7B, Neurod4, Olig2, Olig1, and NCAM) that were upregulated (+) or downregulated (−) in schizophrenia NCCs. Consistently, in control human NCCs, these genes were up- or downregulated by transfected constitutive active nuclear FGFR1(NLS/SP-). These findings are consistent with the model in which increased nFGFR1 gene targeting in schizophrenia leads to gene up- or downdysregulation. This modified figure is based on the results from Narla et al. (2017)

et al. 2003). In addition, studies have demonstrated that both full-length and truncated forms of FGFR1 accumulate in cancer cells and thereby promote metastasis (Chioni and Grose 2012; Coleman et al. 2014; Nguyen et al. 2013).

Reactome and IPA showed that the gene dysregulation in schizophrenia was centered on pathways controlling development of neuronal systems, neural genes,

Table 6.1 Selected gene otology (GO) terms for schizophrenia-dysregulated genes targeted by nFGFR1

GO term	Total genes	Genes dysregulated
Positive regulation of axon extension (GO:0045773)	36	10
Central nervous system neuron development (GO:0021954)	68	14
Positive regulation of axonogenesis (GO:0050772)	69	14
Regulation of cyclin-dependent protein kinase activity (GO:1904029)	99	18
Ensheathment of neurons (GO:0007272)	92	16
Axon ensheathment (GO:0008366)	92	16
Neurotransmitter secretion (GO:0007269)	101	17
Presynaptic process involved in synaptic transmission (GO:0099531)	105	17
Neurotransmitter transport (GO:0006836)	140	22
Glial cell differentiation (GO:0010001)	138	21
Cell cycle arrest (GO:0007050)	156	23
Eye morphogenesis (GO:0048592)	150	22
Regulation of synaptic plasticity (GO:0048167)	139	20
Regulation of synapse structure or activity (GO:0050803)	230	32
Extracellular matrix organization (GO:0030198)	374	52
Regulation of neurotransmitter levels (GO:0001505)	176	24
Negative regulation of nervous system development (GO:0051961)	265	36
Negative regulation of neurogenesis (GO:0050768)	243	33
Regulation of neuron differentiation (GO:0045664)	555	75
Telencephalon development (GO:0021537)	230	30
Regulation of neuron projection development (GO:0010975)	406	52
Positive regulation of neuron differentiation (GO:0045666)	306	39
Negative regulation of neuron differentiation (GO:0045665)	190	24
Positive regulation of neuron projection development (GO:0010976)	230	29
Regulation of neurogenesis (GO:0050767)	669	84
Axon guidance (GO:0007411)	567	71
Regulation of nervous system development (GO:0051960)	755	90
Axonogenesis (GO:0007409)	672	80
Neuron development (GO:0048666)	1024	115
Generation of neurons (GO:0048699)	1629	182
Regulation of MAPK cascade (GO:0043408)	783	74
Regulation of cell motility (GO:2000145)	715	67

extracellular organization, as well as other developmental genes. Examples of the affected pathways included pluripotency regulation, Notch signaling, Wnt/β-catenin signaling, PI3K/AKT signaling, eNOS signaling, VEGF signaling, and L1cam signaling, which play a role in axonal growth, axonal guidance pathway, glutamate

receptor signaling, CREB signaling in neurons, various extracellular matrix pathways, and transcriptional regulation by TP-53 (Table 6.2). Pathways related to the release of dopamine, serotonin, norepinephrine, and glutamate neurotransmitters were also affected. Example of gene activity changes in Wnt pathway in schizophrenia is shown on Fig. 6.4c.

Together, these analyses revealed that the dysregulation of gene expression in NCCs derived from patients with schizophrenia was centered on neuronal genes as well as other developmental genes. The upregulated genes were found to be involved in (TP53-dependent) transcription of cell cycle genes, neuronal development, axon guidance, and cholesterol biosynthesis, whereas downregulated genes were involved in cell junction organization, cell–cell junctions, neurotransmitter receptor binding, and cell-cell communication including glutamate receptor signaling, CREB signaling in neurons, and dopamine degradation (Table 6.2).

Thus as predicted by the watershed hypothesis, one observes a common dysregulation of the fundamental developmental functions occurring already at a preneuronal stage.

The second key point of the watershed hypothesis is that dysregulation observed in schizophrenia would be found around specific pathways. Analysis of the 1349 dysregulated genes revealed many neuronal gene ontologies such as neural crest development, regulation of synaptic plasticity, learning, memory, and synapse organization overrepresentation. In addition to these neuronal functions, ontological groups related to glial function such as myelination, axon ensheathment, regulation of glial cell differentiation, and oligodendrocyte differentiation were also found overrepresented. In addition many of the dysregulated genes are involved in pathways centered around neurotransmitter release, developmental biology, axonal growth, and Notch signaling among others. These results suggest that dysregulation of genes observed in schizophrenia represents targeted dysregulation of pathways rather than a random set of genes.

Overexpression studies on select genes have shown that nFGFR1 can cause schizophrenia-like changes in genes such as TH, DISC, Olig2, Wnt7B, and others (Fig. 6.4d). Taken together these results suggest that nFGFR1 plays a strong role in schizophrenia dysregulation. Recent global studies verify that genes affected by overexpressing nFGFR1 in NCC derived from hESC include the same categories and pathways found to be dysregulated in schizophrenia cells (Fig. 6.4d).

6.8 Role of DNA Topology in Schizophrenia

How can expression of hundreds or thousands of genes, which during development are expressed in a coordinated manner, become simultaneously disrupted? How can complex transcriptional gene networks be replaced by new vastly different gene expression profiles? We have hypothesized that a disruption on the level of chromatin structure may be occurring in schizophrenia during the brain development, and as a result vast multigene programs are becoming dysregulated.

Table 6.2 Ingenuity pathway analysis (IPA) of schizophrenia-dysregulated genes targeted by nFGFR1

Ingenuity canonical pathways	Downregulated	Upregulated
Axonal guidance signaling	10/434 (2%)	41/434 (9%)
p53 signaling	3/98 (3%)	14/98 (14%)
ERK/MAPK signaling	6/187 (3%)	16/187 (9%)
Integrin signaling	8/207 (4%)	15/207 (7%)
Wnt/β-catenin signaling	6/169 (4%)	14/169 (8%)
Cyclins and cell cycle regulation	3/78 (4%)	9/78 (12%)
STAT3 pathway	4/73 (5%)	7/73 (10%)
PDGF signaling	3/77 (4%)	8/77 (10%)
Ephrin receptor signaling	4/174 (2%)	14/174 (8%)
PI3K/AKT signaling	4/123 (3%)	10/123 (8%)
NANOG in embryonic stem cell pluripotency	3/111 (3%)	10/111 (9%)
Actin cytoskeleton signaling	6/216 (3%)	14/216 (6%)
Gap junction signaling	5/155 (3%)	11/155 (7%)
Notch signaling	4/38 (11%)	3/38 (8%)
IGF-1 signaling	1/97 (1%)	10/97 (10%)
Synaptic long-term depression	7/142 (5%)	7/142 (5%)
Dopamine-DARPP32 feedback in cAMP signaling	8/161 (5%)	7/161 (4%)
Protein kinase A signaling	11/386 (3%)	17/386 (4%)
CREB signaling in neurons	9/171 (5%)	6/171 (4%)
Semaphorin signaling in neurons	0/53 (0%)	7/53 (13%)
Neurotrophin/TRK signaling	3/67 (4%)	5/67 (7%)
GDNF family ligand–receptor interactions	3/68 (4%)	5/68 (7%)
TR/RXR activation	1/85 (1%)	8/85 (9%)
ErbB2–ErbB3 signaling	1/57 (2%)	6/57 (11%)
Glutamate receptor signaling	4/57 (7%)	3/57 (5%)
Insulin receptor signaling	3/132 (2%)	9/132 (7%)
JAK/Stat signaling	1/72 (1%)	7/72 (10%)
Synaptic long-term potentiation	7/119 (6%)	4/119 (3%)
HGF signaling	3/105 (3%)	7/105 (7%)
Wnt/Ca+ pathway	2/56 (4%)	4/56 (7%)
EGF signaling	4/56 (7%)	2/56 (4%)
Prolactin signaling	3/73 (4%)	4/73 (5%)
Oct4 in mammalian embryonic stem cell pluripotency	1/46 (2%)	4/46 (9%)
G protein-coupled receptor signaling	7/256 (3%)	10/256 (4%)
NF-κB signaling	5/172 (3%)	7/172 (4%)
RhoA signaling	3/122 (2%)	6/122 (5%)
Neuregulin signaling	3/88 (3%)	4/88 (5%)
Glucocorticoid receptor signaling	2/275 (1%)	14/275 (5%)
Calcium signaling	4/178 (2%)	7/178 (4%)
BMP signaling pathway	0/76 (0%)	5/76 (7%)
cAMP-mediated signaling	4/219 (2%)	8/219 (4%)
Retinoic acid-mediated apoptosis signaling	2/61 (3%)	2/61 (3%)

(continued)

Table 6.2 (continued)

Ingenuity canonical pathways	Downregulated	Upregulated
Sonic hedgehog signaling	0/30 (0%)	2/30 (7%)
Telomere extension by telomerase	0/15 (0%)	1/15 (7%)
nNOS signaling in neurons	2/47 (4%)	0/47 (0%)

Selected IPA pathways and numbers of dysregulated genes relative to all genes in pathway are shown

Throughout cellular development, specific subsets of genes become active and can be found in de-condensed chromatin structures known as euchromatin, while transcriptionally inactive regions are tightly packed into complexes known as heterochromatin (Francastel et al. 2000). Temporal and positional DNA–protein interactions lead to the formation of chromatin topologically associated domains (TADs) within which coordinated regulation and expression of multiple loci take place. TADs contain looped together fragments of the same or different chromosomes, spanning distances that can be greater than 1 Mb.

Changes to chromatin structure occur as the cell transitions from one stage of development to another, such as is observed in ESCs differentiating into NCCs (Meshorer and Misteli 2006). Histone modifications, architectural proteins, and transcription factors together determine gene expression patterns and supervise delineation between active and repressed gene loci. Recent ChIP-seq studies in our laboratory have shown nFGFR1 to bind to genomic sites on every chromosome in both human and mouse ESCs. FGFR1-binding sites are significantly remodeled during retinoic acid (RA)-induced ESC stimulation toward neuronal cell differentiation. Gene ontology (GO) analysis revealed genes targeted by nFGFR1 are primarily involved in the maintenance and development of the stem cells in ESCs, while in RA-induced NCCs, nFGFR1 binds to promoters of genes engaged in the formation of mRNA transcripts and in the development of the nervous system. The nFGFR1 regulation of those genes and of neuronal differentiation was demonstrated by transfecting nuclear-active and dominant-negative nFGFR1 forms in ESC models (Stachowiak et al. 2011b, 2015).

We have hypothesized that nFGFR1, through its widespread binding across the genome, could be involved in dynamic organization of chromatin structure. We have considered two models of how nFGFR1 binding may elicit global gene regulation in brain development and dysregulation in schizophrenia and other developmental disorders. In the *cis* model, nFGFR1 acts by binding to transcription enhancer complexes at the promoter sites of individual regulated genes to influence their activities (Fig. 6.5a). In the *trans* model, nFGFR1 binding brings together distant DNA regions enabling their common regulation or dysregulation. We hypothesize that alternating DNA loops may be extruded by nFGFR1 allowing for the execution of the distinct gene programs.

We have begun testing our hypotheses by focusing on the activation of the HoxA genes, which govern the formation of different CNS regions and body parts. The HoxA gene cluster contains 12 HoxA genes A1, A2, A3, A4, A5, A6, A7, A9, A10,

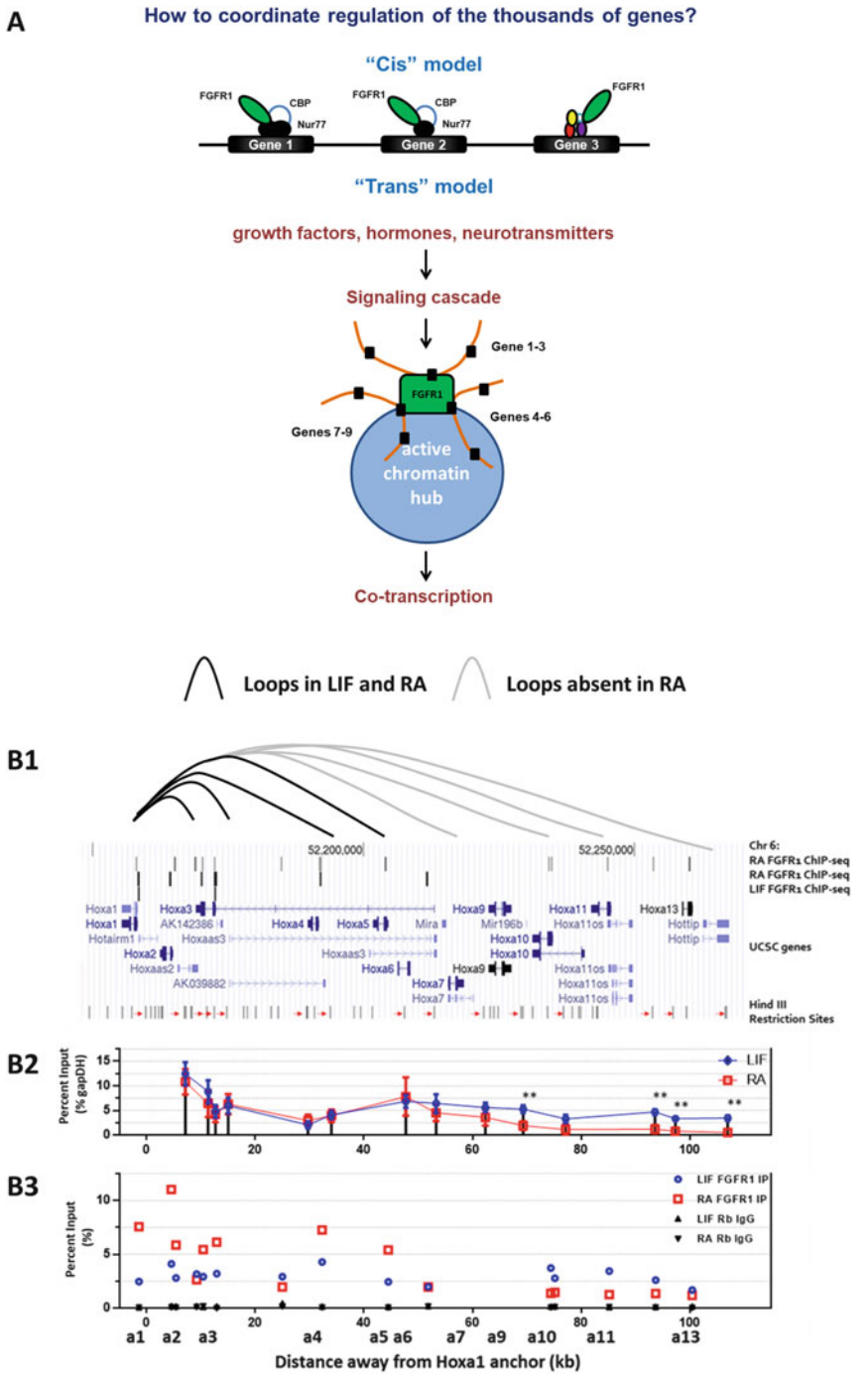

Fig. 6.5 (**a**) Models of global gene regulation by nFGFR1. In the cis model, the regulation of transcription by nFGFR1 occurs at the individual gene sites targeted by nFGFR1. In the trans model, nFGFR1 binds to sites which bring together distal chromatin and forms transcription-

A11, and A13, of which the 3′ genes (HoxA1–HoxA5) are involved in the progressive (head to tail) generation regions of the hindbrain regions and the remaining 5′ genes generate the spinal cord (reviewed in Stachowiak and Stachowiak 2016). nFGFR1 binds to several sites across the HoxA cluster, and during the RA-induced neuronal development, it activates predominantly the 3′ members (HoxA1–HoxA5) of the cluster (Terranova et al. 2015). To analyze the gene interactions within the HoxA cluster, we performed chromatin conformation capture (3C), a PCR-based technique, which estimates proximity between the selected gene loci (Dekker et al. 2002; Hagege et al. 2007). We have recently completed the 3C analysis in the HoxA cluster using HoxA1 as an anchor for measuring its interaction frequencies with downstream HoxA cluster members.

Within an inactive HoxA cluster of the pluripotent mESC, HoxA1 engages in the interactions with all downstream, HoxA2–HoxA13, genes, thus forming loops of different genomic lengths. During RA-induced neuronal differentiation, the HoxA1 locus maintains interactions only with the proximal 3′ HoxA2–HoxA5 genes. The interactions of HoxA1 with HoxA6, HoxA7 HoxA9, HoxA10, HoxA11, HoxA12, and HoxA13 are reduced, and thus the formation of the longer loops no longer occur. These structural changes correlate with nFGFR1 binding, which in RA-treated cells increase at the proximal (3′) HoxA genes but decrease at the distal (5′) HoxA genes. The exclusion of distal HoxA genes from the loops correlates also with their lack of or smaller activation by RA, compared to the proximal HoxA genes. Thus the observed changes in the loop formation isolate differences between the regulations of the hindbrain forming upstream HoxA genes from the spinal cord forming downstream HoxA genes.

These limited findings give backing to our proposed notion that nFGFR1 participates in the formation of the chromatin structures, which enable coordinated gene regulation during brain development and dysregulation in schizophrenia. Our continued experiments aim to identify the nFGFR1-associated chromatin interactions on a genome-wide scale. The results could provide insight into how chromatin topological programs form during development and how they may be disrupted in the schizophrenia leading to the brain malformations discussed in our accompanying chapter.

Fig. 6.5 (continued) associated domains, TADs, with coordinately regulated genes. (**b**) Structural regulation of the HoxA gene cluster—role of nFGFR1. (**b1**) UCSC genome browser tracks containing chromosome location, FGFR1 ChIP-seq binding data, gene location, and Hind III restriction enzyme site tracks. Loops forming between HoxA1 and other Hox genes in pluripotent (LIF) mESC and differentiated NCCs (RA) are indicated. (**b2**) 3C qPCR on nondifferentiated pluripotent mESC (LIF) and differentiated NCCs (RA) measuring the frequency of HoxA1 interacting with downstream HoxA cluster loci. (**b3**) ChIP-qPCR on LIF and RA conditions measuring nFGFR1 binding at ChIP-seq identified loci throughout the HoxA cluster. Control IgG are also indicated

6.9 Summary

In summary, schizophrenia is a developmental disorder characterized by complex aberrations in the structure, wiring, and chemistry of multiple neuronal systems. Over 200 genes, selected by their linkage, association, and expression, have been proposed to contribute to the etiology of the disease. However, there is no single gene whose expression is altered in a majority of schizophrenia patients (Rodriguez-Murillo et al. 2012; Sun et al. 2010). In the proposed transcriptional circuit, INFS integrates incoming developmental signals (St) transmitted by the diverse pathways in which the schizophrenia-linked genes reside. A disruption of any of the individual upstream signal leads to the dysregulation of nFGFR1 which in turn affects the diverse neuro-ontological regulations listed on Fig. 6.6. In addition FGFR1 binds to promoters of the unchanged schizophrenia-linked genes which may lead to their dysregulation as indicated in Fig. 6.6 by the nFGFR1 feedback loops. We propose that the alterations in nFGFR1 interactions with developmental gene networks, miRNA genes, and chromatin topology factors in schizophrenia may underlie the neurodevelopmental pathology of this disease.

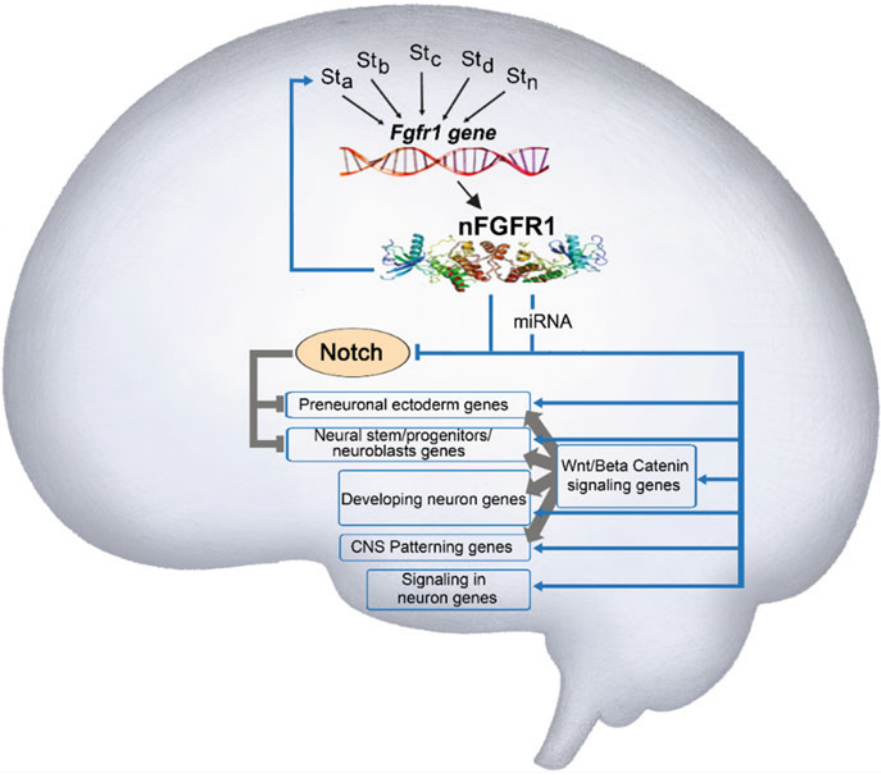

Fig. 6.6 Genetic experiments position the FGFR1 gene at the top of gene hierarchy that directs the development of multicellular animals. FGFR1 governs gastrulation, as well as development of the major body axes, neural plate, central and peripheral nervous systems, and mesoderm by affecting the genes and miRNAs that control the cell cycle, pluripotency, and differentiation (Stachowiak and Stachowiak 2016). This regulation is executed by nuclear protein, nFGFR1, which integrates diverse schizophrenia-linked genes and pathways (Sun et al. 2010). Signals generated by diverse developmental stimuli (St; neurotransmitters, hormones, growth factors, cell contact receptors, etc.,) in embryonic and brain stem cells are propagated by a newly synthesized nFGFR1 protein which translocates into the nucleus and "feeds forward" neurogenic signals to key mRNA and miRNA genes that program and execute different stages of neural development (based on the results of ChIPseq, ChIP, RNAseq, and RNA analyses). For example, nFGFR1 removes the "developmental road block" imposed by the anti-neural Notch1 gene. nFGFR1 targets and activates several master genes that initiate and instruct neural development. Those include proneural Ascl1, and multiple genes in the Wnt pathway. The nFGFR1 binding correlates the activation of genes that stimulate or transduce WNT signals with downregulation of the genes that inhibit Wnt receptors. nFGFR1 binding activates neuronal developmental genes Pax, Id3, Cdx1, IRX3, CREB/CBP signaling genes, and CNS patterning Hox genes. nFGFR1 targets activated axonal guidance genes, and genes involved in synaptic plasticity and development of dopamine and glutamate neurons [based on the Stachowiak and Stachowiak (2016)]. The ST represents diverse signaling pathways in which schizophrenia-linked genes have been found (see Fig. 6.1) and which are also regulated by nFGFR1. In schizophrenia, the mutations of these individual genes, including "weak" copy variations, are proposed to dysregulate this autoregulated genomic circuit and thus lead to broad molecular and developmental dysfunctions [figure is based on information in Narla et al. (2017), Stachowiak and Stachowiak (2016), and Terranova et al. (2015) and linked databases]. Figure drawn by Sun Young Kang

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