A significant effect of the HUWE1 copy number on autistic spectrum disorder and the development of zebrafish

Haojuan Wu
Sichuan University WCSUH: Sichuan University West China Second University Hospital
Yan Liang
Sichuan University West China Hospital
Juan Li
The First People's Hospital of Longquanyi District Chengdu
Xueguang Zhang
Sichuan University WCSUH: Sichuan University West China Second University Hospital
Baoshan Xu
Guangdong Provincial Stomatological Hospital
Xiang Wang
Sichuan University WCSUH: Sichuan University West China Second University Hospital
Juan Cheng
Sichuan University WCSUH: Sichuan University West China Second University Hospital
Andras Dinnyes
BioTalentum Ltd
Jiang Xie
Chengdu Third People's Hospital
wenming Xu (xuwenming@scu.edu.cn)
Sichuan University West China Second University Hospital https://orcid.org/0000-0002-3686-229X

Research

Keywords: HUWE1, Copy number variation (CNV), Autism spectrum disorder (ASD), Neurodevelopmental disorders, zebrafish model

Posted Date: April 14th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-404499/v1

Abstract

Autistic spectrum disorder (ASD) is a complex neurodevelopmental disorder. The copy number variation (CNV) of genes in the X-chromosome is speculated to play a critical role in ASD. The HECT, UBA and WWE domain containing the E3 ubiquitin protein ligase 1 (*HUWE1*) gene is located on the X-chromosome and is associated with neurodevelopment. However, the CNV of *HUWE1* has not been linked to ASD. In this study, we identified increased copy numbers of *HUWE1* in ASD patients compared to the controls. Moreover, an elevated severity of ASD phenotype was observed in patients with more *HUWE1* copy numbers. In vitro, overexpression *HUWE1* could decrease the proliferation of nervous system derived cells and promote their apoptosis, declining the G2/M phase transition. The functional assay showed that *HUWE1*, an E3 ubiquitin protein ligase, regulated the ubiquitination degradation of key mediators in the Wnt/β-catenin signaling pathway, which is involved in neurodevelopment. Consistent with the ASD patients, overexpression of *huwe1* in zebrafish embryos caused defective brain development, small eyes, cardiac edema and a curly spinal cord, supporting the pivotal role of the *HUWE1* CNV in neurodevelopmental disorders. In this study, we demonstrated the role of the *HUWE1* CNV for complement pathway factors in the origin of ASD for the first time. Genetic analysis of the *HUWE1* copy number could inform the clinical counseling of ASD patients.

Introduction

Autistic spectrum disorder (ASD) is considered a neurodevelopmental disorder defined in the DSM-5 (the Diagnostic and Statistical Manual of Mental Disorders 5th ed.) [1] and is characterized by impairment of social interactions and communication, stereotyped and repetitive behaviors and restricted interests [2]. The prevalence of ASD in the United States has increased to 1 in 68 children [3] and has been estimated to be approximately 1% worldwide [4]. However, the etiology of ASD is complex and indistinct. Studies in twins and families have conclusively described ASD as the most 'genetic' of neuropsychiatric disorders, with concordance rates of 82–92% in monozygotic twins compared with 1–10% in dizygotic twins; the sibling recurrence risk is 6% [5, 6]. Although ASD has a heritability of approximately 90%, mutations or copy number variations (CNVs) associated with ASD are rarely reported [5]. The genes related to ASD are found in different chromosomes [7]; the most associated genes are *neuroligin 4 X-linked* (*NLGN4X*) and *neuroligin 3* (*NLGN3*), which are located on the X-chromosome, and the mutations of *NLGN4X* and *NLGN3* have been identified in a few families with ASD [8]. A striking feature of ASD is the excess of affected males, and sex distribution is markedly skewed (male:female = 4:1) [9], suggesting that genes on the X-chromosome might play crucial roles in ASD. Indeed, many genes in X-chromosome are linked to autism (*KIAA1202, PTCHD1, DIA1R, RPS6KA6, ZNF711, ACSL4, DLG3, IL1RAPL2, TBL1X, AFF2, KIAA2022, TRPC5, KLHL15, HCCS, FAM120C, IL1RAPL1, PIR, GABRQ, GPRASP2, SYTL4, CDH6, FAT2, PCDH8, CTNNA3, ANKRD11, SEMA3F, MIDN, SLC7A3, ARHGEF9, MAOB, IL1RAPL1, GLRA2, CASK, RAB39B, RPL10, MECP2, PTCHD1* and *TMLHE*). Importantly, among them, the pathogenic variations of *NLGN4X*, *NLGN3, ribosomal protein L10* (*RPL10*), *trimethyllysine hydroxylase, epsilon* (*TMLHE*), patched domain containing 1 (*PTCHD1*) and *methyl-CpG binding protein 2* (*MECP2*), have been approved by OMIM

database [10–13]. However, these could explain the mechanisms of limited cases. Therefore, it remains necessary to improve the discriminative power of a single candidate gene in human ASD.

HUWE1, a gene located at Xp11.23, encodes an E3 ubiquitin ligase that is involved in various physiological processes by regulating the stability of multiple proteins [14, 15]. Strikingly, *HUWE1* plays a crucial role in the development of the nervous system by regulating neuronal stem cell proliferation via the ubiquitination pathway [16]. It has been reported that Huwe1 could balance proliferation and neurogenesis in the developing brain of embryonic mice and regulate Bergmann glia differentiation in the developing cerebellum of postnatal mice [17, 18]. Notably, *HUWE1* is the most important candidate gene for intellectual disability (ID) because the increased copy number of *HUWE1* has been identified in many patients with ID [16, 19–21]. Furthermore, overexpression of *HUWE1* could affect axon branching of flies by compromising the Wnt/β-catenin pathway [22]. Using next-generation sequencing, possibly deleterious variants of *HUWE1* have been observed in several ASD patients, indicating that *HUWE1* is also associated with ASD [23]. Similar to ID, ASD is also a neurodevelopmental disorder, while the CNV of *HUWE1* has not been investigated in ASD patients thus far.

To evaluate whether the CNV of *HUWE1* is associated with ASD, we investigated the DNA and mRNA levels of *HUWE1* in ASD patients and normal controls in the present study. The enhanced gene dosages of *HUWE1* has been confirmed as a significant risk factor for ASD. In particular, the overexpression of *HUWE1* could suppress the proliferation of mouse neuronal stem cells. Complementally, the *HUWE1* transgenic zebrafish model represented the abnormal development of the brain. In this study, we showed that overexpression of HUWE1 could increase the risk of ASD through affecting the growth and morphology of neuronal stem cells by promoting the ubiquitination degradation of key proteins in the Wnt/β-catenin signaling pathway.

Materials And Methods

Study population

A total of 400 unrelated Han Chinese children ranging from 3 to 12 years of age were recruited from Sichuan Province in southwestern China. The general clinical data and blood samples were obtained from the Third People's Hospital of Chengdu. The subjects included 200 male normal controls and 200 male ASD patients who were diagnosed in accordance with DSM-V-TR criteria, the CARS and ABC for ASD. This study was approved by the Ethics Committee for Clinical Trials and Biomedical Research, West China Second University Hospital, Sichuan University. Informed consent was obtained from the parents or legal guardians of all study subjects.

Fish strains

Zebrafish embryos were obtained from the natural spawning of the wide-type AB strain. Embryos were reared at a 14 h/10 h light-dark (LD) cycle in Holtfreter's solution at 28.5°C and staged according to criteria established by Kimmel et al. [45]. All experiments were performed in accordance with ethical

permissions granted by the National Zebrafish Resources of China and West China Second University Hospital.

Copy number analysis of the HUWE1 gene

Genomic DNA was extracted from peripheral blood lymphocytes using a DNA purification kit (Tiangen Biotech Co., Ltd.). *HUWE1* copy numbers were analyzed using relative quantitative polymerase chain reaction (qPCR). In the analysis, *GAPDH* and *α-tubulin* were used as internal controls in tests I and II, respectively. The primers were designed using Primer 5.0 to perform two independent tests of *HUWE1*-1/*GAPDH* and *HUWE1*-2/*α-tubulin* (Supplementary Material Table S1).

Analysis of HUWE1 mRNA expression levels

Total RNA was extracted from whole blood using RNAiso Plus (D9108A; TaKaRa, Japan). Total RNA (500 ng) was used for reverse transcription with a PrimeScriptRT reagent kit and gDNA Eraser (DRR047A; TaKaRa). Quantitative PCR (qPCR) was performed using SYBR Premix Ex Taq II (RR820A, TaKaRa) on an iCycler RT-PCR detection system (Bio-Rad, CA) to analyze the relative mRNA expression levels of *HUWE1*. The ΔΔCT method was used for data analysis. Each assay was performed in triplicate for each sample. Absolute quantitation of *HUWE1* copy numbers was performed with two independent tests, using *GAPDH* and *α-tubulin* as internal controls. The primers for RT-PCR are listed in Supplementary Materials Table S1.

Zebrafish embryo RNA isolation and real-time PCR (RT-PCR)

RNA was isolated from a pool of embryos at different stages using TRIzol reagent (Gibco BRL, USA). Total RNA (1 μg) was reverse transcribed. The relative mRNA expression levels of *huwe1* were amplified using SYBR Premix Ex Taq II (RR820A, TaKaRa) on an iCycler RT-PCR detection system (Bio-Rad, CA). The *gapdh* gene was used as an internal control. The primers for RT-PCR are listed in Supplementary Materials Table S1. After 40 cycles, amplified cDNAs were analyzed on agarose gels.

Western blotting

The proteins of cultured cells and zebrafish embryos were extracted by a universal protein extraction lysis buffer (BioTeke) containing protease inhibitor cocktail (Roche). The denatured proteins were separated on 10% SDS-polyacrylamide gels and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) for immunoblot analysis. The following antibodies were used for western blotting: anti-β-catenin (1:1000 dilution, ab32572, Abcam), anti-ubiquitin (1:1000 dilution, ab7780, Abcam), anti-DVL1 (1:500 dilution, 27384-1-AP, Proteintech), anti-CK1 (1:500 dilution, 55192-1-AP, Proteintech), anti-GAPDH (1:5000 dilution, ab8245, Abcam), and anti-HA (1:200dilution, sc-805, Santa Cruz Biotechnology).

Immunofluorescence staining

Cells transfected for 48 h were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100 for 10 min, and blocked with 1% BSA for 30 min. After that, the cells were incubated with primary antibodies overnight at 4 ℃ and DyLight 488- or DyLight 594-labeled secondary antibodies at

room temperature for 1 h. To label the cell nuclei, the cells were counterstained with DAPI. Images were acquired using a laser scanning confocal microscope (Olympus, Tokyo, Japan).

Cell counting kit-8 (CCK-8) assays

The cell proliferation was analyzed by a cell counting Kit-8 (Beyotime, C0038). Briefly, the 10 μl CCK-8 solution was added to each well of 96-well plates containing transfected cells. The absorbance of each well was measured at 450 nm using a microplate reader after 2 h of incubation. The relative proliferation was determined as the fold change, which was calculated using the absorbance of each well. The results were normalized by the value of a control.

Cell cycle and apoptosis assays

For the cell cycle assay, the transfected cells were fixed with 70% ethanol overnight at 4°C and then treated with RNaseA (0.02 mg/ml) in the dark for 30 min. Next, the cells were stained with propidium iodide (PI, Sigma-Aldrich, P4864) and analyzed on a COULTER EPICS XL flow cytometer (Beckman, Krefeld, Germany). Cell apoptosis was detected by an Annexin V-Alexa Fluor 647/PI apoptosis detection kit (BD Pharmacy, Franklin Lakes, NJ). The cells were harvested and washed with 1xPBS, resuspended with 200 μl 1×binding buffer, then added 10 μl Annexin V- Alexa Fluor 647 and 5μl PI solution, incubated for 15 min at room temperature in the dark and finally, analyzed by a COULTER EPICS XL flow cytometer.

Generation of the HUWE1-overexpressing zebrafish model

HECT mRNA was synthesized *in vitro* using an mMESSAGE mMACHINE Kit (Ambion, USA). Different doses of *HECT* mRNA (50, 100 and 300 pg) were microinjected into zebrafish embryos at 0 h. The control group was injected with the same volume of 1x PBS. After microinjection, zebrafish embryos with different developmental stages were used in the following experiments.

Alcian blue staining

Alcian blue staining was carried out to investigate the cartilage structures in the head of the *HECT*-overexpressing zebrafish embryos. The method was performed according to previous research[38] and was adapted as follows. After 6 dpf, embryos were euthanized by rapid cooling of the plates on ice and fixed with 4% paraformaldehyde (PFA) for 2 h at room temperature. After washing with 1x PBS, the embryos were incubated with 100 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$ for 10 min. Subsequently, the embryos were stained with 1% alcian blue in 0.1 M HCl for 50 min at room temperature. Then, the alcian blue was removed, and the embryos were fast-washed with 0.1 M HCl. Next, the embryos were washed with 100% ethanol, 80% ethanol/10 mM $MgCl_2$, 50% ethanol/10 mM $MgCl_2$ and 25% ethanol/10 mM $MgCl_2$ for 10 min for each wash. Finally, the embryos were incubated with 25% glycerol and 0.1% KOH for 3 h and then stored in 50% glycerol in 0.1% KOH. The images were acquired by stereomicroscopy (SMZ1000, Nikon).

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software (IBM Company). Student's *t*-test was used to compare the observed indexes between the experimental groups. Pearson's correlation test and linear regression analysis were used to confirm data similarity between the two qPCR assays. A nonparametric Spearman's test was used to investigate the correlation between the scores of CARS and ABC and the relative copy numbers of *HUWE1* and the *HUWE1* mRNA expression levels. A *p*-value less than 0.05 was considered significant

Results

Association of HUWE1 CNV with ASD

The *HUWE1* copy number was analyzed using relative quantitative polymerase chain reaction (qPCR). To ensure the reliability of the results, we performed two independent qPCR analyses amplifying two fragments of *HUWE1* and using *GAPDH* and *α-tubulin* as internal controls. Pearson's correlation analysis showed that the correlation coefficient reached 1.00, suggesting a linear relationship between the data that were obtained from the two tests ($P < 0.001$). This linear relationship was confirmed using the regression coefficient hypothesis test ($P < 0.001$), and the equation was established as $y = 1.145x + 0.222$, in which the independent and dependent variables indicate the data from tests I and II, respectively. The observations suggested a good degree of data similarity between the two qPCR tests. In test I, the mean *HUWE1* copy numbers were greater in the ASD group than that in the control group (Fig. 1a). In test II, the mean *HUWE1* copy numbers were also significantly increased in the ASD group compared to the control group (Fig. 1b). To convincingly confirm the association between *HUWE1* copy numbers and ASD, we investigated the correlation between *HUWE1* copy numbers and the scores of the childhood autism rating scale (CARS) and autism behavior checklist (ABC), which are used to diagnose ASD and to evaluate the severity of ASD. Using the nonparametric Spearman's test, a positive correlation was observed between the *HUWE1* copy numbers and the ABC scores in all subjects in the ASD group but not in the control group (all subjects: ρ coefficient = 0.517, $P < 0.001$; ASD: ρ coefficient = 0.766, $P < 0.001$; control: ρ coefficient = 0.138, $P > 0.05$) (Fig. 1c-e). Similarly, the correlation between *HUWE1* copy numbers and the CARS scores was observed only in all subjects and the ASD group (all subjects: ρ coefficient = 0.499, $P < 0.001$; ASD: ρ coefficient = 0.656, $P < 0.001$; control: ρ coefficient = 0.113, $P > 0.05$) (Fig. 1f-h). According to these findings, the effect of the *HUWE1* CNV on the susceptibility to ASD is clear, and the increased copy numbers of *HUWE1* could enhance the risk of ASD.

The relationship between HUWE1 mRNA expression levels and ASD

Considering that the increased copy number of *HUWE1* is associated with ASD and the positive correlation (ρ coefficient = 0.332, $P < 0.001$) between *HUWE1* copy numbers and *HUWE1* mRNA levels was observed obviously (Fig. 2a) we hypothesized that there must be a significant difference in the expression of *HUWE1* mRNA levels between the ASD group and the control group. Remarkably, the results of Student's *t*-test showed that the *HUWE1* expression level was significantly increased in the ASD group compared to the control group (Fig. 2b, c). A positive correlation was also observed between *HUWE1*

mRNA expression levels and ABC scores (all subjects: ρ coefficient = 0.452, P < 0.001; ASD: ρ coefficient = 0.718, P < 0.001; control: ρ coefficient = 0.075, P > 0.05) (Fig. 2d-f) and CARS scores (all subjects: ρ coefficient = 0.413, P < 0.001; ASD: ρ coefficient = 0.318, P < 0.001; control: ρ coefficient = 0.03, P > 0.05) (Fig. 2g-i) in the ASD group. These data further support the conclusion that the increased gene expression level of *HUWE1* indicates an increased risk of ASD.

Influence of overexpression HUWE1 on neuronal stem cells proliferation

To further clarify the crucial role of *HUWE1* in ASD, we transiently transfected *HUWE1* plasmid into the mouse neuronal stem cells NE-4C and mouse neuroblastoma cells N2a respectively, and the increased HUWE1 protein levels were observed in these overexpressed cells by western blotting (Fig. 3a). Intriguingly, the decreased proliferation in NE-4C and N2a cells transfected with *HUWE1* plasmid was detected obviously (Fig. 3b). Moreover, the increased apoptosis was also found in the HUWE1 overexpressed NE-4C and N2a cells (Fig. 3c). Cell cycle is another factor which affects the speed of cell proliferation. Therefore, we investigated the cell cycle of cells with different treatments, and a declined G2/M phase transition was observed in HUWE1-overexpressing NE-4C and N2a cells (Fig. 3d). These data indicated the negative effect of HUWE1 overexpression on the proliferation of neural related cells. The carriers of the 16p11.2 copy-number variant, which is associated with ASD, have the abnormal neuronal size and dendrite length [24]. Thus, these results formally pointed to the possibility that *HUWE1* might somehow be involved in the growth of nervous system cells, and the elevated *HUWE1* gene dosages might disrupt the neuronal stem cells proliferation, which might contribute to ASD.

HUWE1-mediated ubiquitination of key molecules in the Wnt/β-catenin signaling pathway

HUWE1 is a vital regulator of the Wnt/β-catenin pathway [25]; therefore, we investigated whether HUWE1 is involved in neurogenesis via the Wnt/β-catenin signaling pathway. To obtain a mechanistic insight into the role of HUWE1 in the Wnt/β-catenin pathway, we investigated the interactions of HUWE1 and β-catenin, dishevelled segment polarity protein 1 (DVL1) or casein kinase 1 alpha 1 (CK1) by Co-IP. The bindings of exogenous/endogenous HUWE1 between β-catenin, DVL1 and CK1 were observed in 293T cells, respectively (Fig. 4a-d). Additionally, the interactions between HUWE1 and β-catenin, DVL1 and CK1 were confirmed by immunofluorescence experiments (Fig. 4e). To address the effect of HUWE1 on the expression of β-catenin, DVL1 and CK1, we overexpressed *HUWE1* in 293T cells, and the western blotting results showed a significant reduction in the β-catenin, DVL1 and CK1 protein levels in HUWE1-overexpressing cells compared to the control (Fig. 5a-c). Due to *HUWE1* encoding an E3 ubiquitin ligase, we hypothesized that the function of HUWE1 was involved in the posttranscriptional regulation of β-catenin, DVL1 and CK1 expression. Remarkably, we observed that the overexpression of HUWE1 significantly enhanced the ubiquitination of endogenous β-catenin, DVL1 and CK1 in 293T cells (Fig. 5a-c). It has been reported that Lithium chloride (LiCl) can be as an activator of Wnt/β-catenin pathway in

the treatment of some neurodevelopmental disorders[26, 27]. Therefore, we treated the cells with 1mM LiCl in the culture medium for 24h, and found that LiCl can rescue the inhibitory effect of overexpression *HUWE1* on neuronal stem cells proliferation associated with cells apoptosis and cell cycle (Fig. 3B). These findings strongly supported that HUWE1 functions as a negative regulator of the Wnt/β-catenin signaling pathway that is involved in neurodevelopment.

An elevated huwe1 dosage leads to embryonic developmental defects in zebrafish

To further confirm the pivotal role of *HUWE1* in neurodevelopment, *in vivo* experiments were performed on zebrafish, which is the ideal model organism to investigate early embryonic development. The Zebrafish Information Network (ZFIN) database (http://zfin.org) indicates that the expression of huwe1 is restricted to the brain. *HUWE1* is highly evolutionarily conserved. Notably, human HUWE1 shares an 82.3% protein identity with its orthologs in zebrafish (https://www.ncbi.nlm.nih.gov). Furthermore, we examined the mRNA expression pattern of *huwe1* at different developmental stages with RT-PCR. The zebrafish *huwe1* mRNA reached the peak expression level at 10 h postfertilization (hpf), which is the stage that the brain begins to develop (Fig. 6a). The expression pattern of *huwe1* in zebrafish indicated that *huwe1* might play a crucial role in zebrafish neurodevelopment. Then, the mRNA of HECT domain, the most important functional domain of HUWE1, was synthesized and injected into zebrafish embryos at 0 h at different dosages (50, 100 and 300 pg). The mRNA and protein of the experimental groups (50 and 100 pg) as well as the control group were harvested at the shield stage. The embryos in the 300 pg group were all dead. The RT-PCR and western blotting results showed that the expression level of huwe1 was increased in a dose-dependent manner compared with the control group (Fig. 6b), indicating the efficiency of the microinjection for subsequent experiments. Remarkably, the decreased protein expression levels of ck1, dvl1 and β-catenin were observed in the experimental groups compared with the control group by western blotting (Fig. 6c), suggesting that the effect of huwe1 on the Wnt/β-catenin signaling pathway was also present in the zebrafish.

Subsequently, we observed the zebrafish phenotypes of different treatment groups in different developmental stages. Intriguingly, a developmental delay was observed in the HECT domain-overexpressing groups compared to the control group and was more serious with the increase in the expression level. Abnormal phenotypes were observed in the experimental groups during zebrafish development (Fig. 6d). Specifically, at 10 hpf, embryos injected with HECT mRNA showed obscure or no polster compared to the control, and the normal polster was most prominent near the animal pole in the prospective head region, where the head formation occurs. The regular brain rudiment and the eyes were clearly delineated in the control group at 14 hpf, whereas the shape of the head and size of the eyes were abnormal in the experimental groups. Notably, at 18 hpf, the experimental groups had a small or irregular forebrain, and somites were not noticeable compared to the control group. At 22 hpf, pigmentation was observed in zebrafish from the control group, while little or no pigmentation was observed in the experimental groups. Furthermore, the experimental groups also had an aberrant head morphology. Strikingly, at 3 dpf, the developmental defects were more noticeable in the experimental groups compared to the control group. Specifically, overexpression of HECT resulted in a deformed head, unconsumed yolk

sac, small eyes, cardiac edema or curved spinal cord in zebrafish from the experimental groups (Fig. 7a, b). Moreover, the 100 pg group exhibited more severe phenotypes than the 50 pg group, indicating that *huwe1* impeded embryonic development in a dose-dependent manner, which was consistent with the observations in ASD patients. Expectedly, the LiCl can relieve the developmental malformation of zebrafish caused by injection HECT mRNA. Next, we assessed the head morphology of injected zebrafish by staining cartilage with alcian blue at 5 dpf. Striking differences in the craniofacial skeleton were observed between the experimental groups and control group, such as reduced mandibular length (the distance between ceratohyal cartilage (ch) and Meckel's cartilage (Mk)) and abnormal angulation of the ceratohyal cartilage in the experimental groups (Fig. 7c). Similarly, the anomalous head morphologies could be rescued by LiCl. Thus, all the data from the zebrafish model suggested that overexpression of *huwe1* could result in dose-dependent and consistent alterations in zebrafish morphogenesis, such as small eyes, a deformed head, and a curly spinal cord, indicating the crucial role of *huwe1* in the development of the central nervous system (CNS). Overall, the anomalies were more severe than those observed in ASD patients.

Discussion

ASD is a complex neurodevelopmental disease, and the pathogenesis of autism remains elusive. Several studies have shown that variants of *HUWE1* are related to neurodevelopmental disorders, whereas the specific role of *HUWE1* in ASD and convincing evidence is insufficient. In this study, we demonstrated that the increased gene dosage of *HUWE1* plays a crucial role in the incidence of ASD. Particularly, increases in the copy number and enhanced mRNA levels of *HUWE1* were significantly observed in the ASD group compared to the control group. Furthermore, HECT-overexpressing zebrafish models showed abnormalities in neurodevelopment, supporting that *HUWE1* is the dosage-sensitive gene for which overexpression could increase the risk of ASD. Moreover, our *in vivo* and *in vitro* experiments both showed a key mechanism that HUWE1 could regulate the Wnt/β-catenin signaling pathway via facilitating ubiquitin-mediated β-catenin, DVL1, and CK1 degradation involved in the development of the nervous system.

Carriers of recurrent copy number variation have an increased risk for developing ASD [28–33]. The most prevalent cytogenetic abnormality in ASD is duplications involving the chromosomal region 15q11-15q13, accounting for 1–2% of cases [34, 35]. Considering the clear sex ratio disequilibrium in ASD, CNVs in the X chromosome were also investigated. The X chromosome contains the largest number of genes expressed in the brain, most of which are dosage-sensitive genes [36], yet the known CNVs associated with ASD are limited thus far. *HUWE1* located on the X chromosome is ubiquitously expressed, with high expression levels in human brain tissues. It has been suggested that normal ubiquitin proteasome activity is essential for neuronal cell differentiation [37]. HUWE1, as a key E3 ubiquitin ligase, is fundamental for neurodevelopment [16–18, 38]. Thus, there might be a potential association of *HUWE1* CNVs and ASD.

To systematically investigate *HUWE1* CNVs that confer a risk to ASD, we first specifically assessed *HUWE1* CNVs in 200 unrelated healthy male controls and 200 male patients with ASD. Importantly, our

results indicated that there was a significant difference in the *HUWE1* copy number distribution between ASD patients and normal controls, suggesting that an increased *HUWE1* copy number indicates an increased susceptibility to ASD. Intriguingly, the correlations were observed between the *HUWE1* copy number and the scores of CARS/ABC in ADS patients, suggesting that the *HUWE1* copy number is associated with the severity of ASD. A previous study reported that the size of the copy number has a potential effect on the phenotypic severity [39]. Indeed, ASD patients with more *HUWE1* copy numbers showed more severe phenotypes in communication obstacles and stereotyped and repetitive behaviors (data not shown). We analyzed *HUWE1* mRNA levels in ASD patients and controls, and observed that the average *HUWE1* mRNA level of the ASD group was approximately 1.5-fold greater than that of controls, and the levels were also correlated with disease severity in the male ASD patients. We further investigated HUWE1 protein levels in some ASD patients and controls and observed that the leukomonocyte from ASD patients with *HUWE1* duplication had increased HUWE1 protein levels than leukomonocyte from controls (data not present). The relationship between the *HUWE1* CNV and the expression level is consistent with that of *MECP2*, another dosage-sensitive gene associated with ASD in the X chromosome [13]. The crucial role of the *HUWE1* CNV in ASD should be confirmed by further investigation.

Predictably, the impaired effect of increased *HUWE1* expression levels on the developing nervous system was observed in the zebrafish model. Strikingly, the abnormal phenotypes were dose dependent. To date, only one animal model (*Drosophila melanogaster*) has been used to investigate the increased Huwe1 expression level on the development of the nervous system, which is associated with ID. However, only a disturbed branching phenotype was observed in the *Drosophila melanogaster* model [22]. Huwe1 in *Drosophila melanogaster* only shares a 45.8% protein identity with its orthologs in humans, suggesting that the function of HUWE1 might be different between humans and *Drosophila melanogaster*. Therefore, the abnormal phenotype in the brain was not observed in the *Drosophila melanogaster* model. In our study, we used zebrafish as the HUWE1-overexpressing model because of the high homology of HUWE1 between humans and zebrafish. Furthermore, the functions of many dosage-sensitive genes associated with neurodevelopmental disorders have been validated in the zebrafish model [40–42]. This is the first study to show the crucial role of *HUWE1* in the development of the nervous system in the zebrafish model.

It has been suggested that normal proteasome activity is essential during neuronal cell differentiation [37]. Indeed, our study provided strong evidence that the E3 ubiquitin ligase *HUWE1* plays a central role in neurodevelopment. In Drosophila, the Wnt/β-catenin signaling pathway has been suggested to be associated with axonal branching [22]. Lorenz et al. [43] reported that the Wnt/β-catenin signaling pathway is essential for the proliferation and differentiation of cerebellar granule neuron precursors. Therefore, we hypothesized that *HUWE1* is involved in neurodevelopment by regulating the Wnt/β-catenin signaling pathway. *In vitro*, we observed that overexpressing *HUWE1* promoted the ubiquitination degradation of CK1, β-catenin and DVL1, which are the main mediators in the Wnt/β-catenin signaling pathway. Similar results were observed in the huwe1-overexpressing zebrafish. Disturbances in the Wnt/β-catenin signaling pathway are considered to lead to defects in the differentiation of mesenchymal stem cells, developmental disorders and cancer [44]. In the present study, we first proposed that the imbalance

of the Wnt/β-catenin signaling pathway caused abnormal expression of HUWE1, increasing the risk of ASD.

The abnormalities of neural development were clear in the zebrafish model, but neurobehavioral tests were not carried out because of severe physical deformities. Therefore, to better elucidate the association of *HUWE1* with ASD behaviors, such as social interactions, communication, and stereotyped and repetitive behaviors, a mouse or rat model should be considered for future studies. Furthermore, in HUWE1 expression analysis, we used mRNA expression, but few protein levels to reflect the expression of HUWE1 in blood, because the expression of HUWE1 in brain was not determined due to the sample unavailable. Nevertheless, the good correlation of CNV level and mRNA expression with the parameters related to autistic traits still justify the in-depth molecular study using in vitro and zebrafish model.

Conclusion

In conclusion, the copy number of *HUWE1* is significantly increased in ASD children compared to that in normal controls, which is consistent with the results that the overexpression of HUWE1 leads to developmental defects in zebrafish. All these data indicate that strict regulation of HUWE1 is critical for the appropriate development and maintenance of neuronal function. Future clinical, genetic, and functional studies are warranted to further delineate the phenotypic spectrum and etiology of *HUWE1*-associated disorders. Further cases with *HUWE1* mutations are necessary to determine if a genotype-phenotype correlation can be made.

Abbreviations

ASD：Autistic spectrum disorder；CNV：The copy number variation; HUWE1: the E3 ubiquitin protein ligase 1; NE-4C: mouse neural stem cells; DSM-5: the Diagnostic and Statistical Manual of Mental Disorders 5th ed; CNVs: mutations or copy number variations; NLGN4X: the most associated genes are neuroligin 4 X-linked; NLGN3: neuroligin 3; RPL10: ribosomal protein L10; MECP2: methyl-CpG binding protein 2; qPCR: relative quantitative polymerase chain reaction; CARS: the childhood autism rating scale; ABC: autism behavior checklist; DVL1: dishevelled segment polarity protein 1; CK1: casein kinase 1 alpha 1.

Declarations

Acknowledgements

We thank the study participants who provided the blood samples and clinical information.

Author contributions

HW,YL,and LJ performed the experiments and data analysis. WX and JX collected the blood and the volunteers' clinical information. XZ,XW,BX,and JC performed the PCR analyses. XW carried out the Flow

Cytometric Analysis. AD performed the neuronal induction. HW and WX wrote the manuscript. WX and JX supervised the study.

Funding

This work was supported by National Natural Science Foundation of China (Grant No. 31701085)

Availability of data and materials

Please contact author for data requests.

Declarations

Ethics approval and consent to participate

This study was approved by the ethics committee of West China Second University Hospital (Project number: 2019019).

Consent for publication

Not applicable.

Conflict of Interests

The authors declare no conflict of interest.

Author details

[1]Research Core Facility of West China Hospital , Sichuan University, Chengdu Sichuan, 610041,P.R.China. [2]The First People's Hospital Of Longquanyi District□Chengdu□West China Second University Hospital, Sichuan University. [3]The 2nd affiliated Hospital of Chengdu Medical College Nuclear Industry 416 Hospital, Chengdu 610051, China. [4]Department of Obstetrics/Gynecology, Joint Laboratory of Reproductive Medicine (SCU-CUHK), Key Laboratory of Obstetric, Gynecologic and Pediatric Diseases and Birth Defects of Ministry of Education, West China Second University Hospital, Sichuan University, Chengdu 610041, China. [5]Guangdong Provincial Key Laboratory of Stomatology, Guanghua School of Stomatology, Hospital of Stomatology, Institute of Stomatological Research, Sun Yat-sen University, Guangzhou, Guangdong, China. [6]BioTalentum Ltd., H-2100 Gödöllő, Hungary. [7]Molecular Animal Biotechnology Laboratory, Szent Istvan University, H-2100 Gödöllő, Hungary. [8]The Third People's Hospital of Chengdu, Chengdu 610031, China

References

1. Battle DE. Diagnostic and Statistical Manual of Mental Disorders (DSM). Codas. 2013;25:191-2.

2. WHO Staff, et al. The ICD-10 classification of mental and behavioural disorders: clinical descriptions and diagnostic guidelines Organisation mondiale de la santé, World Health Organization.1992.
3. Prevalence of autism spectrum disorder among children aged 8 years - autism and developmental disabilities monitoring network, 11 sites, United States, 2010. MMWR Surveill Summ. 2014;63:1-21.
4. Elsabbagh M, Divan G, Koh YJ, Kim YS, Kauchali S, Marcín C, et al. Global prevalence of autism and other pervasive developmental disorders. 2012;Autism Res 5:160-79.
5. Freitag CM. The genetics of autistic disorders and its clinical relevance: a review of the literature. Mol Psychiatry. 2007;12:2-22.
6. Abrahams BS,Geschwind DH. Advances in autism genetics: on the threshold of a new neurobiology. Nat Rev Genet. 2008;9:341-55.
7. Freitag CM, Staal W, Klauck SM, Duketis E,Waltes R. Genetics of autistic disorders: review and clinical implications. Eur Child Adolesc Psychiatry. 2010;19:169-78.
8. Jamain S, Quach H, Betancur C, Råstam M, Colineaux C, Gillberg IC, et al. Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism. Nat Genet. 2003;34:27-9.
9. Gillberg C, Cederlund M, Lamberg K,Zeijlon L. Brief report: "the autism epidemic". The registered prevalence of autism in a Swedish urban area. J Autism Dev Disord. 2006;36:429-35.
10. Gong X, Delorme R, Fauchereau F, Durand CM, Chaste P, Betancur C, et al. An investigation of ribosomal protein L10 gene in autism spectrum disorders. BMC Med Genet. 2009;10:7.
11. Celestino-Soper PB, Shaw CA, Sanders SJ, Li J, Murtha MT, Ercan-Sencicek AG, et al. Use of array CGH to detect exonic copy number variants throughout the genome in autism families detects a novel deletion in TMLHE. Hum Mol Genet. 2011;20:4360-70.
12. Chaudhry A, Noor A, Degagne B, Baker K, Bok LA, Brady AF, et al. Phenotypic spectrum associated with PTCHD1 deletions and truncating mutations includes intellectual disability and autism spectrum disorder. Clin Genet. 2015;88:224-33.
13. Ramocki MB, Peters SU, Tavyev YJ, Zhang F, Carvalho CM, Schaaf CP, et al. Autism and other neuropsychiatric symptoms are prevalent in individuals with MeCP2 duplication syndrome. Ann Neurol. 2009;66:771-82.
14. Gu J, Dubner R, Fornace AJ, Jr.,Iadarola MJ. UREB1, a tyrosine phosphorylated nuclear protein, inhibits p53 transactivation. Oncogene. 1995;11:2175-8.
15. Zhong Q, Gao W, Du F,Wang X. Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis. Cell. 2005;121:1085-95.
16. Froyen G, Corbett M, Vandewalle J, Jarvela I, Lawrence O, Meldrum C, et al. Submicroscopic duplications of the hydroxysteroid dehydrogenase HSD17B10 and the E3 ubiquitin ligase HUWE1 are associated with mental retardation. Am J Hum Genet. 2008;82:432-43.
17. Zhao X, D DA, Lim WK, Brahmachary M, Carro MS, Ludwig T, et al. The N-Myc-DLL3 cascade is suppressed by the ubiquitin ligase Huwe1 to inhibit proliferation and promote neurogenesis in the developing brain. Dev Cell. 2009;17:210-21.

18. D'Arca D, Zhao X, Xu W, Ramirez-Martinez NC, Iavarone A,Lasorella A. Huwe1 ubiquitin ligase is essential to synchronize neuronal and glial differentiation in the developing cerebellum. Proc Natl Acad Sci U S A. 2010;107:5875-80.
19. Froyen G, Belet S, Martinez F, Santos-Rebouças CB, Declercq M, Verbeeck J, et al. Copy-number gains of HUWE1 due to replication- and recombination-based rearrangements. Am J Hum Genet. 2012;91:252-64.
20. Madrigal I, Rodríguez-Revenga L, Armengol L, González E, Rodriguez B, Badenas C, et al. X-chromosome tiling path array detection of copy number variants in patients with chromosome X-linked mental retardation. BMC Genomics. 2007;8:443.
21. Santos-Rebouças CB, de Almeida LG, Belet S, Dos Santos SR, Ribeiro MG, da Silva AF, et al. Novel microduplications at Xp11.22 including HUWE1: clinical and molecular insights into these genomic rearrangements associated with intellectual disability. J Hum Genet. 2015;60:207-11.
22. Vandewalle J, Langen M, Zschätzsch M, Nijhof B, Kramer JM, Brems H, et al. Ubiquitin ligase HUWE1 regulates axon branching through the Wnt/β-catenin pathway in a Drosophila model for intellectual disability. PLoS One. 2013;8:e81791.
23. Nava C, Lamari F, Héron D, Mignot C, Rastetter A, Keren B, et al. Analysis of the chromosome X exome in patients with autism spectrum disorders identified novel candidate genes, including TMLHE. Transl Psychiatry. 2012;2:e179.
24. Deshpande A, Yadav S, Dao DQ, Wu ZY, Hokanson KC, Cahill MK, et al. Cellular Phenotypes in Human iPSC-Derived Neurons from a Genetic Model of Autism Spectrum Disorder. Cell Rep. 2017;21:2678-2687.
25. de Groot RE, Ganji RS, Bernatik O, Lloyd-Lewis B, Seipel K, Šedová K, et al. Huwe1-mediated ubiquitylation of dishevelled defines a negative feedback loop in the Wnt signaling pathway. Sci Signal. 2014;7:ra26.
26. Meng L, Wang X, Torensma R, Von den Hoff JW,Bian Z. Lithium inhibits palatal fusion and osteogenic differentiation in palatal shelves in vitro. Arch Oral Biol. 2015;60:501-7.
27. Manji HK, Moore GJ,Chen G. Clinical and preclinical evidence for the neurotrophic effects of mood stabilizers: implications for the pathophysiology and treatment of manic-depressive illness. Biol Psychiatry. 2000;48:740-54.
28. Charbonnel B. What a psychiatrist needs to know about diabetes. Eur Psychiatry. 2005;20 Suppl 4:S330-4.
29. Vorstman JA, Staal WG, van Daalen E, van Engeland H, Hochstenbach PF,Franke L. Identification of novel autism candidate regions through analysis of reported cytogenetic abnormalities associated with autism. Mol Psychiatry. 2006;11:1, 18-28.
30. Sebat J, Lakshmi B, Malhotra D, Troge J, Lese-Martin C, Walsh T, et al. Strong association of de novo copy number mutations with autism. Science. 2007;316:445-9.
31. Szatmari P, Paterson AD, Zwaigenbaum L, Roberts W, Brian J, Liu XQ, et al. Mapping autism risk loci using genetic linkage and chromosomal rearrangements. Nat Genet. 2007;39:319-28.

32. Marshall CR, Noor A, Vincent JB, Lionel AC, Feuk L, Skaug J, et al. Structural variation of chromosomes in autism spectrum disorder. Am J Hum Genet. 2008;82:477-88.
33. Weiss LA, Shen Y, Korn JM, Arking DE, Miller DT, Fossdal R, et al. Association between microdeletion and microduplication at 16p11.2 and autism. N Engl J Med. 2008;358:667-75.
34. Bolton PF, Dennis NR, Browne CE, Thomas NS, Veltman MW, Thompson RJ, et al. The phenotypic manifestations of interstitial duplications of proximal 15q with special reference to the autistic spectrum disorders. Am J Med Genet. 2001;105:675-85.
35. Schroer RJ, Phelan MC, Michaelis RC, Crawford EC, Skinner SA, Cuccaro M, et al. Autism and maternally derived aberrations of chromosome 15q. Am J Med Genet. 1998;76:327-36.
36. Nguyen DK,Disteche CM. High expression of the mammalian X chromosome in brain. Brain Res. 2006;1126:46-9.
37. Nahreini P, Andreatta C,Prasad KN. Proteasome activity is critical for the cAMP-induced differentiation of neuroblastoma cells. Cell Mol Neurobiol. 2001;21:509-21.
38. Vriend J, Ghavami S,Marzban H. The role of the ubiquitin proteasome system in cerebellar development and medulloblastoma. Mol Brain. 2015;8:64.
39. Shen Y, Yan Y, Liu Y, Zhang S, Yang D, Zhang P, et al. A significant effect of the TSPY1 copy number on spermatogenesis efficiency and the phenotypic expression of the gr/gr deletion. Hum Mol Genet. 2013;22:1679-95.
40. Rooryck C, Diaz-Font A, Osborn DP, Chabchoub E, Hernandez-Hernandez V, Shamseldin H, et al. Mutations in lectin complement pathway genes COLEC11 and MASP1 cause 3MC syndrome. Nat Genet. 2011;43:197-203.
41. Beunders G, Voorhoeve E, Golzio C, Pardo LM, Rosenfeld JA, Talkowski ME, et al. Exonic deletions in AUTS2 cause a syndromic form of intellectual disability and suggest a critical role for the C terminus. Am J Hum Genet. 2013;92:210-20.
42. Fan Y, Yin W, Hu B, Kline AD, Zhang VW, Liang D, et al. De Novo Mutations of CCNK Cause a Syndromic Neurodevelopmental Disorder with Distinctive Facial Dysmorphism. Am J Hum Genet. 2018;103:448-455.
43. Lorenz A, Deutschmann M, Ahlfeld J, Prix C, Koch A, Smits R, et al. Severe alterations of cerebellar cortical development after constitutive activation of Wnt signaling in granule neuron precursors. Molecular and cellular biology. 2011;31:3326-3338.
44. Lorenz A, Deutschmann M, Ahlfeld J, Prix C, Koch A, Smits R, et al. Severe alterations of cerebellar cortical development after constitutive activation of Wnt signaling in granule neuron precursors. Molecular and cellular biology. 2011;31:3326-3338.
45. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B,Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn. 1995;203:253-310.

Figures

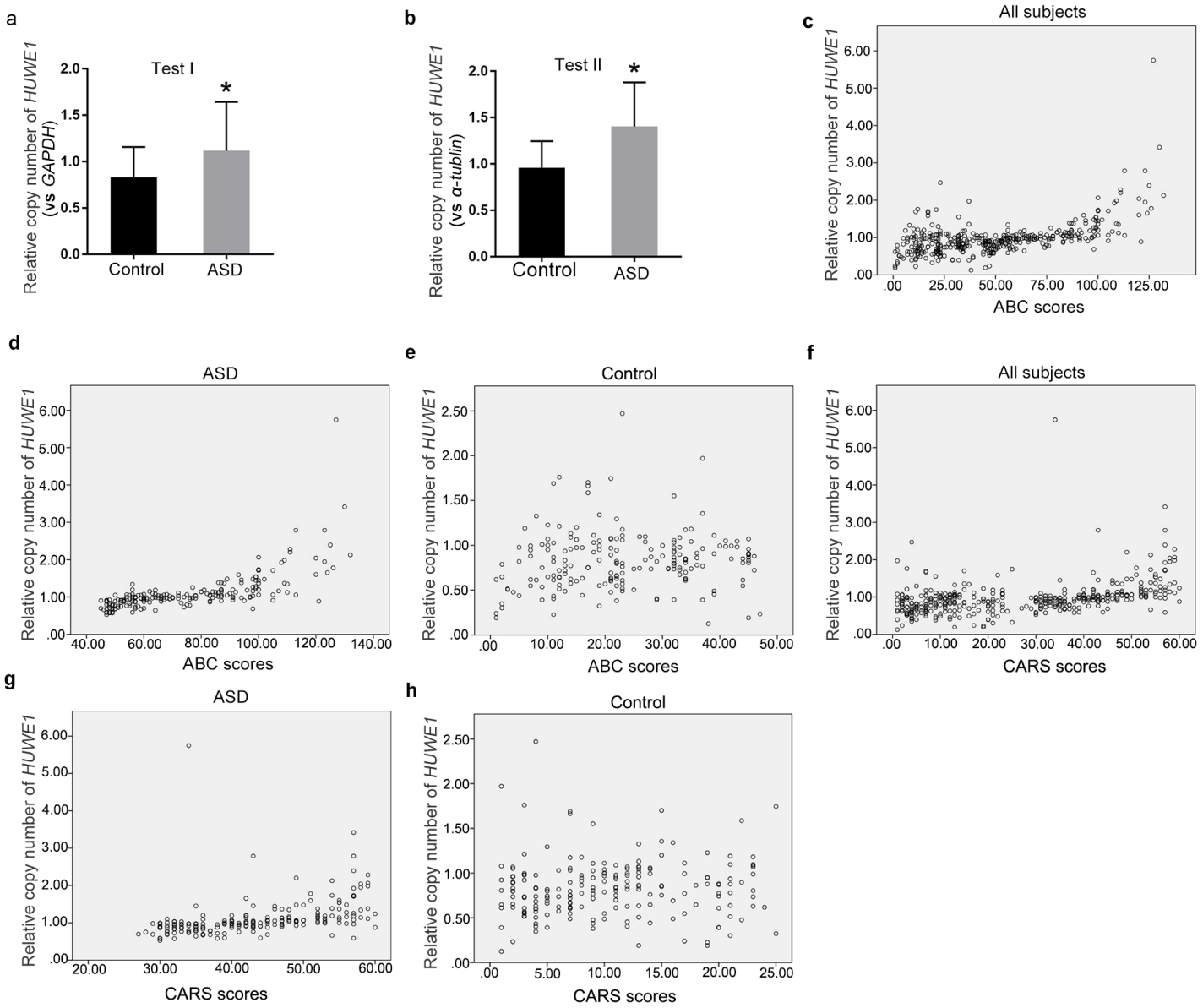

Figure 1

The correlation between the HUWE1 copy number and ASD. (a,b)RT-PCR showed significant differences in the relative copy number of HUWE1 between the ASD group and the control group in test I (a) and teast II (b). The results are presented as the mean ± s.d. (n = 3). * $p < 0.05$ according to Student's t-test. c, A scatter diagram showing a positive correlation between the HUWE1 copy number and the ABC scores of the entire study population, including ASD patients and controls (ρ coefficient = 0.517, P <0.001). d, A scatter diagram showing a positive correlation between the HUWE1 copy number and the ABC scores of the ASD patients (ρ coefficient = 0.766, P <0.001). e, A scatter diagram showing the correlation in the control group. No correlation between the HUWE1 copy number and the ABC scores was observed (ρ coefficient = 0.138, P >0.05). f, A scatter diagram showing a positive correlation between the HUWE1 copy number and the CARS scores of the entire study population, including ASD patients and controls (ρ coefficient = 0.499, P <0.001). g, A scatter diagram showing a positive correlation between the HUWE1

copy number and the CARS scores of the ASD patients (ρ coefficient = 0.656, P <0.001). h, A scatter diagram showing the correlation in the control group. No correlation between the HUWE1 copy number and the CARS scores was observed (ρ coefficient = 0.113, P >0.05).

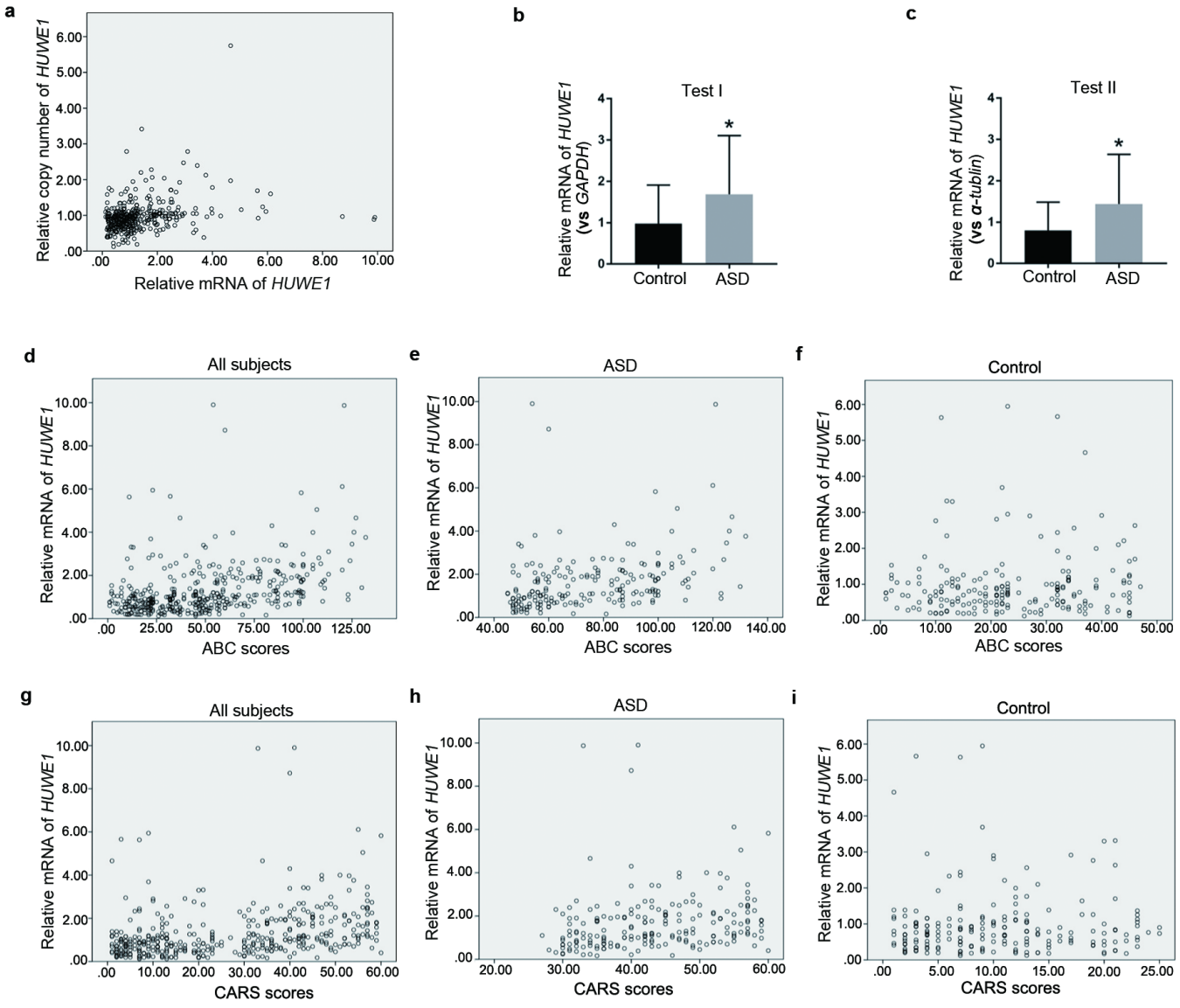

Figure 2

The relationship between HUWE1 mRNA expression levels and ASD. a, A scatter diagram showing a positive correlation between the HUWE1 mRNA levels and HUWE1 copy numbers (ρ coefficient = 0.332, P <0.001). Increased HUWE1 mRNA expression levels were observed in the ASD group compared to the control group in test I (b) and test II (c). The results are presented as the mean ± s.d. (n = 3). * p < 0.05 according to Student's t-test. d, A scatter diagram showing a positive correlation between the HUWE1 mRNA expression levels and the ABC scores of the entire study population, including ASD patients and controls (ρ coefficient = 0.452, P <0.001). e, A scatter diagram showing a positive correlation between the HUWE1 mRNA expression levels and the ABC scores of the ASD patients (ρ coefficient = 0.718, P <0.001).

f, A scatter diagram showing the correlation in the control group. No correlation between the HUWE1 mRNA expression levels and the ABC scores was observed (ρ coefficient = 0.075, P >0.05). g, A scatter diagram showing a positive correlation between the HUWE1 mRNA expression levels and the CARS scores of the entire study population, including ASD patients and controls (ρ coefficient = 0.413, P <0.001). h, A scatter diagram showing a positive correlation between the HUWE1 mRNA expression levels and the CARS scores of the ASD patients (ρ coefficient = 0.318, P <0.001). i, A scatter diagram showing the correlation in the control group. No correlation between the HUWE1 mRNA expression levels and the CARS scores was observed (ρ coefficient = 0.03, P >0.05).

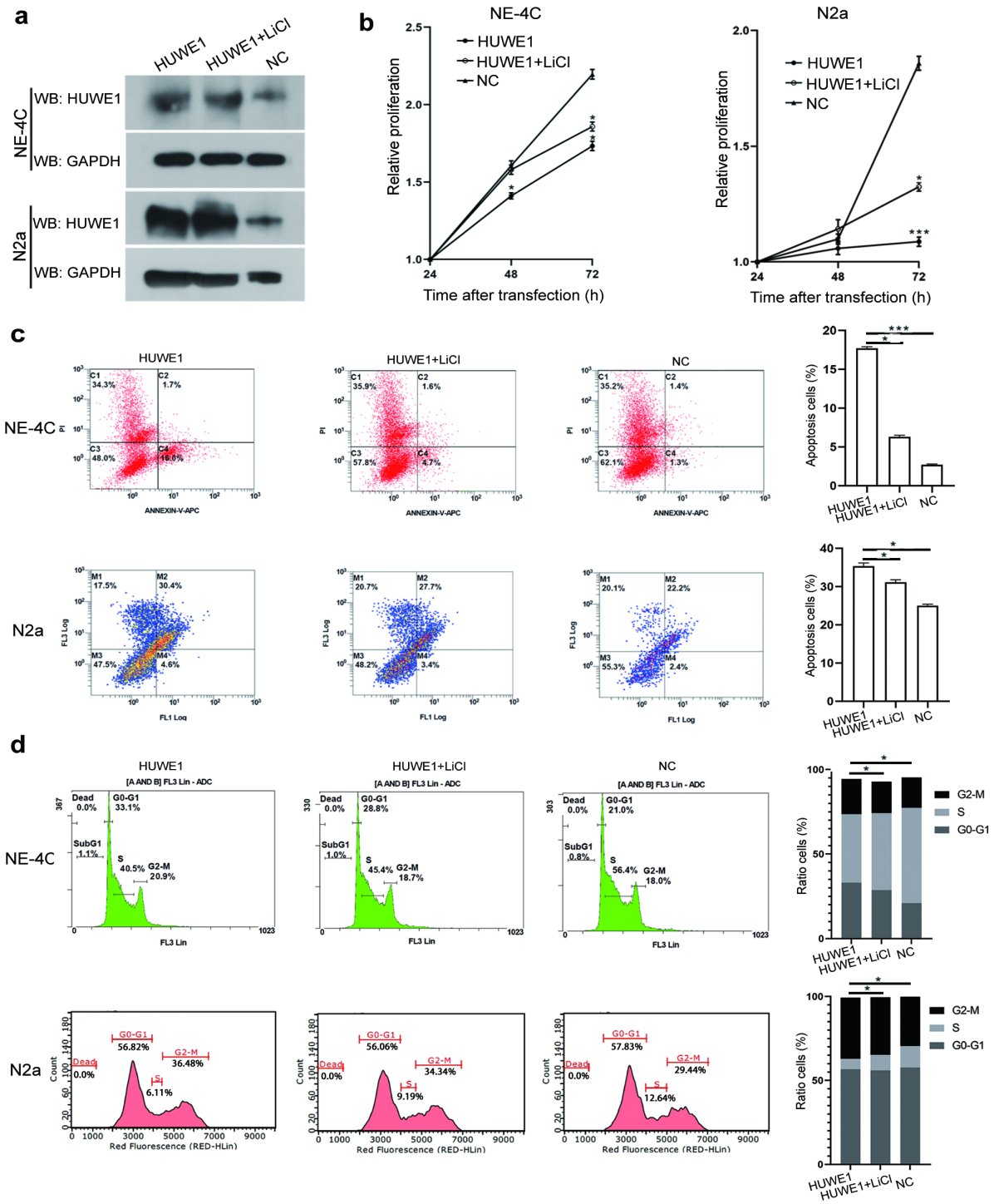

Figure 3

HUWE1 promotes neuronal stem cells proliferation and effects neurons morphology. a, The western blotting showed the overexpressed HUWE1 in NE-4C and N2a cells. b, CCK-8 assays showed that the overexpression of HUWE1 could inhibit NE-4C and N2a cells proliferation, and Licl could rescue the inhibit effect caused by HUWE1. c, Cell apoptosis assays showed that the overexpression of HUWE1 could increase cell apoptosis and Licl could impair this negative influence induced by HUWE1. Columns

showing the percentages of apoptotic NE-4C and N2a cells and the alteration in the percentage of apoptosis cell after different treatments. d, Cell cycle assays showed that HUWE1 could suppress the G2/M phase transition in cell cycle. Columns showing the percentages of G0/G1, S and G2/M phase cells of NE-4C and N2a cells and the alteration in the percentage of G2/M phase population of the two cells after different treatments. Data are presented as the mean ± S.D. (n = 3, *p < 0.05 and ***p < 0.01).

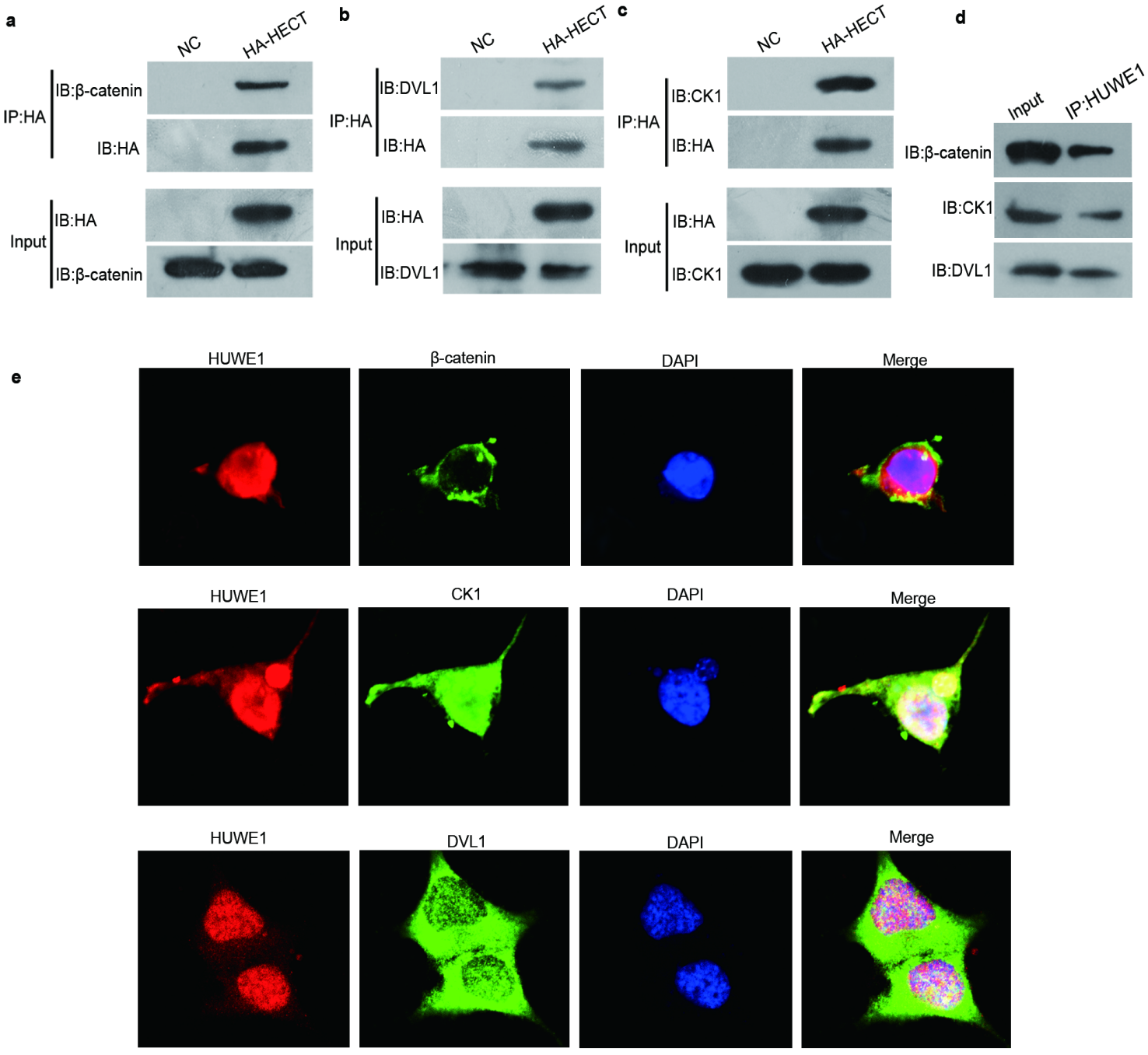

Figure 4

HUWE1 physically interacts with β-catenin, CK1 and DVL1. a, The Co-IP results showed that HA-HECT, the domain of HUWE1, interacted with β-catenin in 293T cells. b, Exogenous HA-HECT could bind to CK1. c, An interaction between HA-HECT and DVL1 was observed in 293T cells by Co-IP. d, The interaction of

endogenous HUWE1 with β-catenin, CK1 and DVL1. e, Immunofluorescence assays showed the colocalization of HA-HECT and β-catenin, CK1 or DVL1.

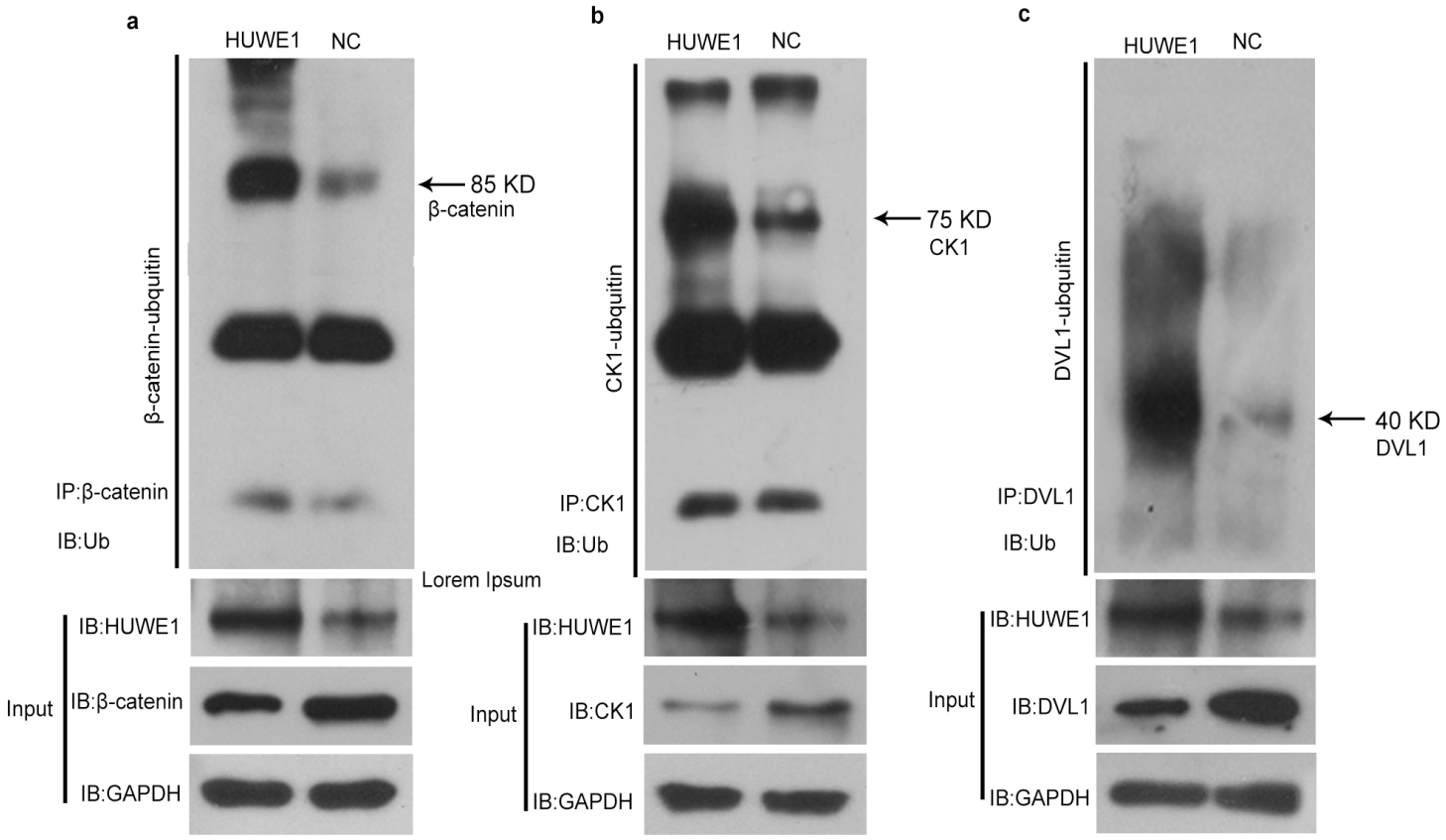

Figure 5

HUWE1 induces the ubiquitin-mediated degradation of β-catenin, CK1 and DVL1. a, Overexpression of HUWE1 promoted a notable decrease in the β-catenin protein level by ubiquitin-mediated degradation. b, HUWE1 increased CK1 ubiquitination degradation. c, DVL1 was degraded by HUWE1 through ubiquitination pathway.

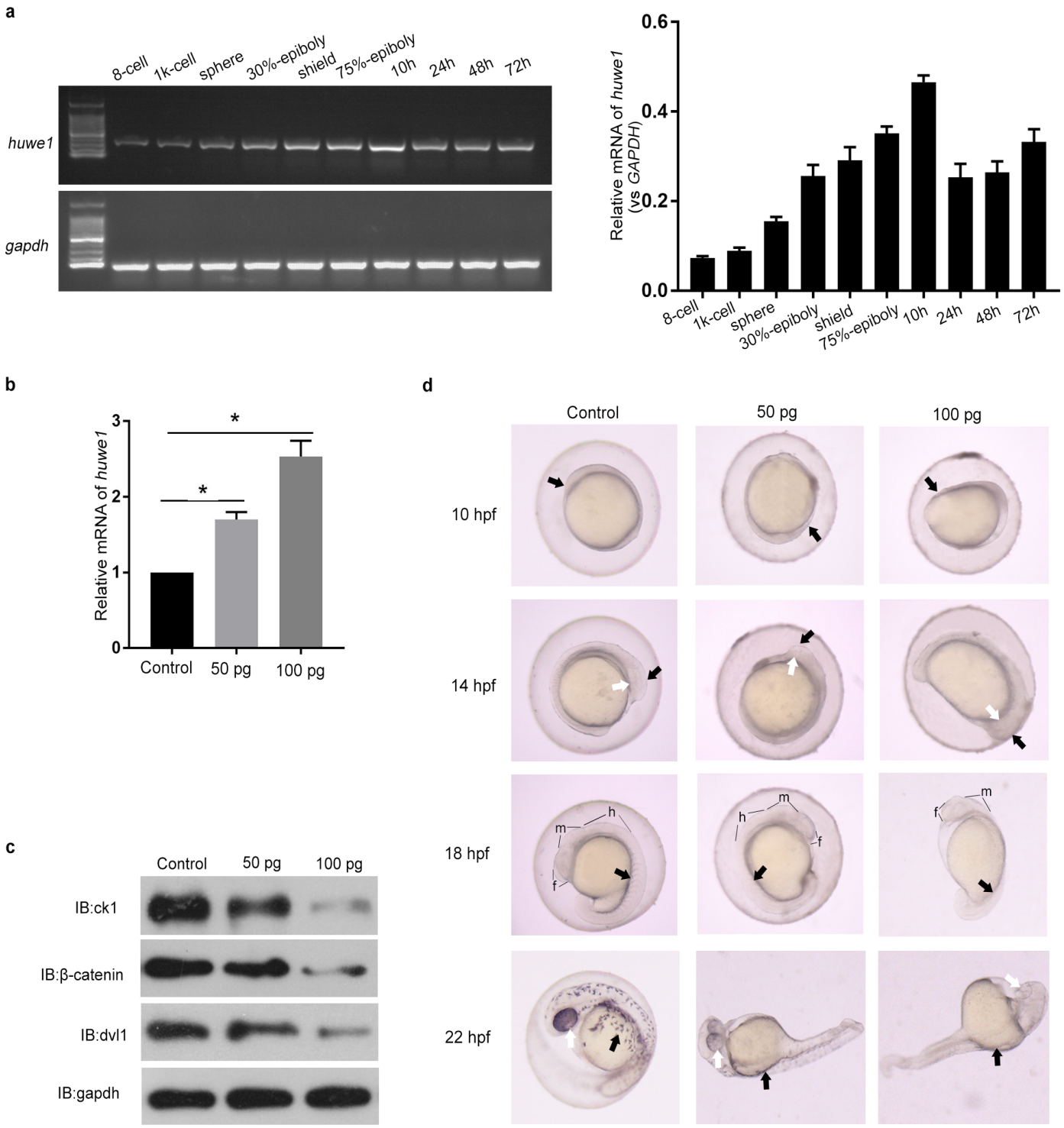

Figure 6

Overexpression of HECT leads to developmental defects in zebrafish. a, Huwe1 expression in the zebrafish embryo at various timepoints postfertilization. A high expression level of Huwe1 was observed at 10 hpf by RT-PCR. b, The overexpression efficiency of 50 pg and 100 pg HECT was analyzed by RT-PCR. The results are presented as the mean ± s.d. (n = 3). * $p < 0.05$ according to Student's t-test. c, Overexpression of HECT induced β-catenin, ck1 and dvl 1 degradation in vivo. d, Abnormalities were

observed in developing zebrafish embryos with HECT overexpression. At 10 hpf, polster (black arrowhead) was absent in the 50 pg and 100 pg groups. At 14 hpf, defective development in the head (black arrowhead) and eyes (white arrowhead) was observed in the huwe1-overexpressing groups. At 18 hpf, the experimental groups showed smaller forebrains (f) than the control group, and the somites (black arrowhead) were also absent. f, forebrains; h, hindbrain; m, midbrain. At 22 hpf, small eyes (white arrowhead) and pigmentation defects (black arrowhead) were observed in the experimental groups.

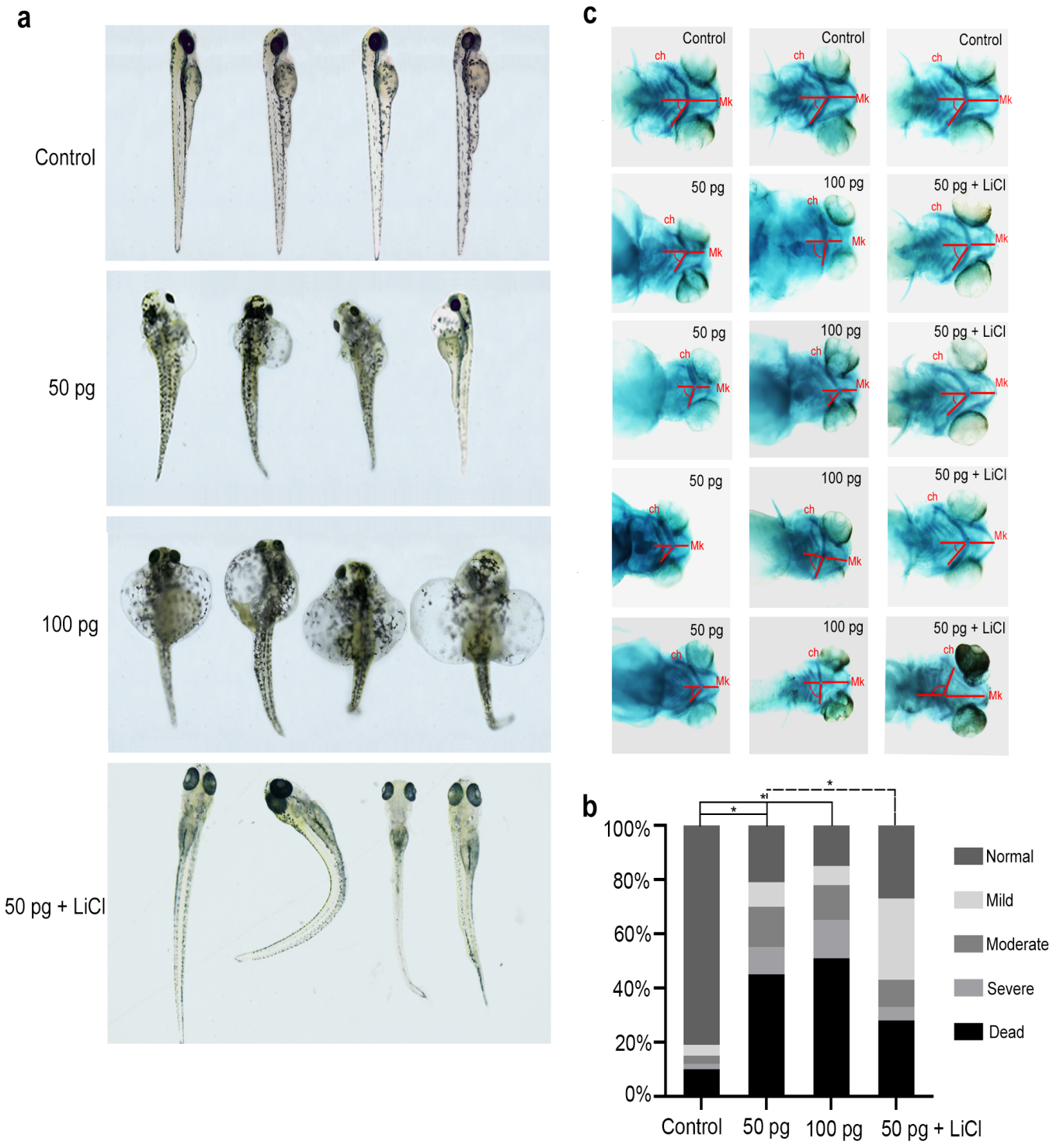

Figure 7

Overexpression of HECT caused polymorphic abnormalities in zebrafish. a, The gross morphology of the zebrafish at 3 dpf. The HECT-overexpressing groups showed abnormal phenotypes, including an unconsumed yolk sac, cardiac edema, small eyes, a deformed head, and a curly spinal cord. LiCl can partially rescue the abnormality of zebrafish caused by overexpression HECT mRNA. b, The proportions of morphological classification in different treated groups were shown in the stacked-bar graphs. *p < 0.05 according to Chi-square test. c, Alcian blue staining showed a reduced distance between ch and Mk and abnormal angulation of the ceratohyal cartilage in the experimental groups.

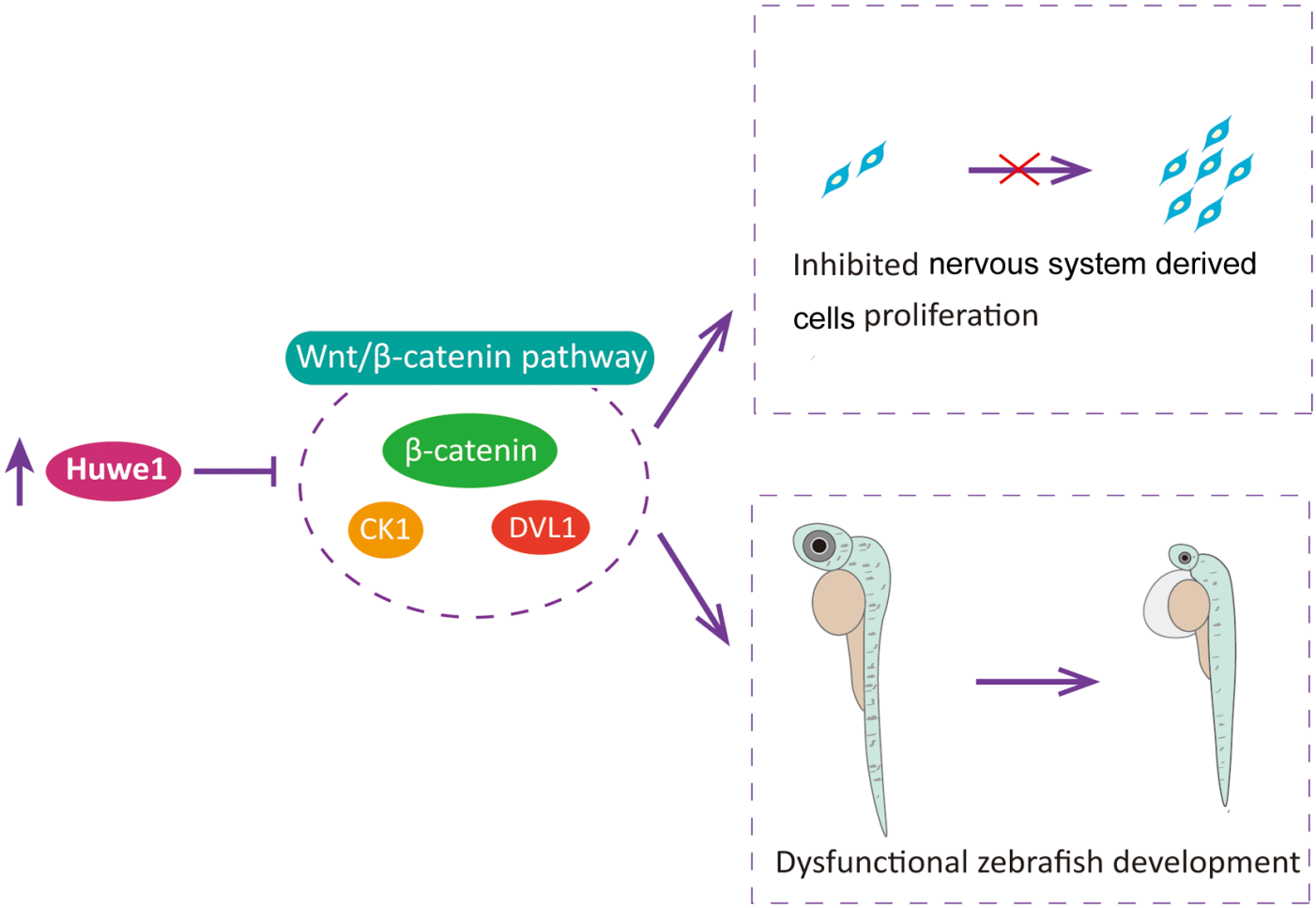

Figure 8

Proposed model for the mechanisms of action underlying the inhibition of neurodevelopment by HUWE1 overexpression. HUWE1 disrupts the neural development in ASD patients as well as zebrafish by promoting ubiquitin-dependent degradation of the key factors (β-catenin, CK1 and DVL1) in Wnt/β-catenin signaling pathway to inhibit nervous system related cells proliferation.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMaterialsTableS1.docx
- coverletter.docx