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Abnormal X Chromosome Inactivation in Females with Major Psychiatric Disorders

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Abstract

Mania, major depression and schizophrenia are severe brain disorders. No biological hallmark has been identified for any of these disorders. Here, we report that abnormal X chromosome inactivation often presents with a variety of major psychiatric disorders in a significantly large portion of female patients from the general population. X chromosome inactivation is well preserved in human lymphoblastoid cells. Expression of *XIST*, *KDM5C,* and other genes on X chromosome is significantly higher in the lymphoblastoid cells of female patients than in those of female healthy controls. We found that *XIST* and *KDM5C* expression can be used as a potential diagnostic hallmark for this sub-population of patients. Preliminary studies also suggest an increased *XIST* expression in postmortem brains from female patients with schizophrenia, bipolar disorder, and major depression. An increased gene dosage from some genes on X chromosome may contribute to development of psychiatric disorders since functional disomy of partial X chromosome have been suggested to cause mental retardation and other developmental abnormalities. Mutation of *KDM5C* gene was reported to cause X-linked syndromic mental retardation. We propose that abnormal X chromosome inactivation could play a causal role in development of major psychiatric disorders in females. Correction of abnormal X chromosome inactivation may prevent and/or cure major psychiatric disorders in female patients in future.

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Introduction

Schizophrenia, major depression and bipolar disorder are severe brain disorders. Biological basis of these major psychiatric disorders has been little understood. Our previous studies found that inhibition of protein translation may contribute to pathogenesis of major psychiatric disorders in a rare Scottish family (Ji et al., 2014). On the other hand, excessive protein translation has been suggested in fragile X syndrome and autism (Santoro et al., 2012). We speculate that abnormal protein translation may contribute to a wide range of mental disorders not only in rare families, but also in the general population. It is impossible to conduct direct measurement of protein translation activity in human brain. However, some of genes affect protein translation in both lymphocytes and neurons; for example, mutation of *FMR1* causes excessive protein translation in both lymphoblastoid cells and brains (Bhakar et al., 2012; Gross and Bassell, 2012). Therefore, we analyzed protein translation activity in psychiatric patients' lymphoblastoid cells. We observed a significantly larger variation of protein translation activities in the patients than in the controls. Surprisingly, all variations of protein translation activities came from the female patients. These findings prompted us to investigate functions of the X chromosome in the female patients' cells. We examined X chromosome inactivation (XCI) in the female patients' lymphoblastoid cells. Expression of *XIST* and several other genes on the X chromosome was investigated. We extended our analyses of XCI to female patients with a different psychiatric disorder, recurrent major depression. Finally, we conducted a preliminary study of *XIST* expression in the postmortem brains of patients with schizophrenia, bipolar disorder, and major depression.

Results

Abnormal protein translation activity in patients with severe mania and psychosis

Protein translation activity was examined in the lymphoblastoid cells of psychiatric patients. To synchronize cell growth, lymphoblastoid cells were first serum-starved for 8 h before re-stimulated with

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normal concentration of serum. Protein translation activity was measured using the SUnSET (surface sensing of translation) method (Ji et al., 2014; Schmidt et al., 2009) 8 h after serum re-stimulation. Different protein translation activities were observed between cell lines (Figure 1A). After analyzing 26 healthy controls and 28 patients with severe mania and psychosis, we found a significantly larger variation of protein translation activities in the lymphoblastoid cells from the patients (Figure 1B). To rule out whether variations of protein translation activities measured by SUnSET may come from differential puromycin uptake between cell lines, we compared the intracellular concentrations of puromycin using a modified quantification method (Fujiwara et al., 1982). We selected two cell lines (B25 and B27) with a marked difference in SUnSET (Figure 1C). Concentration of intracellular puromycin was quantified by its effectiveness to block the interaction between anti-puromycin antibody and puromycin-containing proteins on the membrane in Western blot. Western blot signal was gradually decreased by an increasing amount of cell lysate added to the blocking solution (Figure 1C). There was no difference between the blocking activities of B25 and B27 cell lysate, indicating that there is a comparable concentration of intracellular puromycin between the two cell lines. We conclude that different SUnSET intensities between B25 and B27 likely came from differential activities of protein synthesis. We also conducted SUnSET experiments in proliferating lymphoblastoid cells. There is a high correlation of SUnSET intensities between the proliferating cells and the synchronized cells (data not shown). No correlation was found between individuals age and protein translation activity (Figure S1A). When the SUnSET data were sub-grouped according to gender, however, we found that all variations of protein translation activities came from the female patients (Figure 1D). To avoid potential bias in the normalization of SUnSET data between gels, we re-ran females' SUnSET on the same Western blot gel. Larger variation of protein translation activities was confirmed in the female patients (Figure S1B).

XCI dysregulation in female patients with severe mania and psychosis.

Restriction of abnormal protein translation activities to the female patients prompted us to

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investigate potential dysregulation of sex chromosome functions. We examined XCI in female lymphoblastoid cells by quantifying expression of *XIST* (Brown et al., 1992), *TSIX* (Migeon et al., 2002), *FTX* (Chureau et al., 2002), and *JPX* (Chureau et al., 2002) genes. These genes produce non-coding RNAs (ncRNA) and are localized at the core of the X chromosome inactivation center (XIC). Their mouse orthologs play central roles in establishing XCI (Brockdorff et al., 1992; Chureau et al., 2011; Lee et al., 1999; Tian et al., 2010). We found that expression of *XIST* is significantly higher ($p = 0.001$, FDR corrected for multiple comparisons) in the female patients than in the female controls (Figure 2A). In contrast to high *XIST*expression, *TSIX* expression is significantly lower ($p < 0.01$, FDR corrected for multiple comparisons) in the patients' cells (Figure 2B). We also observed a trend of high expression of *FTX* ($p < 0.1$, FDR corrected for multiple comparisons) in the patients (Figure 2C), but no difference was found in *JPX* expression (Figure 2D). In mouse, *Xist* expression is negatively regulated by *Tsix* and positively regulated by *Ftx* and *Jpx* (Chureau et al., 2011; Lee et al., 1999; Tian et al., 2010). Consistent with the negative role of mouse *Tsix*, human *TSIX* expression is lower in the patients' cells that display higher *XIST* expression. However, there is only a modest linear negative correlation between human *XIST* and *TSIX* expressions (Figure 2E). *TSIX* may play different roles in human from its ortholog *Tsix* in mice (Migeon et al., 2002). There is a modest negative correlation between human *TSIX* and *FTX* expression (Figure S2A), consistent with the opposite roles proposed for mouse *Tsix* and *Ftx* genes in the regulation of *Xist* expression in mice. Taken together, altered expression of *XIST* and its possible regulator genes within XIC indicates an abnormal XCI in the lymphoblastoid cells of the female patients.

Abnormal XCI may affect expression of genes outside of XIC on the X chromosome. We randomly selected a few genes that are either completely inactivated by or escapees from XCI. *PGK1*, *G6PD* and *HPRT1* genes have been known to be inactivated by XCI, and *KDM5C*, *KDM6A* and *RPS4X* genes are the well-established escapees from XCI in human lymphoblastoid cells (Johnston et al., 2008). We found that expression of *KDM5C* is significantly higher ($p < 0.05$, FDR corrected for multiple comparisons) in the female patients than in the female controls (Figure 3A). There is a trend of high expression of *KDM6A*, *PGK1* and *G6PD* genes ($p < 0.1$, uncorrected) in the patients' cells before

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correction of multiple comparisons (Figure 3B, 3C, 3D). No difference was found in expression of either *HPRT1* or *RPS4X* gene between the patients and the controls (Figure S2B, S2C). *KDM5C* and *KDM6A* genes encode different histone H3 lysine-specific demethylases that play key roles in chromatin remodeling (Iwase et al., 2007; Lan et al., 2007; Lee et al., 2007; Tahiliani et al., 2007). A strong positive correlation (Pearson's coefficient, $r = 0.88$) was observed between *KDM5C* and *KDM6A* expressions (Figure 3E), suggesting a potentially functional coordination in the regulation of genome-wide chromatin remodeling and gene expression. Interestingly, *XIST* and *KDM5C* expression also display a strong positive correlation (Figure 3F)(Pearson's coefficient, $r = 0.78$), indicating that their co-regulation may relate to XCI. We are interested in exploring whether a combination of *XIST* and *KDM5C* expression can be used as a potential diagnostic marker to separate the patients from the controls. According to calculation of reference range in clinical blood tests, we calculated the reference ranges of *XIST* and *KDM5C* expressions in the control females to define "normal" distribution. The upper limit of reference range is defined as 1.96 standard deviations above the mean (dashed line, Figure 3F). Out of 13 patients, 8 have *XIST* and/or *KDM5C* expression above their reference ranges.

To investigate whether potential medication history may alter expression of *XIST*, *TSIX, KDM5C* and *KDM6A* genes in patients' lymphoblastoid cells, we examined expression of these genes after treatment of clozapine (CLZ) and valproic acid (VPA) in the lymphoblastoid cells. None of these genes displayed altered expression after a combined treatment of the antipsychotics CLZ and the mood stabilizer VPA (Figure S3A, S3B, S3C, S3D). These data suggested that patients' medication history (if any) unlikely have any effect on the expression of *XIST*, *KDM5C* and other genes in their lymphoblastoid cells. To support our conclusion, expression of *KDM5C* and *KDM6A* genes was not altered in the lymphoblastoid cells of male patients in comparison with the healthy male controls (Figure S4), assuming that male patients also had similar medication history as the females. Neither *XIST* nor *KDM5C* expression is affected by age (Figure S5A, S5B). We investigated whether abnormal XCI may be responsible for the large variation of protein translation activities in the female patients' lymphoblastoid cells. Unfortunately, there is no correlation between *XIST* expression and protein translation activities

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(SUnSET) (Figure S5C). A weak correlation between *KDM5C* expression and SUnSET was observed (Figure S5D). We speculate that other modifier genes on autosomes, which conceivably vary between individuals, may also be involved in the regulation of protein translation.

XCI dysregulation in female patients with recurrent major depression.

We are particularly interested in the finding of abnormal XCI in the lymphoblastoid cells of the female patients with mania and psychosis. To investigate whether abnormal XCI presents in different psychiatric disorders, we examined expression of *XIST*, *TSIX*, *KDM5C, KDM6A, PGK1, and G6PD* genes in the lymphoblastoid cells of female patients with recurrent major depression. These genes had displayed significant or trend of increases of expression in patients with mania and psychosis. We found that expression of *XIST* is significantly higher ($p < 0.001$, FDR corrected for multiple comparisons) in the female patients with recurrent major depression than in the female healthy controls (Figure 4A). In contrast to significant reduction of *TSIX* expression in the female patients with mania and psychosis, down-regulation of *TSIX* expression was not significant in the lymphoblastoid cells of patients with recurrent major depression (Figure 4B). The small sample size of the patients may not have sufficient statistical power to detect the difference. Expression of *KDM5C* is also significantly higher ($p < 0.001$, FDR corrected for multiple comparisons) in the patients with recurrent major depression (Figure 4C). Consistent with a trend of high *KDM6A* expression in patients with mania and psychosis, we observed a significantly higher expression of *KDM6A* ($p < 0.05$, FDR corrected for multiple comparisons) in the lymphoblastoid cells of patients with recurrent major depression (Figure 4D). However, no difference was found in either *PGK1* or *G6PD* expression between the patients and healthy controls (Figure S6A, S6B). As expected, a strong positive correlation (Pearson's coefficient, $r = 0.85$) was observed between *KDM5C* and *KDM6A* expressions (Figure 4E). *XIST* expression is also highly correlated with *KDM5C* expression (Pearson's coefficient, $r = 0.85$)(Figure 4F). After calculating the reference ranges of *XIST* and *KDM5C* expression in the healthy controls, 8 out of 10 patients have *XIST* expression above its

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reference range.

Since *XIST* does not encode a protein, we examined expression of KDM5C and KDM6A proteins in female patients with recurrent major depression. Western blot analyses confirmed a significantly higher level of KDM5C protein ($p < 0.01$) in the patients' cells than in the controls' cells (Figure 5A, 5B). A modest correlation between the levels of *KDM5C* RNA and KDM5C protein was observed (Pearson's coefficient, $r = 0.53$)(Figure 5C). Conceivably, protein expression is not solely controlled by the level of RNA expression (Schwanhausser et al., 2011). Expression of KDM6A protein is more difficult to detect (Figure S6C). A significantly higher level of KDM6A proteins was also observed in the patients ($p < 0.05$)(Figure S6D). No correlation between *KDM6A* RNA and KDM6A protein expression was detected (Figure S6E). We also measured protein translation activity in the patients' cells using SUnSET. A significantly larger variation of protein translation activity was also observed in the cells of female patients with recurrent major depression (data not shown).

A steady-state level of *XIST* expression

For a potential diagnostic marker, it is important that expression of *XIST* and *KDM5C* genes needs to be robust and reproducible. To address this question, we collected batches of cells from different cell passages and analyzed expression of these genes. The level of *XIST* expression is very stable between different cell passages (Figure 6A). There is a high correlation of *XIST* expression (Pearson's coefficient, $r = 0.88$) between the two different cell passages. More variation was observed in *KDM5C* expression between the two different cell passages (Figure 6B). However, there is still a strong correlation (Pearson's coefficient, $r = 0.6$). In contrast to *XIST* and *KDM5C* expression, *KDM6A* expression appeared to be more affected by different cell cultures (Figure S7A). Among the three genes, *XIST* expression displayed the most robust consistency between different cell cultures.

To address potential lack of representativeness of the small sample size of the female controls, we enlarged the control sample size by including more healthy females. Because we ran out of female

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European Caucasian controls, we analyzed expression of *XIST*, *KDM5C* and *KDM6A* genes in a group of healthy females with mixed ethnic backgrounds. There is no difference in either *XIST* or *KDM5C* expression between the European Caucasian female controls and the female controls with mixed ethnic backgrounds (Figure S7B, S7C). A slightly higher expression of *KDM6A* gene was observed in the controls with mixed ethnic backgrounds (Figure S7D). *KDM6A* expression appears to be more susceptible to variation of different cell cultures and different ethnic backgrounds. Since there is no ethnic effect on either *XIST* or *KDM5C* expression, we combined the two groups of healthy female controls together for further analysis. We have demonstrated that patients with either mania and psychosis or recurrent major depression display the same abnormal XCI. Therefore, we combined the two groups of patients together and compared with the combined controls. As expected, *XIST* expression displays a narrow distribution in the combined controls (Figure 6C). A significantly higher *XIST* expression was observed in the combined patients ($p < 10^{-6}$, FDR corrected for multiple comparisons). Females with triple XXX chromosomes have high XIST expression, but we did not find triple XXX chromosome in either controls or patients with high XIST expression (Figure S7E). There is also a significantly higher *KDM5C* expression in the combined patients ($p < 10^{-4}$, FDR corrected for multiple comparisons)(Figure 6D). With an increased size of healthy controls, the reference ranges are more accurately calculated for both *XIST* and *KDM5C* expressions. 16 out of 23 patients displayed high *XIST* (11 patients) and/or *KDM5C* (12 patients) expression above their reference ranges (Figure 6E). Considering that the patients and the controls are randomly collected from the general population, it is likely that a significantly large sub-population of female patients from the general population may have abnormal XCI.

In a limited number of postmortem human brains, we examined expression of *XIST*, *TSIX, KDM5C*, and *KDM6A* genes in cortical RNA from female patients with schizophrenia, bipolar disorder, and major depression (kindly provided by Stanley Medical Research Institute). Due to small sample sizes, there is no significant difference in expression of any of these genes between control and patient groups.

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However, several patients display high *XIST* expression above the upper limit of its reference range calculated from a limited number of female controls (Figure 6F). Despite the small sample size of the controls, its upper limit of reference range (1.279) is very close to the one calculated from the combined control lymphoblastoid cells (1.26), suggesting little variation of *XIST* expression among healthy individuals. In contrast to strong correlations between different gene expressions in lymphoblastoid cells, no correlation between *XIST*, *KDM5C*, and *KDM6A* expressions was observed (data not shown). *TSIX* expression in the 6 patients that have the highest *XIST* expression is significantly lower than that in the rest of population (Figure S7F), supporting potentially abnormal XCI in these patients' brains. It should be cautious that many confounding factors, such as tissue and cell heterogeneity, RNA degradation, disease conditions etc, could complicate RNA expression analysis in the postmortem brains. More human postmortem brains are needed to confirm the findings in future.

Discussion

Lymphoblastoid cells have been used to identify differential gene expressions as potential biomarkers for psychiatric disorders. However, these studies have been questioned for their relevance to psychiatric disorders due to lack of justification of commonality between lymphoblastoid cells and brain. XCI is unique. It occurs early during embryogenesis in all somatic cells to ensure the same gene dosage from X chromosome between males and females (Lee and Bartolomei, 2013). XCI is very stable and well preserved in human lymphoblastoid cells (Johnston et al., 2008; Zhang et al., 2013), providing a convenient cell model to study XCI effects in human disease (Amir et al., 2000). Therefore, we consider that abnormal XCI observed in patients' lymphoblastoid cells reflects the same abnormality of XCI in all somatic cells of the patients including the brain. Our preliminary studies of a limited number of postmortem human brains support the conclusion.

In mouse, *Xist* is a master gene in the initiation of XCI (Kay et al., 1993; Penny et al., 1996; Plath

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et al., 2002). *Tsix*, *Ftx*, and *Jpx* genes, localized in X chromosome inactivation center (XIC), have been demonstrated to regulate expression of *Xist* (Chureau et al., 2011; Lee et al., 1999; Tian et al., 2010). In human lymphoblastoid cells, we observed a significantly higher *XIST* expression in the patients with either mania and psychosis or recurrent major depression. Alteration of *TSIX* and *FTX* expression was less robust, but consistent with the regulatory roles proposed for their mouse orthologs. However, there may be species difference in the mechanisms of regulation; for example, human *TSIX* was reported to be co-expressed with *XIST* from the same inactivated X chromosome in contrast to mouse *Tsix* that expresses only in the active X chromosome (Migeon et al., 2002). Alteration of gene expression caused by abnormal XCI spreads beyond XIC. Out of 6 randomly selected genes outside of XIC, *KDM5C* and *KDM6A* genes displayed significantly higher expression in the patients. At first glance, the results appear paradoxical. Increased expression of *XIST* that is critical for both initiation and maintenance of gene silencing (Yildirim et al., 2013) should theoretically have more suppression of gene expression on X chromosome. We offer two possible explanations for the paradoxical high *XIST* expression (Figure 7). First, excessive expression of *XIST* may be a consequence of deficient XCI that causes up-regulation of all gene expression. It is also possible that excessive *XIST* is a compensatory response to deficient X chromosome inactivation. Second, excessive *XIST* may titrate protein repressor complexes away to impair XCI via insufficient coating of X chromosome and thereby increase expression of the escapee genes *KDM5C* and *KDM6A*. Indeed, it was reported that human *XIST* RNA can be mis-localized (Clemson et al., 1998). We also observed a trend of higher expression of *PGK1* and *G6PD* genes in patients with mania and psychosis, consistent with the notion that more genes on the X chromosome may increase their expression. Could increase of gene dosage from the X chromosome contribute to development of psychiatric disorders? It is well known that gene over-dosage causes human diseases, e.g. Down syndrome. For the X chromosome, there are numerous reports of functional disomy of partial X chromosome in pathogenesis of mental retardation and other developmental abnormalities (Lahn et al., 1994; Sanlaville et al., 2005). Therefore, we posit that over-dosage of some genes on the X chromosome could cause major psychiatric disorders in females. Interestingly, mutation of *KDM5C* gene was reported

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to cause X-linked syndromic mental retardation (Tahiliani et al., 2007), however, functions of its over-expression remain unknown. Considering that both *KDM5C* and *KDM6A* genes encode different histone H3 lysine-specific demethylases, they could conceivably contribute to development of psychiatric disorders via regulation of chromatin remodeling and gene expression (Peter and Akbarian, 2011). KDM6A can also demethylate trimethylated lysine 27 of histone H3 (H3K27me3), one of the most notable epigenetic silencing markers for inactive X chromosome (Plath et al., 2003). Over-expression of KDM6A may reduce H3K27me3 on Xi to impair XCI in the patients. High correlation between *XIST* and *KDM5C* gene expressions is intriguing. It will be difficult to imagine the high correlation without assuming that *XIST* may continuously play active roles in maintenance of XCI, which is consistent with recent findings of mouse *Xist* functions (Yildirim et al., 2013). Additionally, it will be hard to conceive a steady-state level of *XIST* expression in individual cell lines across different cell passages if *XIST* is dispensable after the establishment of XCI. However, the role of *XIST* expression is less understood in the maintenance than in the initiation of XCI. It is likely that *TSIX, FTX,* and *JPX* genes play a less role in the maintenance of *XIST* expression because of lack of robust correlations between *XIST* and any of these genes. It should be kept in mind that X chromosomal genes outside of XIC are also subject to regulation by cell-specific transcription factors in addition to *XIST*. Therefore, XCI can be studied in lymphoblastoid cells, but XCI downstream molecular pathways leading to mental disorders may be better investigated in human brain.

Abnormal XCI has been observed in patients with either mania and psychosis or recurrent major depression. How could the same molecular abnormality result in markedly different clinical symptoms? One explanation could be that psychiatric disorders are not distinct individual diseases, but a spectrum of mental disorders. For example, some patients display both mania and depression. Another explanation is that abnormal XCI may interact with other modifier genes on autosomes and even environment to result in different clinical symptoms. It has been assumed that there are common genetic risk factors between men and women. Surprisingly, we showed that a large subpopulation of female psychiatric patients from

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the general population may actually have a different genetic cause or pathway from men. This subpopulation of female patients may be better defined biologically as a "XIST disorder" rather than being categorized into different psychiatric disorders according to their clinical symptoms. Identification of a subpopulation of "XIST disorder" with a common genetic and biological causality will accelerate scientific research, and provide objectivity in clinical diagnosis as well as a biological target for development of treatment.

Many questions remain to be answered. We found abnormal protein translation in the lymphoblastoid cells of female patients. Does abnormal XCI contribute to abnormal protein translation activities in female patients' lymphoblastoid cells? We failed to observe a correlation between *XIST* expression and protein translation activity. If factors other than XCI are responsible for protein translation abnormalities, why male patients' lymphoblastoid cells do not display abnormal protein translation activities? We observed a weak correlation between *KDM5C* expression and protein translation activity, indicating that abnormal XCI may indirectly contribute to dysregulation of protein translation activities in female patients' cells. Unlike *XIST*, protein translation activities are subject to regulation by other cell-specific factors and therefore may not directly relate to protein translation in brain.

How many genetic factors regulate *XIST/Xist* expression in initiation of XCI, a fundamental biological process during embryogenesis, remain incompletely understood (Lee, 2011). It was reported that mouse *Xist* expression quickly reaches a high level to initiate XCI and maintains the same high level across later development (Buzin et al., 1994) and between different adult tissues (Kay et al., 1993; Ma and Strauss, 2005). Such a steady-state level of *Xist* expression is also supported by our cell culture studies of human *XIST* across different cell passages. If the level of *XIST* expression is a stable character of an individual, measurement of *XIST* expression in lymphoblastoid cells or any other types of somatic cells may provide an objective test for diagnosis of "XIST disorder", and even at childhood to predict the risk of developing mental illness at adolescence or later. To fully evaluate the feasibility of *XIST* as a potential diagnostic marker, more controls and patients are needed. It will also be interesting to know

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whether abnormal XCI presents in females with neurodevelopmental disorders (e.g. autism) and other human diseases. If abnormal XCI is a major genetic and biological causality, correction of abnormal XCI may prevent and/or cure major psychiatric disorders in this subpopulation of female patients in future.

Materials and Methods

Lymphoblastoid cell culture

Studies on human lymphoblastoid cells were conducted under a University of California San Diego IRB-approved protocol. All lymphoblastoid cell lines were kindly provided by Dr. John R. Kelsoe at UCSD. The cells were in their early passages (< 20 passages)(Oh et al., 2013). Demographic information of the subjects is summarized in Table SI. All female patients except one have family history with one or more relatives suffering from psychiatric disorders. There is no significant difference in age between healthy controls and patients when blood was taken to generate lymphoblastoid cells by transformation with Epstein-Barr virus (EBV). A second group of healthy female controls with mixed ethnic backgrounds were included to expand the size of controls and to examine potential effect of ethnic background on *XIST* expression. Lymphoblastoid cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium media containing 10% fetal bovine serum (Life Technologies, CA) with penicillin and streptomycin (Life Technologies, CA) at 37°C in a humidified atmosphere of 5% CO2.

Protein translation activity

Lymphoblastoid cells were serum-starved for 8 h to synchronize cell growth. 8 h after addition of serum, SUnSET was conducted to measure instant protein translation activity as described (Ji et al., 2014). Patient and control samples were loaded alternately on PAGE gels regardless of age and gender. Because of differential overall intensities between different gels in western blot analyses, signal of each sample was first normalized against the average intensity of all samples from the same gel. After normalization,

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samples were analyzed according to diagnosis, gender, and age. To confirm the larger variation of protein translation activity in female patients,, female samples were re-run on the same Western blot gel to rule out potential bias introduced by normalization between gels.

Quantification of intracellular puromycin concentration

Puromycin-labeled protein used to quantify intracellular concentration of puromycin in lymphoblastoid cells was prepared as follows: HEK293T cells were labeled with puromycin for 20 minutes (Ji et al., 2014). Cells were collected and sonicated in passive lysis buffer (Promega, WI) with 1X protease inhibitor cocktail (P8340, Sigma). Protein concentration was measured with Bradford (Abs 595nm) method with Coomassie Plus Protein Assay (Thermo Scientific, IL). B25 and B27 lymphoblastoid cells were incubated with puromycin according to SUnSET protocol (Ji et al., 2014). The cells were spun down to remove supernatant and washed twice before lysis. Quantification of intracellular puromycin concentration by Western blot: 15 ug puromycin-labeled proteins were loading on 4-20% gradient Tris-Glycine gel. After electrophoresis, proteins were transferred onto PVDF membranes. After 1 hour blocking with 5% of nonfat milk in TBST buffer (pH 7.5, 10 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20) at room temperature, the membrane were cut into individual lanes. Each lane was incubated overnight with anti-puromycin antibody (dilution at 1:200,000; 12D10, Millipore) in the presence of either B25 or B27 cell lysate. After washing three times, the blot was incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (dilution at 1:10,000, Cell signaling, MA) for 1.5 hr at room temperature. Pierce ECL Western blot substrate (Pierce, IL) was used to visualize the puromycin signal. Quantification of Western blot bands was conducted using Image J.

RNA from postmortem human brains

We received 57 BA7 cortical RNA samples out of 60 brains from Stanley Medical Research Institute (SMRI), consisting of 15 each diagnosed with schizophrenia, bipolar disorder, or major depression, and 15 unaffected controls. 21 out of 57 samples were females. The four groups were matched by age, sex,

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race, postmortem interval, pH, hemisphere, and mRNA quality (http://www.stanleyresearch.org/dnn/Default.aspx?tabid=196).

Quantitative real-time RT-PCR

Total RNA was extracted from lymphoblastoid cells with TRIzol reagent (Invitrogen, Carlsbad, CA). Primers were selected to amplify exons without alternative splicing in order to quantify all RNA isoforms. All PCR primers were first confirmed to specifically amplify the target genes after sequencing. cDNA was synthesized from 5ug of total RNA using Superscript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) with random hexamers. SYBR Green real-time PCR was used to quantify relative expression of all genes with a comparative Ct method. The standard curve of PCR amplification was examined. All amplifications had R^2 >0.99. Amplification efficiency for each pair of primers was determined using known molecular concentration of template DNA. Each sample had 4 amplification replica wells. After amplification, Ct was set in the exponential amplification phase of the curve as recommended by the manufacturer's protocol (Bio-Rad CFX384). Variation of cycles between amplification replica wells was smaller than 0.5 cycles. β-actin was used as a reference control for normalization. In most cases, relative expression of each gene in each sample was presented as a percentage relative to the mean of the control group. The following primers were used in real-time QPCR:

1. β-actin

Forward primer: 5' TTCTACAATGAGCTGCGTGTG3'

Reverse primer: 5' GGGGTGTTGAAGGTCTCAAA3'

2. XIST

Forward primer: 5' GGATGTCAAAAGATCGGCCC3'

Reverse primer: 5' GTCCTCAGGTCTCACATGCT3'

3. TSIX

Forward primer: 5' TGTGCCAGGTAATAGAAACACA3'

Reverse primer: 5' ACCTGCATTTGTGGGATTGT3'

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4. KDM5C

Forward primer: 5' GGGTCCGACGATTTCCTACC3'

Reverse primer: 5' GCGATGGGCCTGATTTTCG3'

5. KDM6A

Forward primer: 5' CGCTTTCGGTGATGAGGAAA3'

Reverse primer: 5' GTCCTGGCGCCATCTTCAT3'

6. FTX

Forward primer: 5' GTGATCTGGACAAAGGAACAGG3'

Reverse primer: 5' CATTTCCCGTGAACACCCG3'

7. G6PD

Forward primer: 5' CATGGTGCTGAGATTTGCCA3'

Reverse primer: 5' CCTGCACCTCTGAGATGCAT3'

8. JPX

Forward primer: 5' ACTGACACTGGTGCTTTCCT3'

Reverse primer: 5' GGACTCCCAGGCATTGCTAT3'

9. HPRT1

Forward primer: 5' AGTGATGATGAACCAGGTTATGA3'

Reverse primer: 5' GCTACAATGTGATGGCCTCC3'

10. PGK1

Forward primer: 5' GAATCACCGACCTCTCTCCC3'

Reverse primer: 5' GGGACAGCAGCCTTAATCCT3'

11. RPS4X

Forward primer: 5' AGGTCCTCTTTCCTTGCCTA3'

Reverse primer: 5' GAGCAAACACACCGGTCAAT3'

Western Blot

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Lymphoblastoid cells were collected by centrifugation and sonicated in passive lysis buffer (Promega, WI) with 1X protease inhibitor cocktail (P8340, Sigma). Protein concentration was measured with Bradford (Abs 595nm) method with Coomassie Plus Protein Assay (Thermo Scientific, IL). 50 µg proteins were loaded for Western blot analysis (Ji et al., 2014). Rabbit anti-KDM5C (cat. 39230, dilution at 1:7,500, Active Motif, Carlsbad), rabbit anti-KDM6A (cat. 61516, dilution at 1:7,500, Active Motif, Carlsbad), and mouse anti-β-actin (sc-47778, dilution at 1:10,000, Santa Cruz) were purchased as primary antibodies. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (dilution at 1:10,000, Cell signaling, MA) or HRP-conjugated anti-rabbit IgG (dilution at 1:10,000, Santa Cruz, CA). Pierce ECL Western blot substrate (Pierce, IL) was used for chemiluminescence visualization. Quantification of Western blot bands was conducted using Image J.

Data analysis

All data were first tested for normal distribution using the Kolmogorov−Smirnov test. *F*-tests were used to analyze difference between the standard deviations of the two samples. Unpaired, two-tailed student's *t*-test was used for statistical analyses between two samples. Correction of multiple comparisons were conducted by using false discovery rate (FDR)(http://www.sdmproject.com/utilities/?show=FDR).

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Contributions: X.Z. conceived, analyzed, and wrote the studies. B.J. performed and analyzed the research. J.R.K. provided all lymphoblastoid cell lines for the studies.

Conflict of Interest:

The authors declare no conflict of interest.

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Figure Legends

Figure 1. Variation of protein translation activities in patients' lymphoblastoid cells. (A) Protein translation activity was measured in individual lymphoblastoid cell lines using SUnSET. Sample loading was shown by β-actin protein expression after stripping the membrane. C1-C26: healthy European Caucasian controls; B1-B28: European Caucasian patients with mania and psychosis. **(B)** SUnSET intensity of individual samples was normalized by β-actin. To avoid differential overall intensity between the gels, intensity of each sample was presented by a percentage relative to the average intensity of all samples from the same gel. A significantly larger variation of protein translation activity was observed in the patients than in the controls ($p < 0.01$, *F*-test). Each dot represents a human subject (black=healthy controls; red=patients). CTRL: controls; BP: bipolar patients with mania and psychosis. **(C)** Comparison of intracellular puromycin concentration. Two cell lines (B25 and B27) with a marked difference in SUnSET activity were selected. Equal amount of puromycin-labled protein was loaded on each lane. B25 and B27 cell lysates were used to block interaction between membrane-bound puromycin-labeled proteins and anti-puromycin antibodies in the Western blot blocking solution. Increase of either cell lysate had a similar magnitude of blocking effect, suggesting a comparable concentration of intracellular puromycin despite their remarkable difference in SUnSET. **(D)** SUnSET data were analyzed according to gender. There was no difference between male controls and male patients. A significantly larger variation of protein translation activity was observed in female patients ($p < 0.01$, *F*-test). Each dot represents a human subject (black=healthy controls; red=patients). CTRL: controls; BP: bipolar patients with mania and psychosis.

Figure 2. Abnormal expression of XCI regulators in X chromosome inactivation center. Each dot represents a human subject (black=healthy controls; red=patients). CTRL: controls; BP: bipolar patients with mania and psychosis. **(A)** A significantly higher *XIST* RNA expression was observed in the patients than in the controls ($t(23)=-4.23$, $p = 0.001$, unpaired, two-tailed student's *t*-test, FDR corrected for

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multiple comparisons). **(B)** Consistent with its negative role, there is a significantly lower expression of *TSIX* in the patients (t(23)=3.43, $p < 0.01$, unpaired, two-tailed student's *t*-test, FDR corrected for multiple comparisons). **(C)** Consistent with its positive role, a trend of high expression of *FTX* was detected in the patients (t(23)=-2.00, $p < 0.1$, unpaired, two-tailed student's *t*-test, FDR corrected for multiple comparisons). **(D)** No difference was observed in *JPX* expression between the controls and patients (t(23)=-0.24, ns, unpaired, two-tailed student's *t*-test, FDR corrected for multiple comparisons). **(E)** A modest negative linear correlation was present between human *XIST* and *TSIX* expressions in all samples (Pearson's coefficient, $r = -0.43$).

Figure 3. Increased expression of genes outside of X chromosome inactivation center. Each dot represents a human subject (black=healthy controls; red=patients). CTRL: controls; BP: bipolar patients with mania and psychosis. **(A)** A significantly higher *KDM5C* expression (t(23)=-2.89, $p < 0.05$, unpaired, two-tailed student's *t*-test, FDR corrected for multiple comparisons) was observed in the patients. There was a trend of high expression in *KDM6A* **(B)**(t(23)=-2.00, $p < 0.1$, unpaired, two-tailed student's *t*-test), *PGK1* **(C)**(t(23)=-1.84, $p < 0.1$, unpaired, two-tailed student's *t*-test), *G6PD* **(D)**(t(23)=-1.89, $p < 0.1$, unpaired, two-tailed student's *t*-test) in the patients before correction of multiple corrections. **(E)** A high correlation between *KDM5C* and *KDM6A* RNA expressions was observed in all samples (Pearson's coefficient, $r = 0.88$). **(F)** There is a high correlation between *KDM5C* and *XIST* RNA expressions (Pearson's coefficient, $r = 0.78$). Reference ranges of *XIST* and *KDM5C* expression in healthy controls were calculated as an interval between which 95% of values of a reference group fall into. The upper limit of reference range is defined as 1.96 standard deviations above the mean. *XIST* and *KDM5C* expression above the upper limit of their reference ranges are shaded with light green and light purple respectively.

Figure 4. Abnormal XCI in patients with recurrent major depression. Each dot represents a human subject (black=healthy controls; red=patients). CTRL: controls; MDR: patients with recurrent major

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depression. **(A)** There is a significantly higher *XIST* expression (t(21)=-4.62 , $p < 0.001$, unpaired, two-tailed student's *t*-test, FDR corrected for multiple comparisons) in the patients than in the controls. **(B)** Down-regulation of *TSIX* expression was not statistically significant (t(21)=1.35, ns, unpaired, two-tailed student's *t*-test, FDR corrected for multiple comparisons). A significantly higher expression of *KDM5C* **(C)**(t(21)=-4.79 , $p < 0.001$, unpaired, two-tailed student's *t*-test, FDR corrected for multiple comparisons) and *KDM6A* **(D)**(t(21)=-2.51, $p < 0.05$, unpaired, two-tailed student's *t*-test, FDR corrected for multiple comparisons) were observed. **(E)** Consistent with previous results, a high correlation between *KDM5C* and *KDM6A* RNA expression was found (Pearson's coefficient, $r = 0.85$). **(F)** As expected, there is a high correlation between *KDM5C* and *XIST* RNA expressions (Pearson's coefficient, $r = 0.85$). Reference ranges of *XIST* and *KDM5C* expression in healthy controls were calculated as previously described. *XIST* and *KDM5C* expression above the upper limit of their reference ranges are shaded with light green and light purple respectively.

Figure 5. Increased expression of KDM5C protein in patients' cells. Each dot represents a human subject (black=healthy controls; red=patients). CTRL: controls; MDR: patients with recurrent major depression. The Western blot analyses were the last experiments conducted among all reported studies. During the experiments, we found an additional patient cell line from -80 ^{o}C freezer, which was included in the Western blot analyses. **(A)** Western blot analyses of KDM5C protein expression in the lymphoblastoid cells of patients with recurrent major depression. A single band at 180 kD, the calculated size of human KDM5C protein, was detected. C: controls; D: recurrent major depression. β-actin was used as an internal control for normalization. **(B)** Consistent with increased RNA expression, a significantly higher KDM5C protein expression was found in the patients (t(22)=-2.85 , $p < 0.01$, unpaired, two-tailed student's *t*-test). **(C)** A modest correlation between *KDM5C* RNA and KDM5C protein expression was observed across all samples (Pearson's coefficient, $r = 0.53$).

Figure 6. A steady-state level of *XIST* expression across different ethnicities and between cells and

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tissues. (A) *XIST* and *KDM5C* gene expression was examined in the first batch of lymphoblastoid cells (*XIST*-1 and *KDM5C*-1). After more than a month of continuous cell culture, the same set of genes was examined again in the second batch of cells with different cell passages (*XIST*-2 and *KDM5C*-2). *XIST* and *KDM5C* expression was only normalized against β-actin without being further presented as a percentage relative to the mean of the controls in order to compare absolute amount of RNA transcripts between different cell passages. A high correlation of *XIST* expression was observed between the two batches of cells (Pearson's coefficient, $r = 0.88$). There is a relatively strong correlation in *KDM5C* expression between the two batches (Pearson's coefficient, $r = 0.6$)**(B)**. **(C)** To enlarge the sample size of healthy female controls, 23 more healthy females with mixed ethnic background were included in analysis of *XIST* expression. Since both groups of patients display the same abnormal XCI, they were combined in analyses. Significantly higher *XIST* **(C)**($t(57)=-6.06$, $p < 10^{-6}$, unpaired, two-tailed student's *t*-test, FDR corrected for multiple comparisons) and *KDM5C* **(D)**($t(57)=-4.51$, $p < 10^{-4}$, unpaired, two-tailed student's *t*-test, FDR corrected for multiple comparisons) expressions were found in the combined group of patients than in the combined group of the healthy controls. **(E)** As expected, a high correlation between *XIST* and *KDM5C* expression was observed (Pearson's coefficient, $r = 0.7$). Reference ranges of *XIST* and *KDM5C* expression in the combined healthy controls were calculated as previously described. *XIST* and *KDM5C* expression above the upper limit of their reference ranges are shaded with light green and light purple respectively. **(F)** *XIST* expression was quantified in postmortem human brains. Several patients have high *XIST* expression above the upper limit of its reference range calculated from the healthy control brains. Its upper limit (1.279) of the reference range is very close to that (1.26) of the reference range calculated from the combined controls' lymphoblastoid cells.

Figure 7. A hypothetical role of *XIST* in maintenance of XCI. Insufficient amount of *XIST* has been shown to impair maintenance of XCI. We propose that excessive *XIST* may be either a consequence of deficient XCI or a causality of deficient XCI. If excessive *XIST* is a consequence, it could be either a passive result of deficient XCI or a compensatory response to deficient XCI. In theory, deficient XCI

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caused by insufficient *XIST* may also present in female patients with mental disorders.

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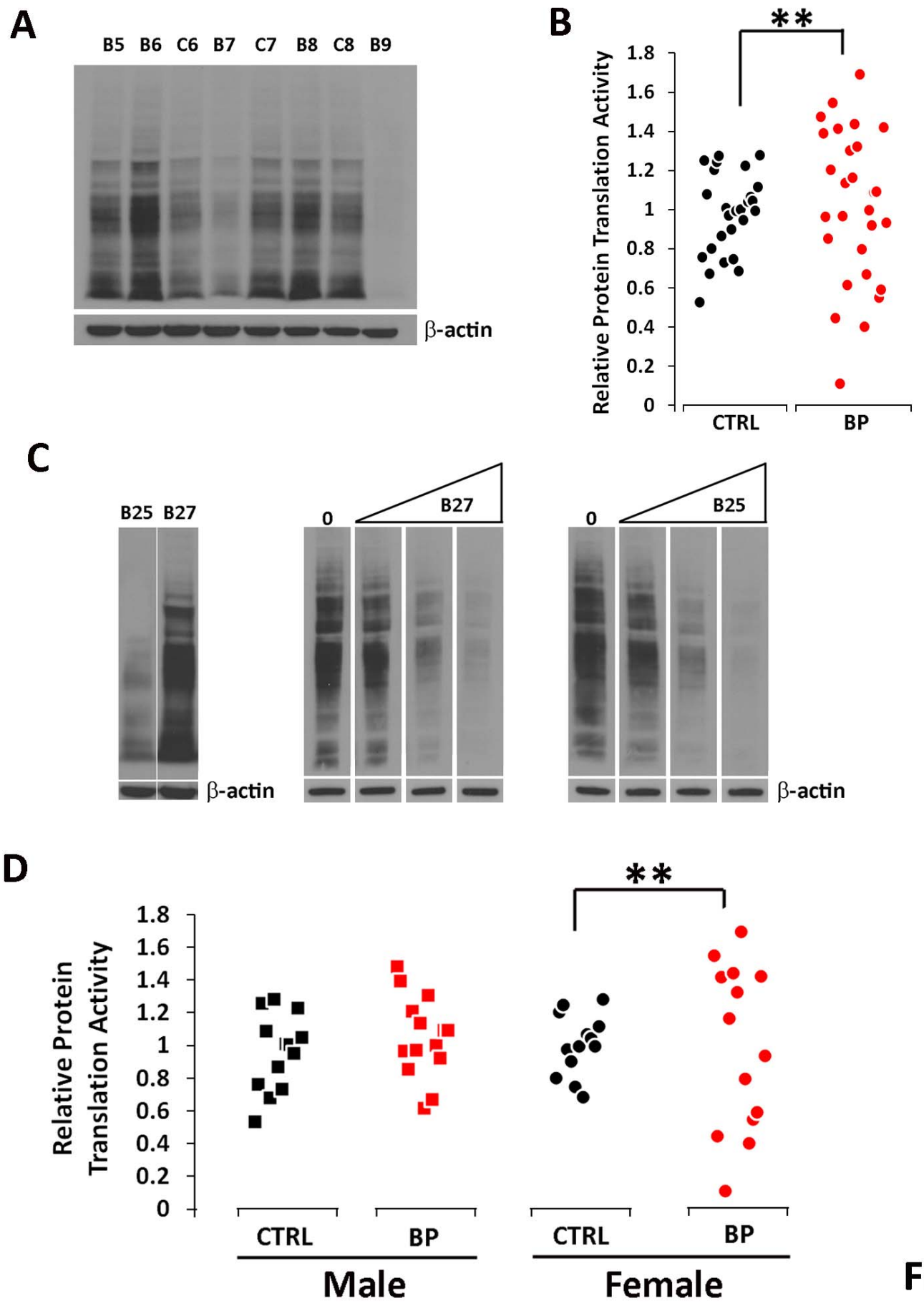

Fig 1

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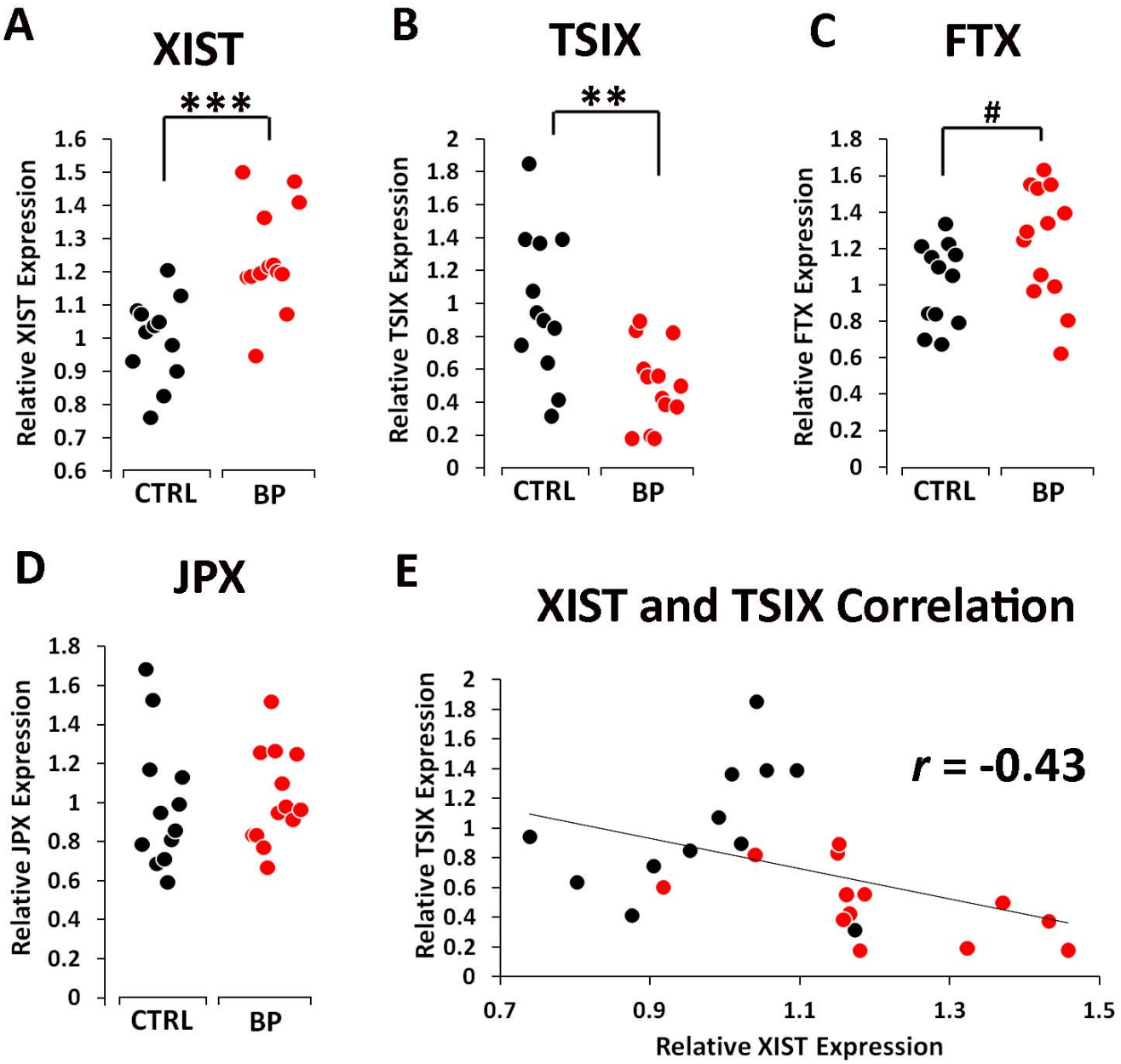

Fig 2

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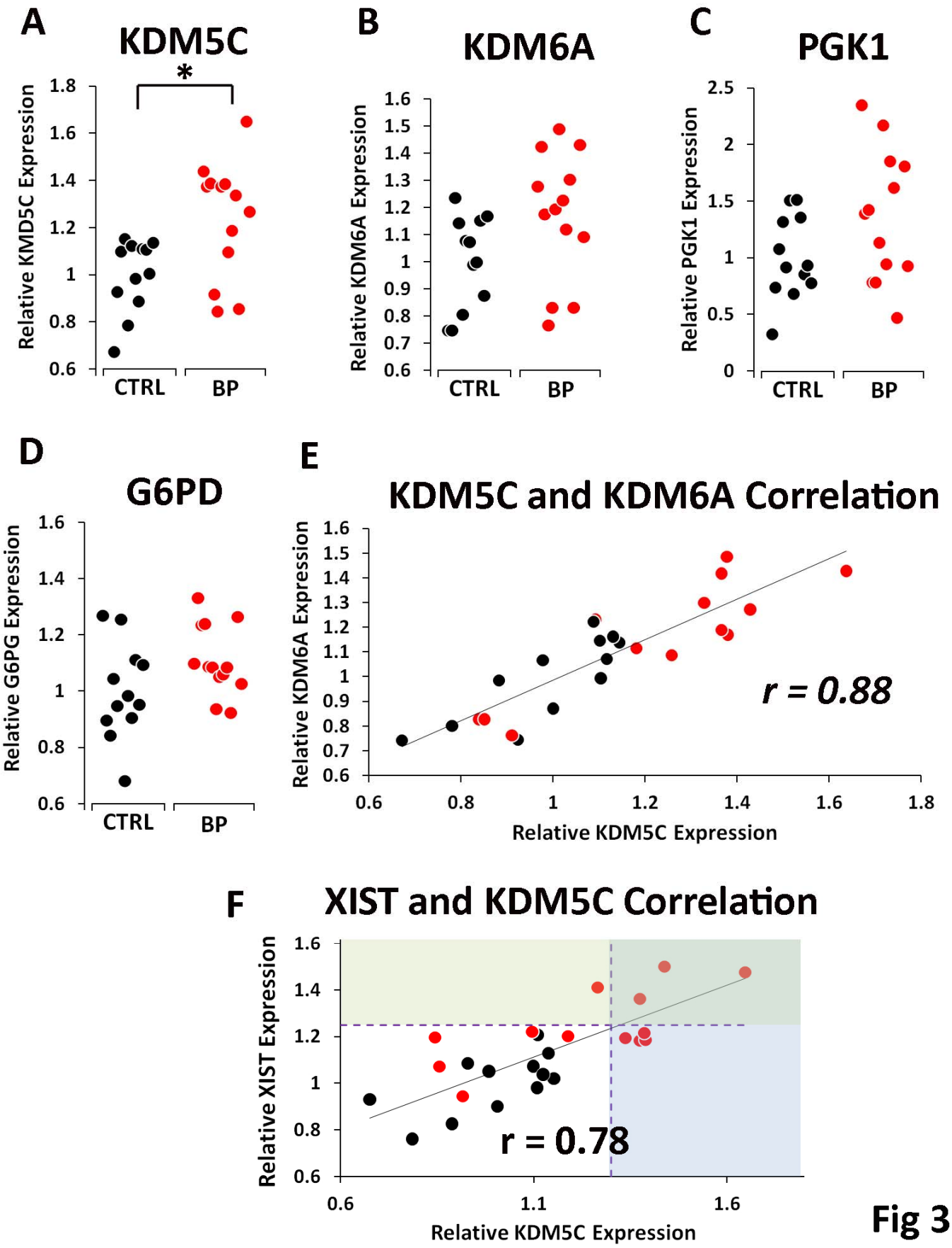

Fig 3

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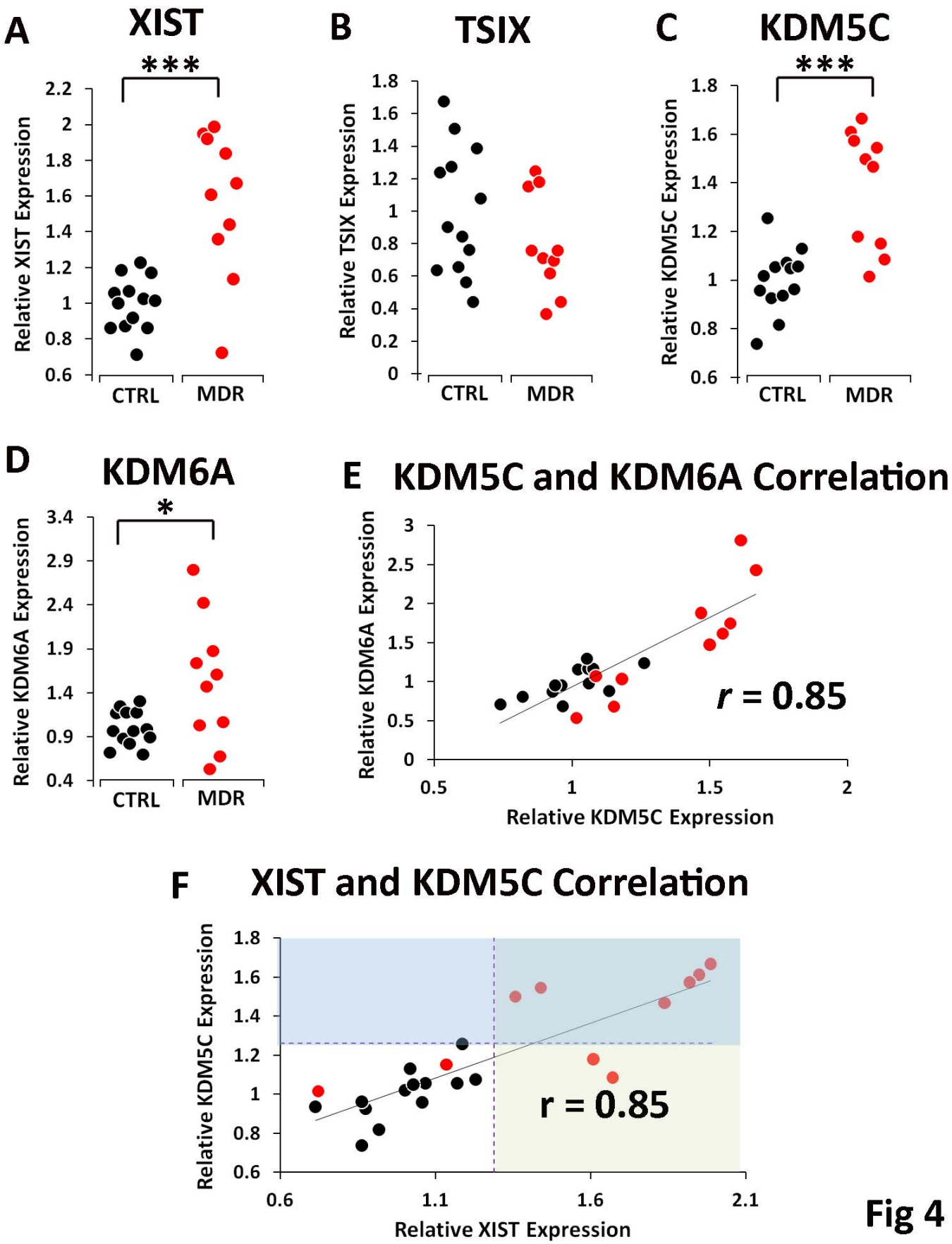

Fig 4

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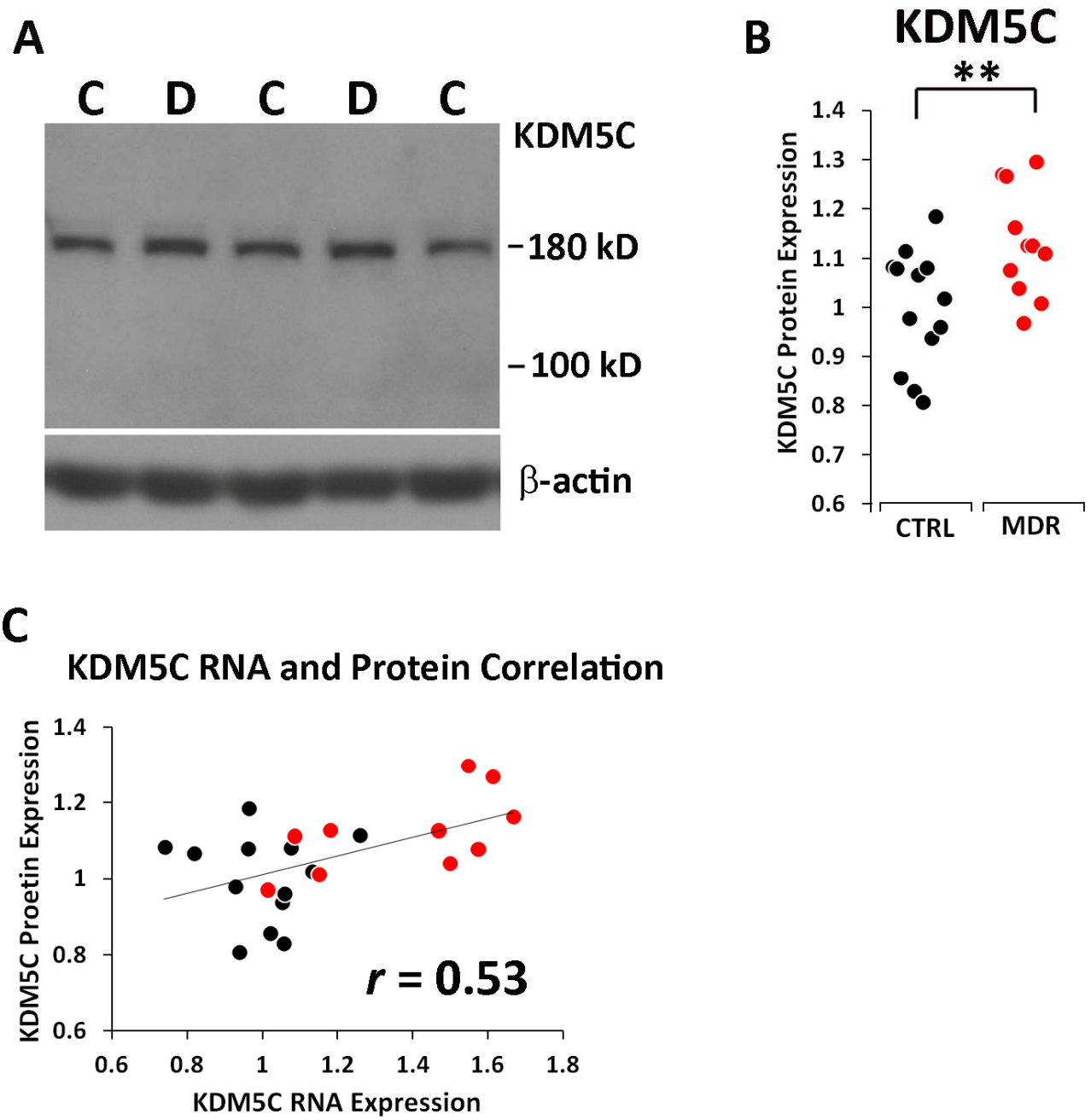

Fig 5

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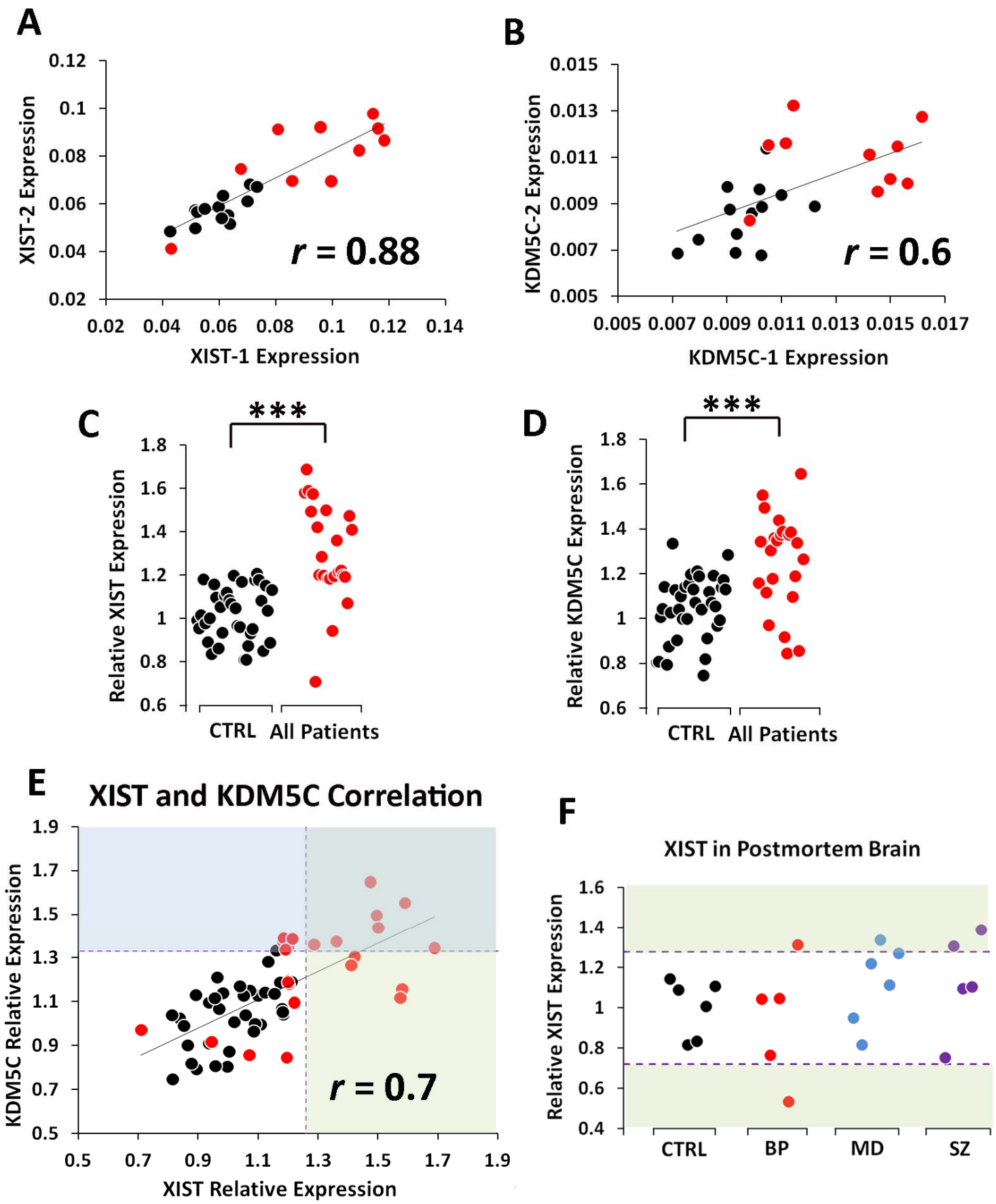

Fig 6

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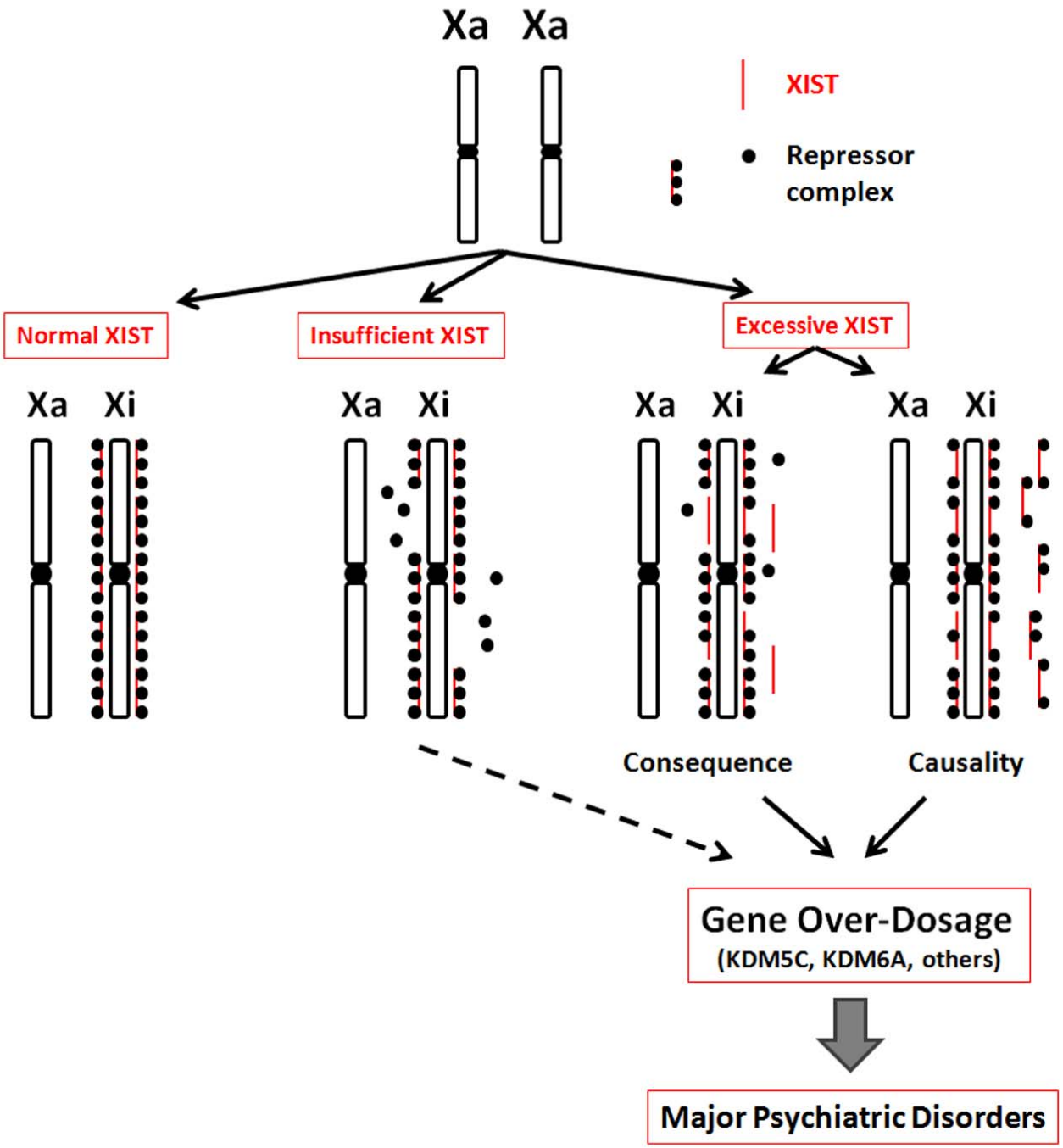

Fig 7