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**Report**

**ARCN1 Mutations Cause a Recognizable Craniofacial Syndrome Due to**

**COPI-mediated Transport Defects**

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**Abstract**

Cellular homeostasis is maintained by the highly organized cooperation of intracellular trafficking systems including COPI, COPII, and clathrin complexes. COPI is a coatomer protein complex responsible for intracellular protein transport between the endoplasmic reticulum and the Golgi apparatus. The importance of such intracellular transport mechanisms is underscored by the various disorders caused by mutations in the COPII coatomer complex, including skeletal disorders such as cranio-lenticulo-sutural dysplasia and osteogenesis imperfecta. In this article, we report a clinically recognizable craniofacial disorder characterized by facial dysmorphisms, severe micrognathia, rhizomelic shortening, microcephalic dwarfism, and mild developmental delay due to loss-of-function heterozygous mutations in *ARCN1*, which encodes the coatomer subunit delta of COPI. *ARCN1* mutant cell lines were revealed to have endoplasmic reticulum stress, suggesting the involvement of ER stress response in the pathogenesis of this disorder. Since *ARCN1* deficiency causes defective type 1 collagen transport, reduction of collagen secretion represents the likely mechanism underlying the skeletal phenotype that characterizes this condition. Our findings demonstrate the importance of

COPI-mediated transport in human development, including skeletogenesis and brain growth.

In eukaryotic cells, secretory and membrane proteins are generally synthesized on rough endoplasmic reticulum (ER). Vesicle trafficking is required for the correct intracellular transport of these proteins. There are three main intracellular protein trafficking systems: COPI, COPII, and clathrin systems<sup>1</sup>. These transport systems are composed of heteromeric proteins forming a lattice-like protein complex that coats vesicles for intracellular transport. COPI is a heptameric protein complex composed of alpha-COP, beta-COP, beta-prime-COP, gamma-COP, delta-COP, epsilon-COP, and zeta-COP subunits<sup>1,2</sup>. COPI plays an important role in retrograde transport from the Golgi apparatus to the ER; however, recent reports also implicate COPI in anterograde transport from the ER to Golgi apparatus<sup>1-3</sup>. For performing highly complex cellular functions, intracellular transport achieved by COPI plays cardinal roles in cellular homeostasis.

Disruption of intracellular protein transport in human disease has been documented in various genetic disorders. COPII plays major roles in anterograde transport from the ER to the Golgi apparatus<sup>1</sup>, and mutations in COPII components are associated with several genetic disorders. These disorders include lipid absorption



disorder (MIM: #246700)<sup>4</sup>, skeletal disorders (such as cranio-lenticulo-sutural dysplasia (MIM: #607812)<sup>5</sup> and osteogenesis imperfecta (MIM: #616294)<sup>6</sup>), hematological disorders (such as congenital dyserythropoietic anemia type II (MIM: #224100)<sup>7</sup> and combined deficiency of Factor V and Factor VIII (MIM: #227300, #613625)<sup>8,9</sup>), Cowden syndrome, and thyroid cancer (MIM: #616858)<sup>10</sup>. However, in contrast to the findings for COPII, disease-causing germline mutations in COPI components had not been described until lately. Recently, germline mutations of *COPA* encoding the alpha-COP subunit of COPI were described in individuals with hereditary autoimmune-mediated lung disease and arthritis, demonstrating that the disruption of COPI transport can cause human disease (MIM: #616414)<sup>11</sup>. Here, we report a clinically recognizable genetic disorder that we identified in four individuals; this disorder was characterized by facial dysmorphisms, severe micrognathia, rhizomelic shortening, microcephalic dwarfism and mild developmental delay caused by germline loss-of-function mutations in the *Archain 1* (*ARCN1*), which encodes the coatamer subunit delta of COPI.

Four individuals with *ARCN1* mutations were independently enrolled in our study following approval granted by the institutional review boards of The University of

Tokyo, Nagano Children's Hospital, KK Women's & Children's Hospital, Singapore, and Genetics department of the CHU and University of Liège, Belgium. Informed consent was obtained from the guardians of the subjects for genetics and molecular studies.

Shared clinical features among these four individuals are facial dysmorphisms including severe micrognathia, rhizomelic shortening, and microcephalic dwarfism (see

Supplemental Note, Figure 1, Table I). Subject 1 was referred to the Genetics Clinic of

Nagano Children's Hospital at the age of 1 month with the chief complaints of facial

dysmorphism and intrauterine growth retardation. On physical examination, several

facial dysmorphisms, including a prominent forehead, downslanted palpebral fissures,

and severe micrognathia, were noted. In addition, shortening of upper arms and legs,

and small joint laxity were observed (Figure 1A). A skeletal survey revealed widening of

the metaphysis and a wide femoral neck (Figure 1B). Subject 2, a boy with

developmental delay and mild autism, was first seen in the Genetics Clinic, KK

Women's & Children's Hospital, at the age of 1 year and 8 months. Subject 2 had

micrognathia, scaphocephaly, and hypotelorism, as well as small joint laxity (Figure 1C).

The clinical history of Subject 3 was partly reported previously by Verloes et al.<sup>12</sup>. At the

age of 25 years, his weight was 98kg, his length was 152cm (-3.5 SD), and his head circumference was 50 cm (-5 SD) (Figure 1D). Subject 4, the daughter of Subject 3, shared common features with Subject 3, which included intrauterine growth retardation followed by postnatal growth failure (length 79.5 cm, -4 SD), microcephaly (head circumference 45 cm, -5 SD), and dysmorphic features including microretrognathia, hypotelorism, shortening of upper arms and legs, and muscular hypertrophy (Figure 1E).

Exome sequencing was performed using genomic DNA extracted from peripheral blood cells. Exome sequencing of the probands revealed heterozygous *ARCN1* loss-of-function mutations in these four probands (Figure 2). The Subject 1's mutation in *ARCN1* resulted in a premature stop codon in exon 2 (NM\_001655:exon2:c.260C>A;p.Ser87\*). Exome sequencing of Subject 2 revealed two *de novo* mutations in *ARCN1* and *SYT1*. The *ARCN1* mutation was a frameshift variant: NM\_001655:c.633del; p.Val212Trpfs\*15 predicted to create a frameshift in exon 4 starting at codon Val212 and ending in a stop codon 14 positions downstream (Figure 2A and Figure S2B). The *SYT1* (NM\_001135805.1:c.697G>A; p.Asp233Asn) variant is

a missense variant that substitutes Aspartic acid at codon 233 with Asparagine (Figure S3C). Subjects 3 and 4 were found to have a common *ARCN1* mutation, which was NM\_001655:c.157\_158del, leading to p.Ser53Cysfs\*39 (Figure S2C).

In order to evaluate the effect of loss-of-function mutations of *ARCN1*, genome editing was performed using the CRISPR/Cas9 system on the colon cancer-derived HCT116 cell line. After the introduction of the *ARCN1* mutation into these cells, single cell cloning was performed using the limiting dilution technique. The gRNA empty vector (ID41824) and hCas9 vector (ID41815) were obtained from Addgene (Cambridge, MA, USA). The gRNA target sequence was cloned into the gRNA empty vector using Phusion polymerase (M0530S: New England Biolabs) and the Gibson assembly system (E5510S: New England Biolabs). The gRNA target sequence was CTAAGGCTCTTCTCAAGAG; it is located at exon 2 of *ARCN1*. Cas9 and gRNA vectors were transfected into cell lines by electroporation using a Neon electroporation system (ThermoFisher Scientific). Genomic DNA was extracted with NucleoSpin tissue kits (Macherey-Nagel, Duren, Germany). The presence of the *ARCN1* mutation was confirmed by Sanger sequencing. A total of 68 clones were

screened and 5 clones with *ARCN1* mutations were identified. None of the clones possessed biallelic loss-of-function mutations of *ARCN1*, suggesting the importance of *ARCN1* in maintaining cell viability. Two independent clones possessing 1-bp duplication leading to a frameshift that resembled the mutations identified in Subject 1 (*ARCN1*: c.262dupA (NM\_001655)) were used for subsequent experiments (Figure S2D). Analysis using the ExPASy translate tool indicated that this 1-bp duplication leads to p.Arg88Lysfs\*5. Similar to the mutation found in four individuals with *ARCN1* mutations, this frameshift mutation in the HCT116 cell line likely causes a loss-of-function effect, since the mRNA in the case of this 1-bp insertion is predicted to undergo nonsense-mediated decay.

To test if such a truncating mutation caused actual dosage reduction of *ARCN1*, we quantified *ARCN1* mRNA using quantitative RT-PCR. Introduction of frameshift mutations in exon 2 resulted in reduction in *ARCN1* mRNA levels, confirming that truncating mutations cause reduction in the levels of *ARCN1* transcript (Figure 2B). Reduction of *ARCN1* mRNA level was also observed in the skin fibroblast samples obtained from Subjects 3 and 4 (Figure S4A).

In order to test the level of *ARCN1*, HCT116 cells with and without *ARCN1* mutations were lysed with SDS sample buffer for immunoblotting. Western blotting of HCT116 mutant clones demonstrated reduction of *ARCN1* compared to the wild type clones (Figure 2C). Mild reduction of *ARCN1* was also demonstrated in the skin fibroblast sample obtained from Subject 4 (Figure S4B). These observations confirmed that truncating mutations of *ARCN1* cause reduction of *ARCN1* mRNA and protein levels.

Given the phenotypic overlap of the probands with *ARCN1* mutations and collagenopathies such as Stickler syndrome (MIM: #108300), we hypothesized that reduction of *ARCN1* may have caused intracellular collagen transport defects. To evaluate the effect of loss-of-function *ARCN1* mutations, siRNA-mediated gene knockdown (KD) of *ARCN1* was performed on a control skin fibroblast cell line, GM02036, since skin fibroblast cell line synthesize type I collagen<sup>13,14</sup>. The greatest reduction in *ARCN1* mRNA and *ARCN1* protein levels was achieved 4 days after *ARCN1* KD (Figure 3A and Figure 4A).

Disruption of intracellular trafficking can cause the accumulation of protein,

leading to an ER stress response. When the rate of protein synthesis exceeds the capacity of protein folding and protein degradation machinery, the ER stress response is induced, which may result in cell death if prolonged<sup>15</sup>. Therefore, the degree of the ER stress response was evaluated in the skin fibroblast cell line after *ARCN1* KD.

Overexpression of stress response genes such as *ATF4*, *DDIT3*, and *HSPA5* serves as a marker of ER stress. Quantitative RT-PCR analysis demonstrated the upregulation of all these ER stress response genes after *ARCN1* KD (Figure 3B). Therefore, reduction in *ARCN1* levels triggered the ER stress response. In order to evaluate the consequence of ER stress, cells were treated with thapsigargin (2  $\mu$ M and 5  $\mu$ M)<sup>16</sup> and tunicamycin (2  $\mu$ g/ml and 5  $\mu$ g/ml)<sup>17,18</sup> for 17 hours; then, the total cellular lysates and RNA were obtained. Thapsigargin is an inhibitor of the ER Ca<sup>2+</sup>ATPase and induces ER stress, and tunicamycin inhibits the initial step of glycoprotein synthesis in ER and induces ER stress. Artificial induction of ER stress by the addition of thapsigargin/tunicamycin triggered *ARCN1* overexpression, although the amount of *ARCN1* remained unchanged, suggesting increased *ARCN1* turnover during the ER stress response (Figure 5A and 5B). Collectively, these results suggest that *ARCN1*

plays a major role in ameliorating the induction of the ER stress response. Evaluation of the Subject 4 derived skin fibroblast sample further confirmed the presence of ER stress, indicated by the cytosolic accumulation of BiP, which is an ER stress marker (Figure S4C and 5). Therefore, reduction in *ARCN1* levels triggered the ER stress response.

Next, the amount of type 1 collagen was evaluated in the control skin fibroblasts with *ARCN1* KD. Interestingly, *ARCN1* KD caused the accumulation of type 1 collagen in the total cellular lysates (Figure 4A). Type 1 collagen secretion from skin fibroblasts was then quantitated by performing trichloroacetic acid (TCA) precipitation of culture supernatants. Immunoblotting performed on culture supernatants demonstrated the reduction of secreted type 1 collagen in the culture media, suggesting that *ARCN1* reduction caused intracellular accumulation of type 1 collagen due to defective intracellular protein transport (Figure 4A). In order to confirm that collagen accumulation was a result of defective intracellular transport, 10  $\mu$ M brefeldin A (BFA), an ER-Golgi transport inhibitor, was added to the culture media, and total cellular lysates were obtained 23 hours after treatment<sup>18</sup>. Addition of BFA caused the accumulation of intracellular type 1 collagen, similar to what was observed with *ARCN1* KD (Figure 4B).



Interestingly, BFA treatment reduced the amount of *ARCN1* in the cells (Figure 4B).

Type 1 collagen is encoded by two genes, *COL1A1* and *COL1A2*. The alpha 1 chain of type 1 collagen is encoded by *COL1A1* and the alpha 2 chain of type 1 collagen is encoded by *COL1A2*. Quantitative RT-PCR demonstrated a slight reduction in *COL1A1* mRNA with *ARCN1* KD, while the level of *COL1A2* mRNA remained unchanged with *ARCN1* KD (Figure 4C). Addition of thapsigargin/tunicamycin did not cause accumulation of type 1 collagen, suggesting that the ER stress response does not cause the collagen transport defect (Figure 5B and Figure S6). These experiments indicate that *ARCN1* is directly responsible for the intracellular transport of type 1 collagen and that the collagen transport defect is not secondary to the ER stress response.

In this paper, we report a clinically recognizable genetic disorder characterized by facial dysmorphisms, micrognathia, rhizomelic shortening, microcephalic dwarfism, and mild intellectual disability due to heterozygous *ARCN1* loss-of-function mutations. We propose the name “*ARCN1*-related syndrome” to denote this condition. The *ARCN1* is approximately 30 kb in size and located on chromosome

11q23.3 with 10 exons (Figure 2A). Germline mutations of *ARCN1* have not been previously reported. The Exome Aggregation Consortium (ExAC) website gives a probability of loss-of-function intolerance score of 1 to *ARCN1*, the highest score, given the absence of loss-of-function *ARCN1* mutations in their cohort<sup>19</sup>. Therefore, *ARCN1* likely represents a dosage-sensitive gene in humans. To the best of our knowledge, germline chromosomal microdeletions spanning the *ARCN1* region have not been reported in the medical literature. Copy number variations within the *ARCN1* locus have also not been reported in the Database of Genomic Variants.

The clinical phenotype of *ARCN1*-related syndrome includes severe micrognathia, microcephalic dwarfism, joint laxity, and mild developmental delay (Table1). There is some phenotypic overlap between *ARCN1*-related syndrome and Stickler syndrome such as micrognathia, short stature and joint laxity, although neurological, ocular and audiology features differ. Stickler syndrome is caused by loss-of-function heterozygous mutations in *COL2A1* and other collagen genes<sup>20</sup>. Given the type of the mutations identified in Stickler syndrome, it is presumed that generalized reduction in type II collagen production leads to a clinical phenotype of Stickler

syndrome such as micrognathia and short stature. This clinical overlap prompted us to evaluate the role of *ARCN1* in intracellular collagen transport where we demonstrated the importance of *ARCN1* in this context. Phenotypic resemblance between *ARCN1*-related syndrome and Stickler syndrome could be explained by the reduction in collagen secretion from the cells. The importance of *ARCN1*-associated intracellular trafficking has been documented in many biological processes such as immune function and influenza virus infection<sup>21</sup>. The inability to create an *ARCN1*-null cell line with CRISPR/Cas9 underscores the importance of *ARCN1* in cell viability. Our findings highlight the unexpected importance of COPI transport in skeletogenesis, particularly in mandibular bone formation. Previously, similar pathological disease mechanisms resulting in skeletal dysplasia were implicated in COPII transport defects such as cranio-lenticulo-sutural dysplasia and osteogenesis imperfecta<sup>6,22</sup>.

*COL2A1* mutations also cause other skeletal dysplasias such as achondrogenesis type II/hypochondrogenesis (MIM: #200610), and spondyloepiphyseal dysplasia (MIM: #183900). These disorders are collectively termed type II collagenopathies<sup>23</sup>. Many of these conditions are caused by heterozygous mutations in

*COL2A1*. However, the effects of *COL2A1* mutations vary in different type II collagenopathies. As a result, there is a wide phenotypic spectrum of type II collagenopathies. Interestingly, some *COL2A1* missense mutations found in type II collagenopathies cause abnormal protein folding of type II collagen, leading to the intracellular accumulation of mutant collagen, which elicits an ER stress response<sup>24</sup>. Furthermore, the artificial induction of ER stress in chondrocytes causes skeletal dysplasia in mice<sup>25</sup>. Therefore, it is proposed that ER stress itself is involved in the pathogenesis of skeletal dysplasia. Hence, in *ARCN1*-related syndrome, the ER stress response may also play a role in the resultant skeletal phenotype. However, our *ARCN1* KD experiments indicate that defective COPI transport could play a direct role in intracellular collagen accumulation in *ARCN1*-related syndrome, rather than the skeletal phenotype, which is secondary to ER stress. This finding is consistent with a previous report that demonstrated the involvement of COPI in intracellular collagen transport<sup>26</sup>. In order to deepen the understanding of the role that ER stress and COPI transport play in skeletogenesis, further studies are warranted.

The ER stress response is elicited even during normal embryogenesis<sup>27,28</sup>.

Given the role of *ARCN1* in the ER stress response, reduction of *ARCN1* likely triggers an exaggerated ER stress response, and such an enhanced ER stress response may lead to cell death. In addition to the direct influence of ER-Golgi transport defects secondary to *ARCN1* mutations, such cell death likely contributes to the pleiotropic phenotype of *ARCN1*-related syndrome.

Mice with a homozygous missense mutation in *Arcn1*, that is, *nur17* mice, have been previously created by N-ethyl-N-nitrosourea (ENU) mutagenesis<sup>29</sup>. The *nur17* mouse harbors a homozygous missense mutation in exon 10 of *Arcn1* and is characterized by the presence of dilute coat color, neurological defects, and low body weight; however, the skeletal phenotype was not characterized in the original report. Mild loss-of-function effects were presumed to mediate disease mechanisms in the *nur17* mutant mouse. There are several similarities between *Arcn1* mutant mice and the probands described in these articles, such as low body weight and the neurological phenotype including ataxia due to cerebellar degeneration. Interestingly, Subject 4 was found to have ataxia and cerebellar atrophy. Hence, *nur17* mice may serve as an animal model for *ARCN1*-related syndrome, although skin color and hair abnormalities

were not detected in individuals with *ARCN1* mutations.

In addition to the skeletal phenotype, another feature typical of *ARCN1*-related syndrome is mild intellectual disability. There are several lines of evidence supporting a role for COPI transport in brain function. First, COPI interacts with a subset of RNA molecules, and COPI has been implicated in intracellular RNA transport in neuronal cells<sup>30</sup>. Second, the COPI system is known to transport molecules that play major roles in neurotransmitter release such as SNARE proteins<sup>31-33</sup>. In fact, the importance of intracellular trafficking has been well known in neuronal function and homeostasis because the abnormalities of intracellular trafficking are linked to the pathogenesis of various neurodegenerative disorders such as Alzheimer's disease<sup>34</sup>. Abnormal ER-Golgi trafficking is demonstrated in Dyggve-Melchior-Clayson syndrome (MIM: #223800) associated with developmental delay and microcephaly<sup>35</sup>. Furthermore, the presence of ER stress during embryogenesis is well documented in the central nervous system, supporting a role for ER stress in brain development<sup>27,28</sup>. Therefore, the findings strongly suggest that COPI is involved in neuronal function. The neurological phenotype of the *nur17* mouse model further supports the notion that

defective *ARCN1* function impairs brain function<sup>29</sup>.

In addition to intellectual disability, Subject 2 experienced seizures. However, it remains to be determined whether the seizures were due to *ARCN1* mutations since Subject 2 also has a *de novo* missense *SYT1* variant, which was not previously reported. *SYT1* encodes synaptotagmin1, which plays important roles in synaptic vesicle recycling, and there have been two recent reports associating *SYT1* mutations in a child with a dyskinetic movement disorder and developmental delay, and another child with developmental delay, facial dysmorphisms and anomalous EEG patterns<sup>36,37</sup> (Table S3). Therefore, it is possible that the neurological phenotype of Subject 2 is partially due to *SYT1* mutation. However, since all four probands with *ARCN1* mutations demonstrated comparable degree of developmental delay/intellectual disability, *ARCN1* mutations likely plays a major role in its neurological phenotype, and *ARCN1* is probably required for normal brain growth and cognitive development.

In conclusion, we report a recognizable craniofacial disorder, *ARCN1*-related syndrome, characterized by facial dysmorphisms, micrognathia, rhizomelic shortening, microcephalic dwarfism, and mild intellectual disability. Intracellular COPI transport has

been implicated in various biological processes and our results strongly indicate the importance of COPI transport in skeletogenesis, particularly in mandibular bone formation, during embryogenesis.

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The *ARCN1* was submitted as a candidate gene to GeneMatcher by three independent research groups in Japan, Singapore, and France<sup>38</sup>. The authors appreciate the GeneMatcher website in facilitating the collaboration. No conflict of interests to disclose.



## Figure Legends

**Figure 1.** Clinical features of the probands with *ARCN1* mutations. The guardians of the probands provided consent for the publication of their photographs. (A) Physical features of Subject 1. Note downslanted palpebral fissures and micrognathia. (B) Skeletal survey of Subject 1. Note micrognathia, widening of the metaphysis and a wide femoral neck. (C) Facial features of Subject 2. (D) Physical features of Subject 3. Note rhizomelic shortening of the limbs. (E) Physical features of Subject 4. Note micrognathia and rhizomelic shortening of the limbs.

**Figure 2.** Effects of *ARCN1* mutation. (A) Structure of *ARCN1* and the location of *ARCN1* mutations identified. Subject 1 has a stop mutation in exon 2 (NM\_001655:exon2:c. 260C>A:p.Ser87\*). Subject 2 has a frameshift mutation in exon 4: NM\_001655:c.633del; p.Val212Trpfs\*15. Subjects 3 and 4 have a common *ARCN1* mutation in exon 2, NM\_001655:c.157\_158del, leading to p.Ser53Cysfs\*39. (B) mRNA level of *ARCN1* in HCT116 cell lines with and without *ARCN1* mutation. RNA extraction was performed with TRIzol reagent (Ambion: 15596-018) and Nucleospin RNA kits (Macherey-Nagel, Duren, Germany). The RNA obtained was reverse transcribed with a

SuperScript VILO cDNA Synthesis Kit (Invitrogen: 11754-050). The PCR primer sets used for quantitative RT-PCR are listed in Table S2. WT: wild type. *ARCN1* expression level was normalized against *GAPDH*. Relative *ARCN1* expression level to the control sample (WT#1) is demonstrated. Data represents mean  $\pm$  2 s.d. \*\*\*  $P < 0.001$ , two-tailed *t* test;  $n=3$  technical replicates per group. Three biological replicates for each sample demonstrated consistent results. (C) Protein level of *ARCN1* in HCT116 cell lines with and without *ARCN1* mutation. The *ARCN1* antibody (ab96725) and histone H3 antibody (ab1791) were from Abcam. Three biological replicates for each sample demonstrated consistent results.

**Figure 3.** ER stress response with the reduction of *ARCN1*. Skin fibroblast *ARCN1* KD experiments were performed 3 times, and consistent results were obtained. Two different siRNAs (ThermoFisher Scientific: *ARCN1*HSS100628 (KD1) and *ARCN1*HSS100629 (KD2)) were used in the study. Stealth siRNA was transfected using Lipofectamine RNAiMAX reagent (ThermoFisher Scientific) according to the manufacturer's instructions. For *ARCN1* KD in skin fibroblasts, two siRNA transfections were performed 2 days apart; 4 days after the initial siRNA transfection, RNA and

cellular lysates were harvested. (A) *ARCN1* mRNA level with *ARCN1* knock down. KD: knock down. Data represents mean  $\pm$  2 s.d, \*\*\*  $P < 0.001$ , two-tailed *t* test; n=3 technical replicates per group. Quantitative PCR was run in triplicates, and mean and standard deviation was calculated. *ARCN1* expression level was normalized against *GAPDH* expression. Relative gene expression level to the control sample is demonstrated. (B) Elevation of ER stress response genes expression upon *ARCN1* KD. Data represents mean  $\pm$  2 s.d. \*\*\*  $P < 0.001$ , two-tailed *t* test; n=3 technical replicates per group. Gene expression level was normalized against *GAPDH* expression. Relative gene expression level to the control sample is demonstrated.

**Figure 4.** Collagen transport defects with *ARCN1* KD. (A) Reduction of *ARCN1* caused accumulation of type 1 collagen in total cellular lysates and reduction of type 1 collagen secretion. The type 1 collagen antibody (AB758) was from Millipore<sup>13</sup>. For the measurement of culture supernatant type 1 collagen, skin fibroblast cell numbers were counted, and equal numbers were plated in culture flasks. The cells were changed to serum-free culture media for the final 24 hours, the time point at which the medium was harvested. One-tenth the volume of TCA was added and placed on ice for 30 minutes.

The samples were centrifuged and precipitates were washed with ethanol. After the ethanol wash, the supernatants were dried and the remaining cell pellets were dissolved with SDS sample buffer for immunoblotting. TCA precipitation was performed 4 times, and consistent results were obtained. Two bands of type 1 collagen represent the bands for alpha 1 and alpha 2 type 1 collagen. Top larger band represents alpha 1 type 1 collagen and lower smaller band represents alpha 2 type 1 collagen. (B) Brefeldin A (BFA; Sigma-Aldrich B5936)) treatment caused the intracellular accumulation of type 1 collagen. 10 uM of BFA was used. DMSO was added for control. BFA treatment experiment was performed three times, and consistent results were obtained. (C) *COL1A1* and *COL1A2* expression with *ARCN1* KD. Slight reduction of *COL1A1* expression was elicited with *ARCN1* KD, however, the gene expression level of *COL1A2* remained unchanged. Gene expression level was normalized against *GAPDH* expression. Relative gene expression level to the control sample is demonstrated. Data represents mean  $\pm$  2 s.d. \*  $P < 0.05$ , \*\*\*  $P < 0.001$ , two-tailed *t* test; n=3 technical replicates per group.

**Figure 5.** Artificial induction of ER stress caused *ARCN1* mRNA overexpression. (A)

Treatment with tunicamycin (Sigma-Aldrich, T7765) and thapsigargin (Sigma-Aldrich, T9033) triggered overexpression of *ARCN1*. *ARCN1* expression level was normalized against *GAPDH* expression. Relative gene expression level to the control sample is demonstrated. Data represents mean  $\pm$  2 s.d. \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , two-tailed *t* test;  $n = 3$  technical replicates per group. (B) The amount of ARCN1 remained unchanged with tunicamycin and thapsigargin treatments. The alpha tubulin antibody (T6074) was from Sigma-Aldrich. Two different concentrations of tunicamycin treatment (2 $\mu$ g/ml (Tuni2) and 5 $\mu$ g/ml (Tuni5)) and thapsigargin treatment (2 $\mu$ M (Tg2) and 5 $\mu$ M (Tg5)) were used. DMSO was added for control. Two biological replicates demonstrated the consistent results.

	Subject 1	Subject 2	Subject 3	Subject 4
IUGR	(+)	(+)	(+)	(+)
Micrognathia	(+)	(+)	(+)	(+)
Cleft palate	(-)	(-)	(-)	(+)
Tracheostomy	(+)	(+)	(-)	(-)
Congenital heart disease	VSD	(-)	(-)	(-)
Cryptorchidism	NA	(+)	(-)	NA
Short stature	(+)	(+)	(+)	(+)
Rhizomelic shortening	(+)	(-)	(+)	(+)
Joint laxity	(+)	(+)	(-)	(+)
Developmental delay	(+)	(+)	(+)	(+)
Autism	(-)	(+)	(-)	(-)
Seizure	(-)	(+)	(-)	(-)
Microcephaly	(+)	(-)	(+)	(+)
<i>ARCN1</i> mutation	p.Ser87*	p.Val212Trpfs*15	p.Ser53Cysfs*39	

Table 1: Clinical features of *ARCN1*-related syndrome. IUGR: intrauterine growth retardation. VSD: ventricular septal defect,

## Web Resources

Database of Genomic Variants (<http://dgv.tcag.ca/dgv/app/home>)

Exome Aggregation Consortium (ExAC) website (<http://exac.broadinstitute.org/>)

ExPASy translate tool (<http://web.expasy.org/translate/>)

GeneMatcher (<https://genematcher.org/>)

OMIM: Online Mendelian Inheritance in Man (<http://www.omim.org>)

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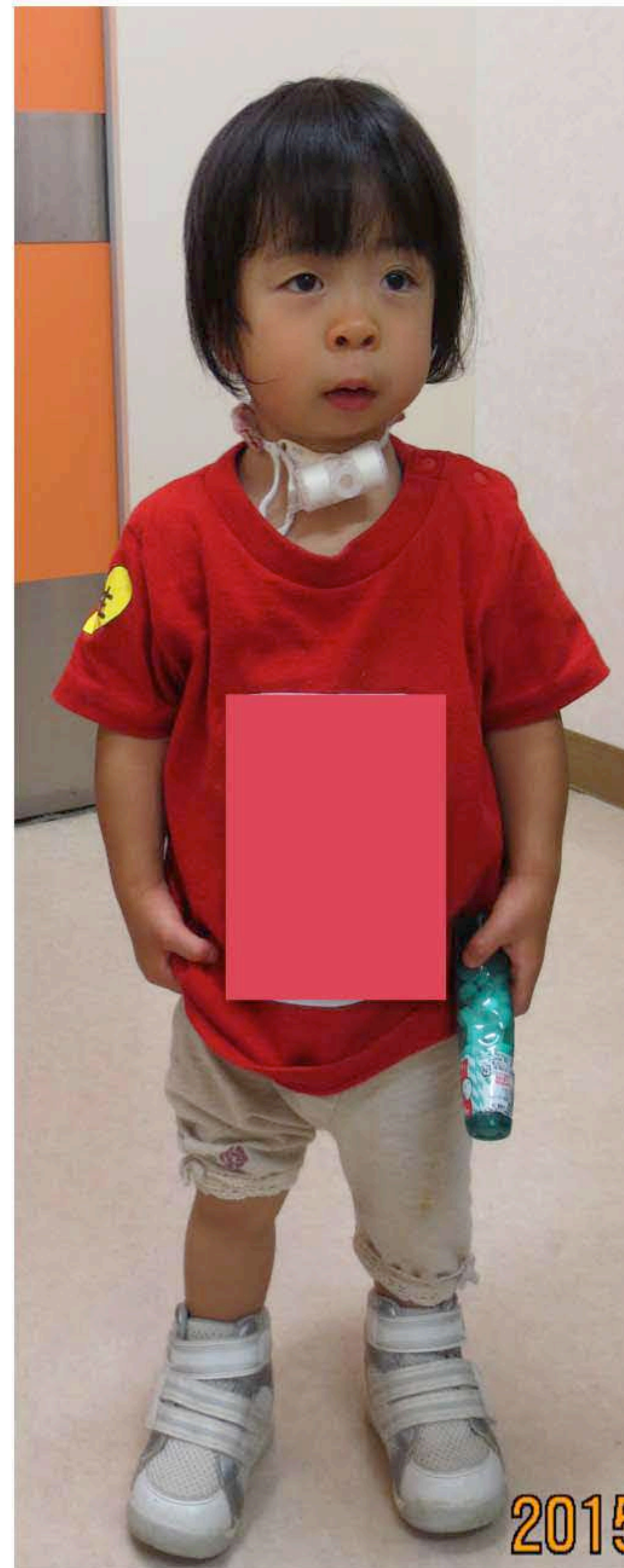
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Figure 1

(a)



(b)



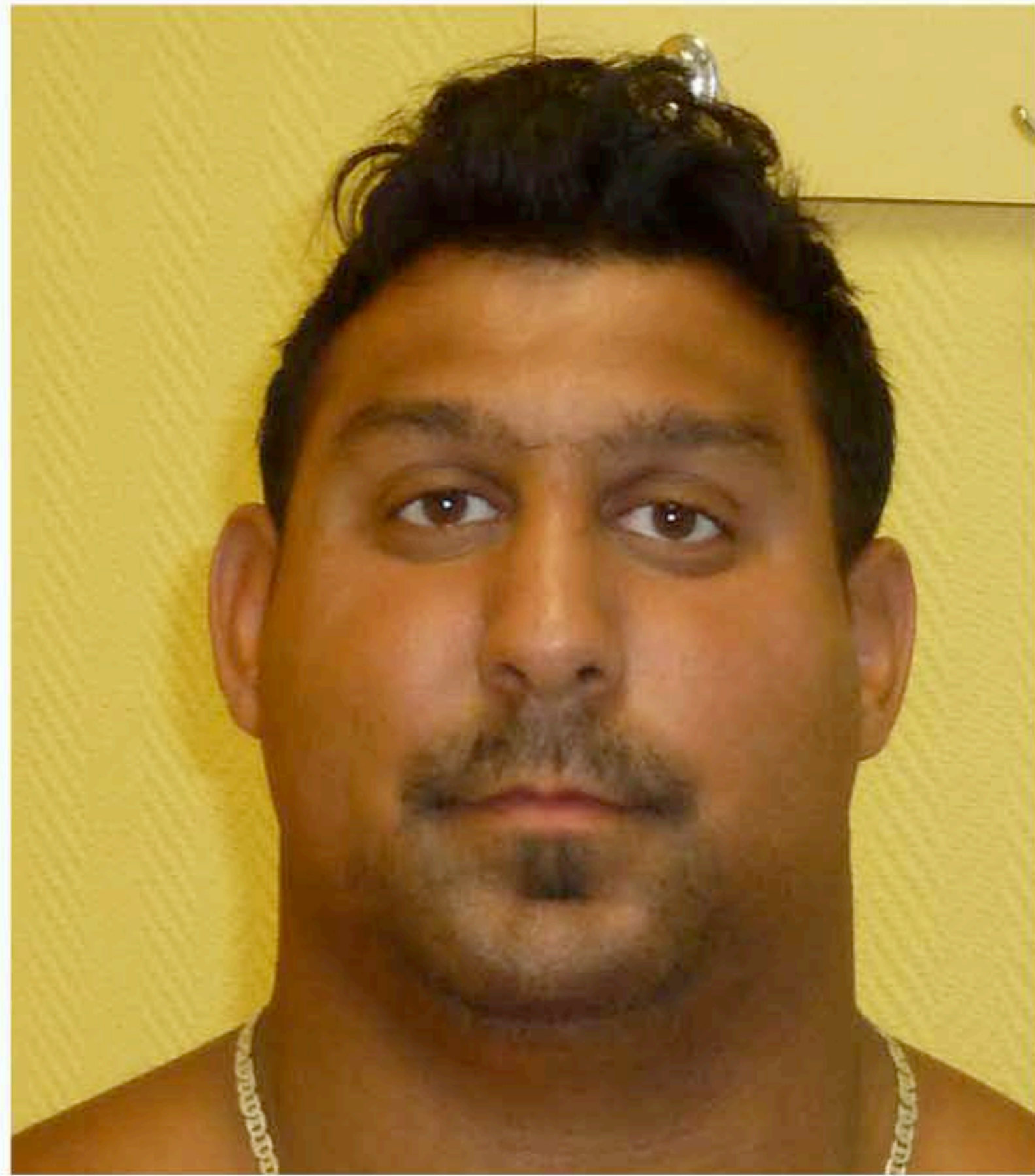


Figure 1

(c)



(d)



(e)

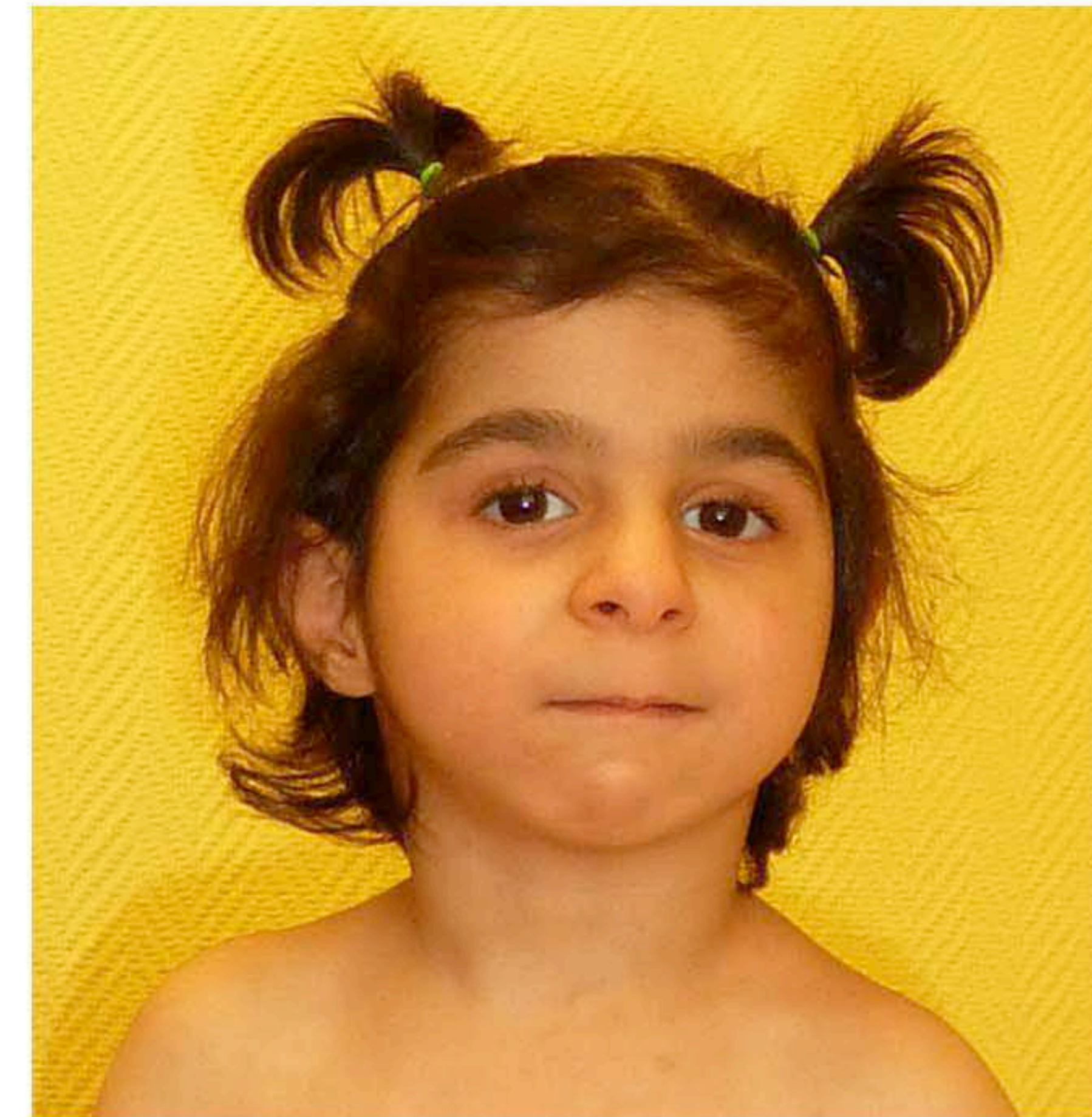
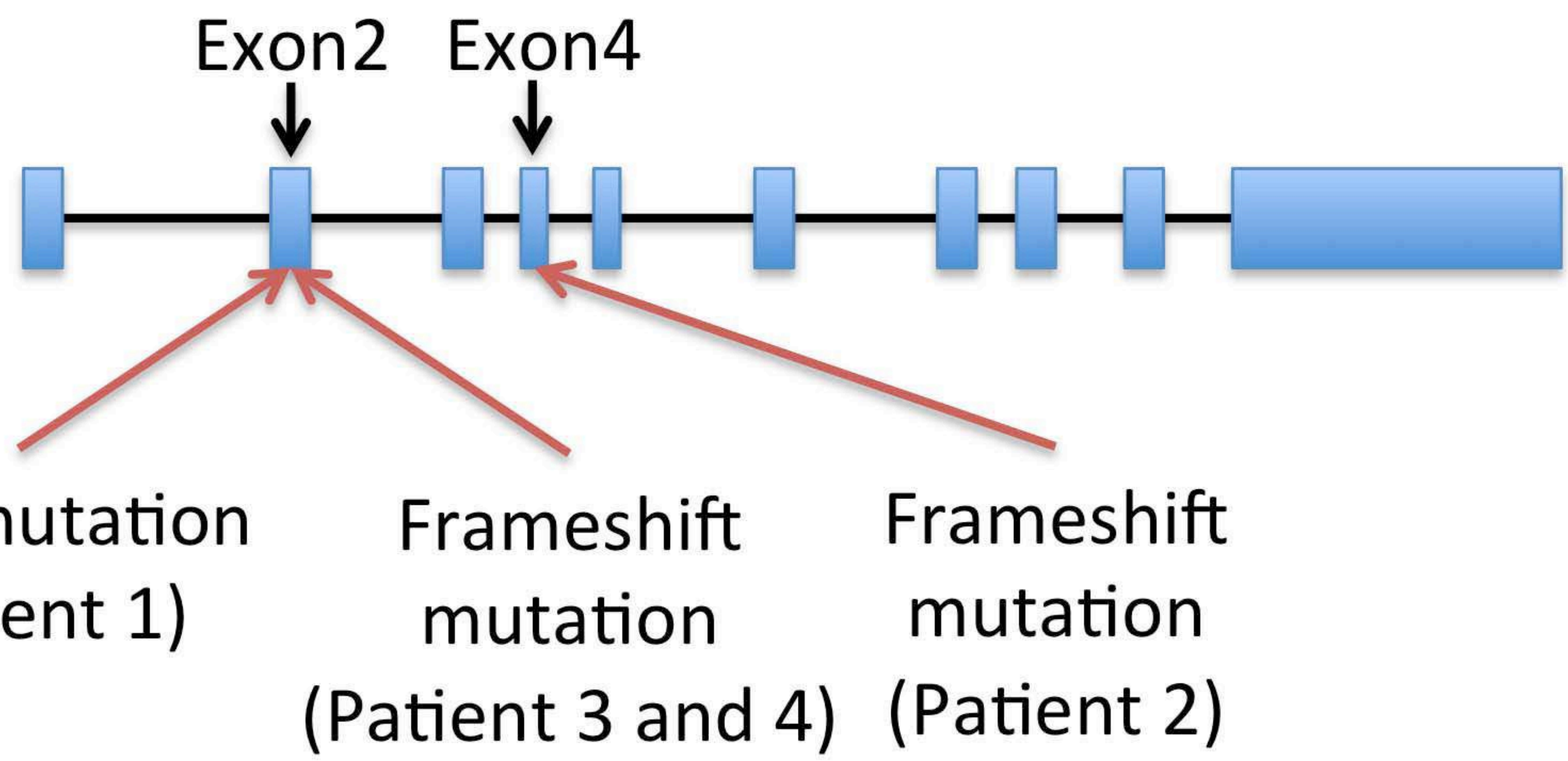


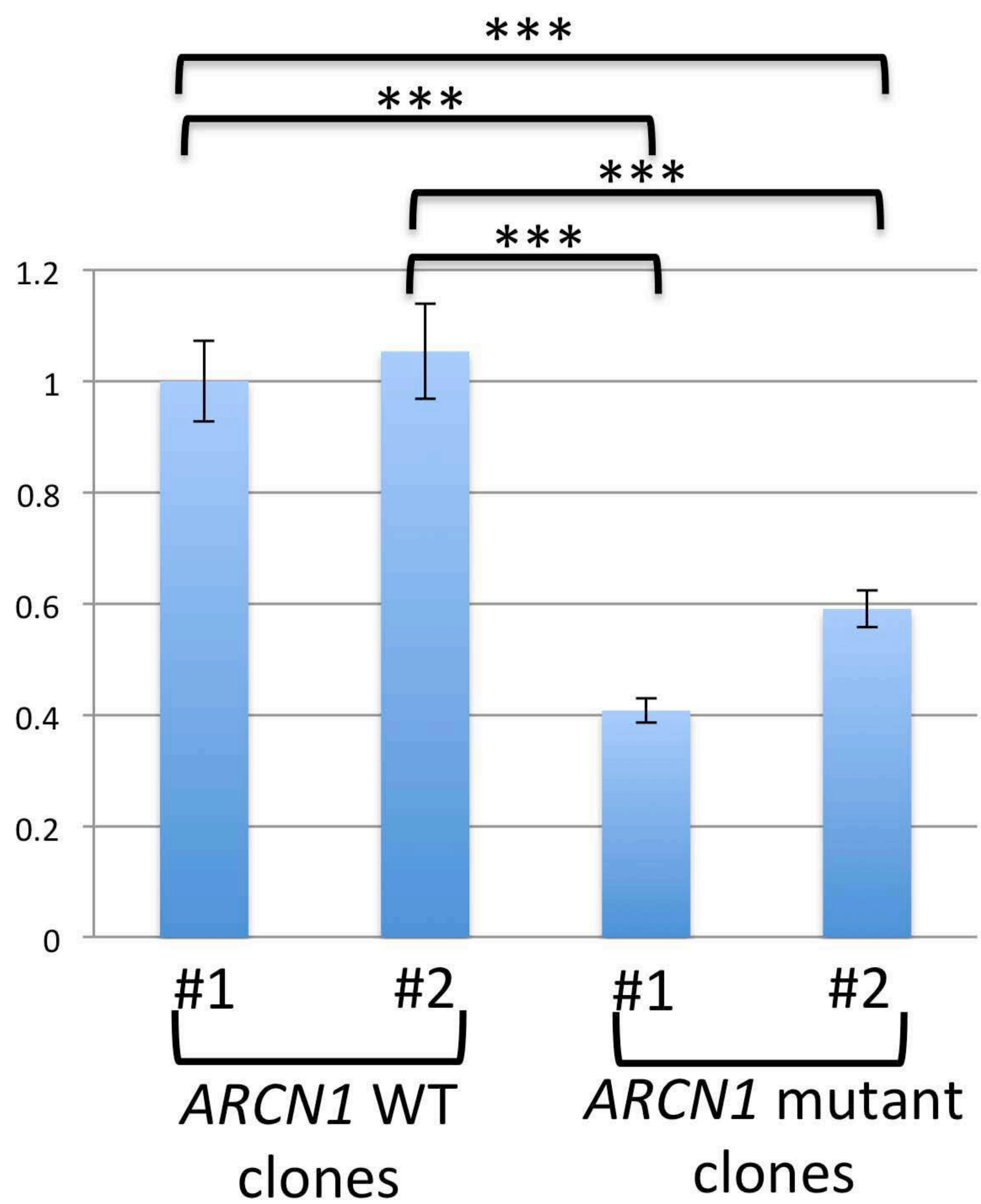


Figure 2

(a)



(b)



(c)

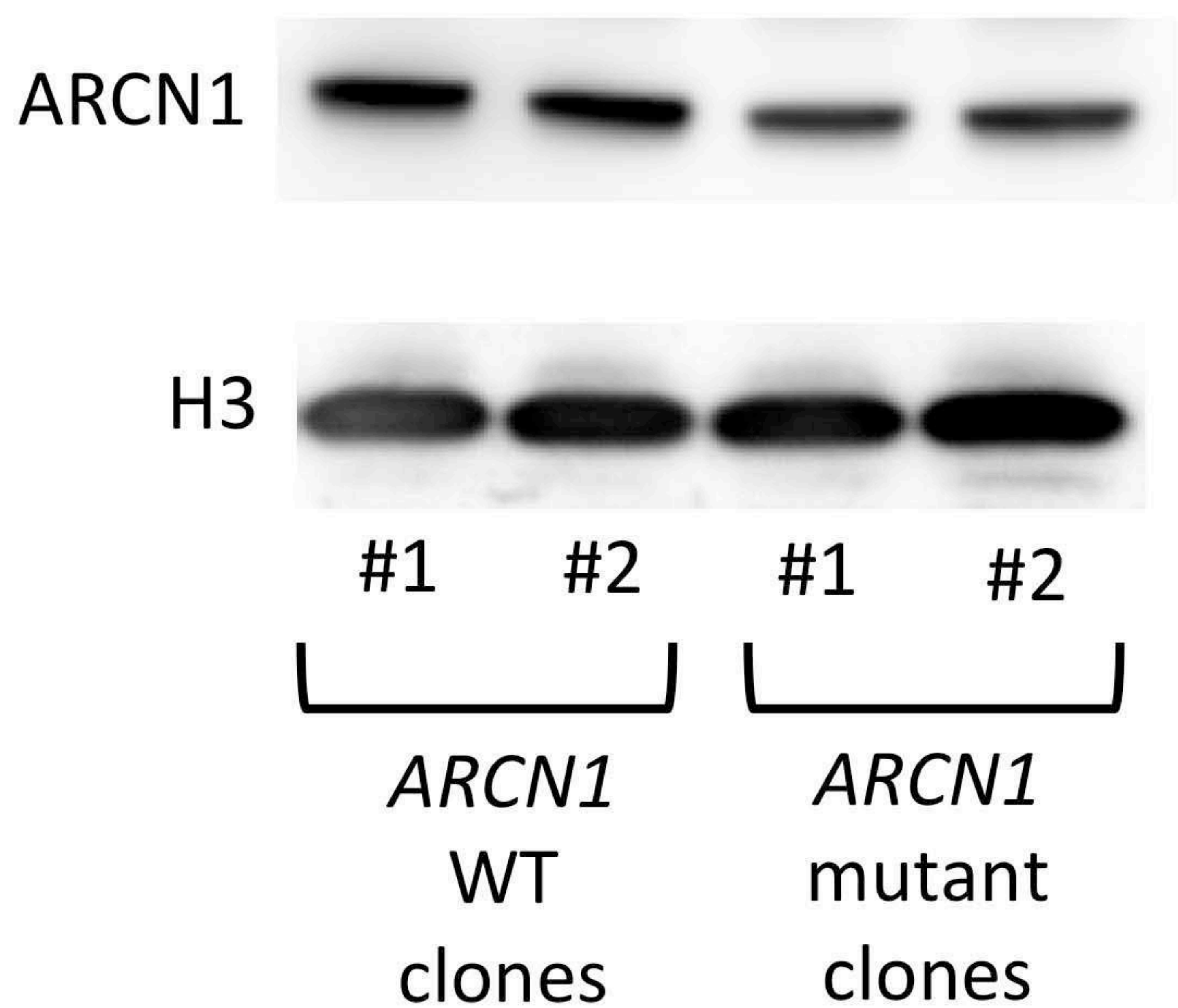




Figure 3

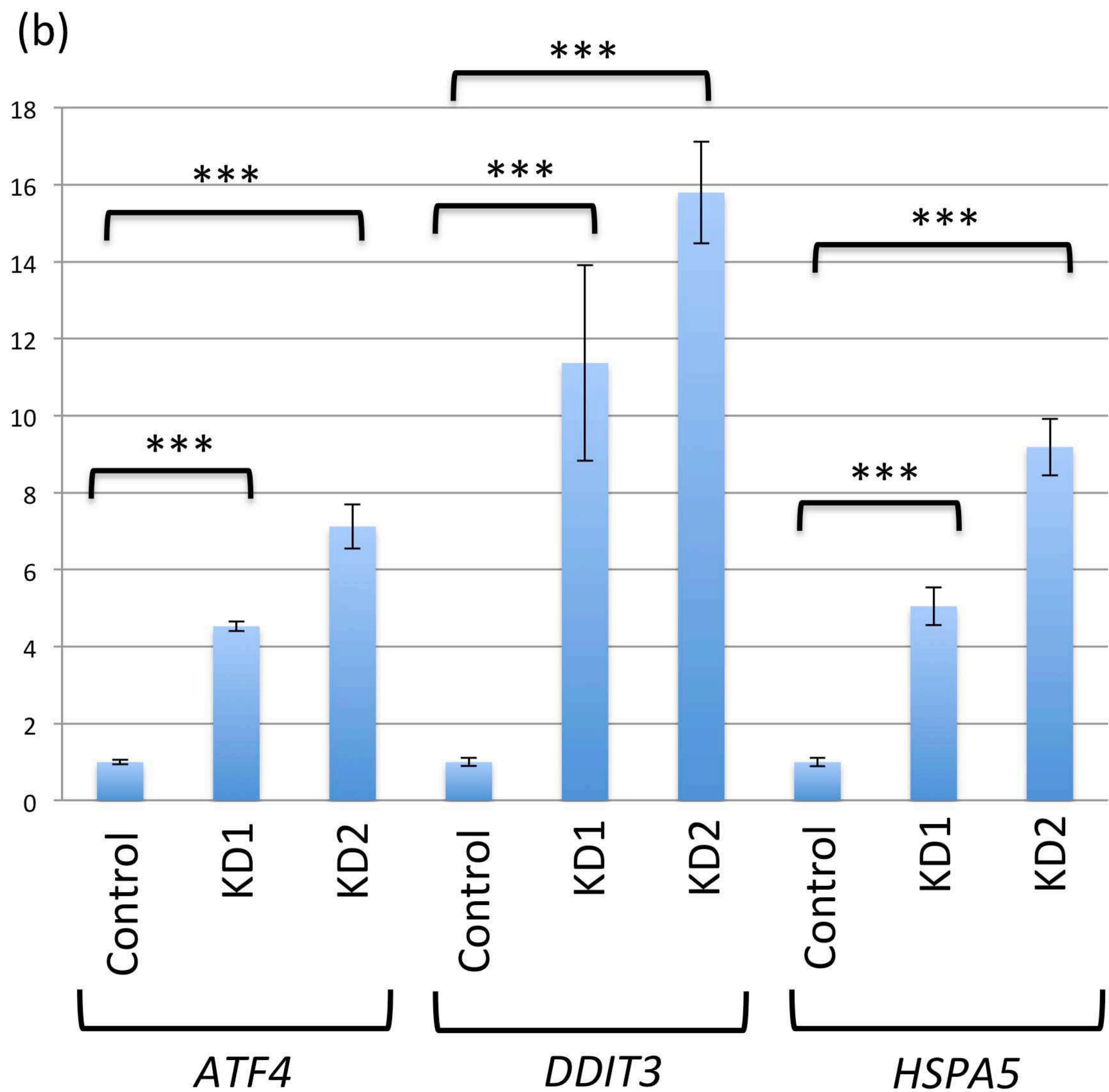
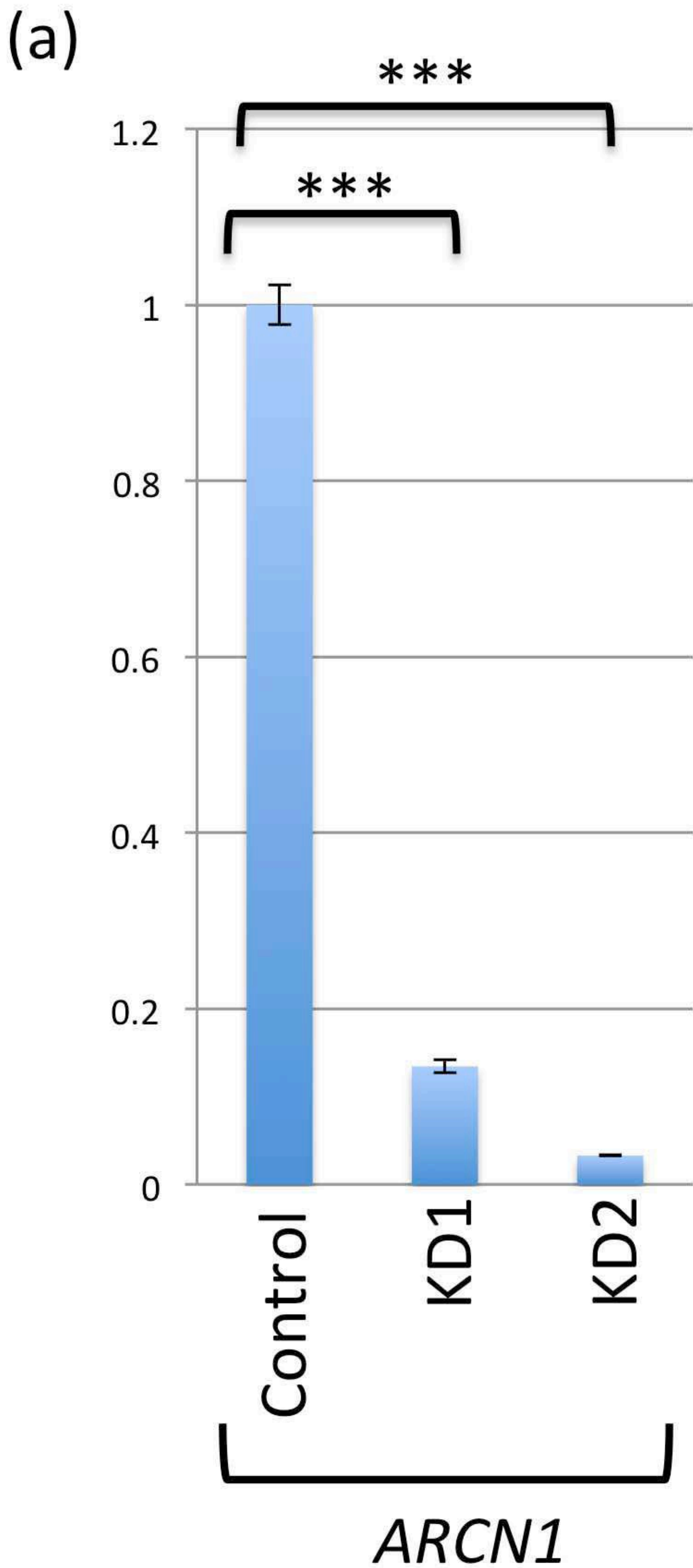




Figure 4

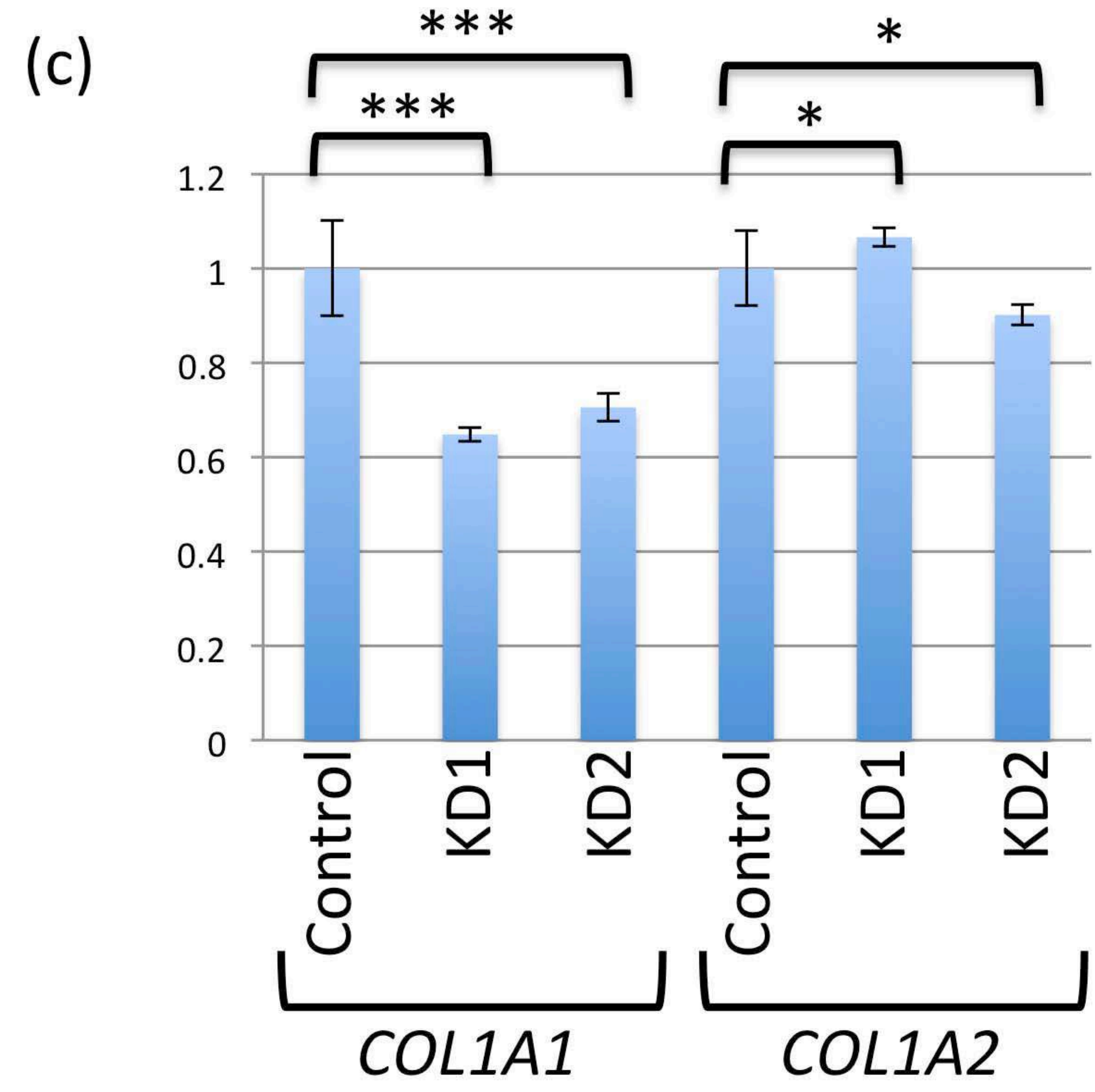
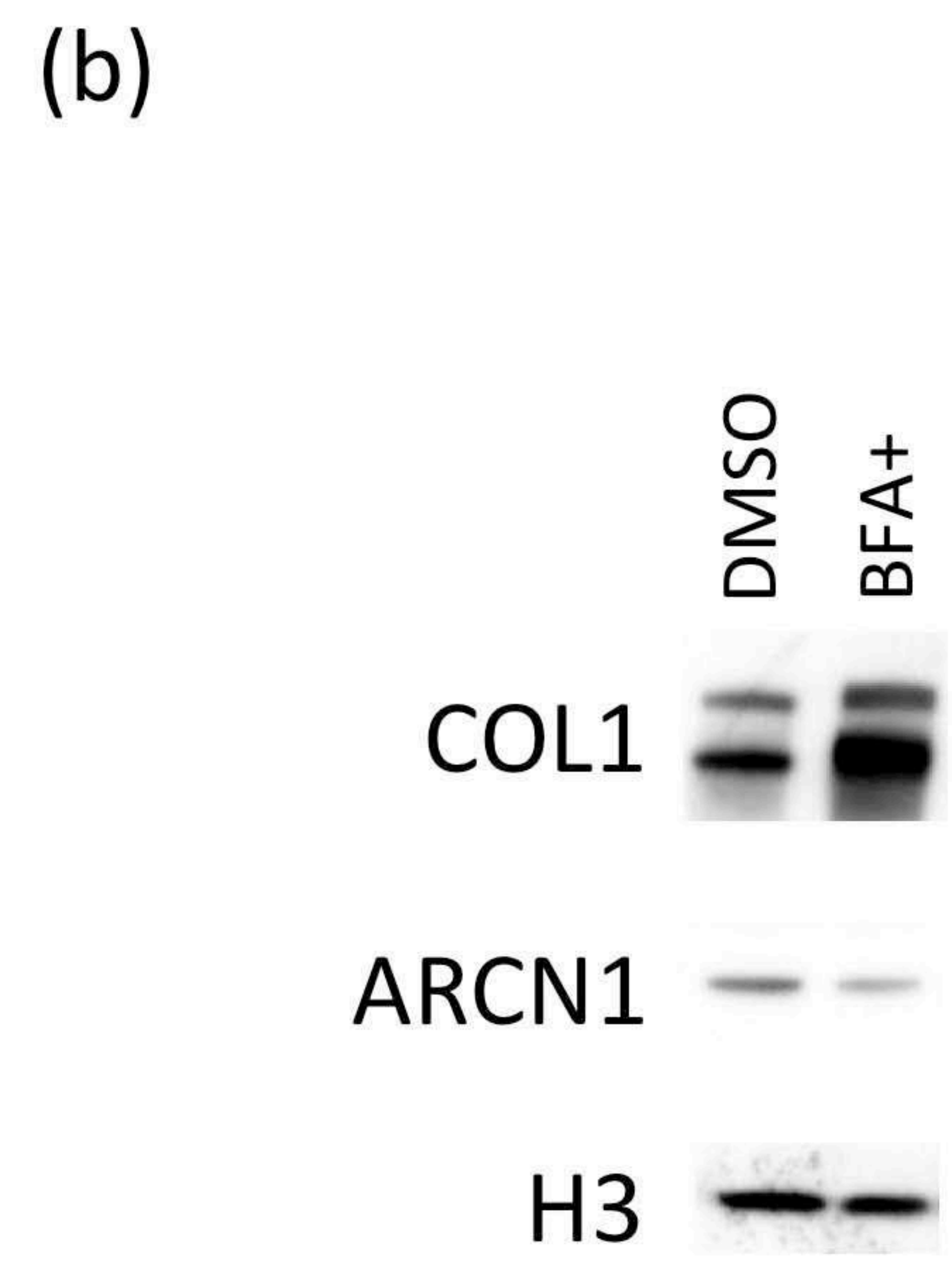
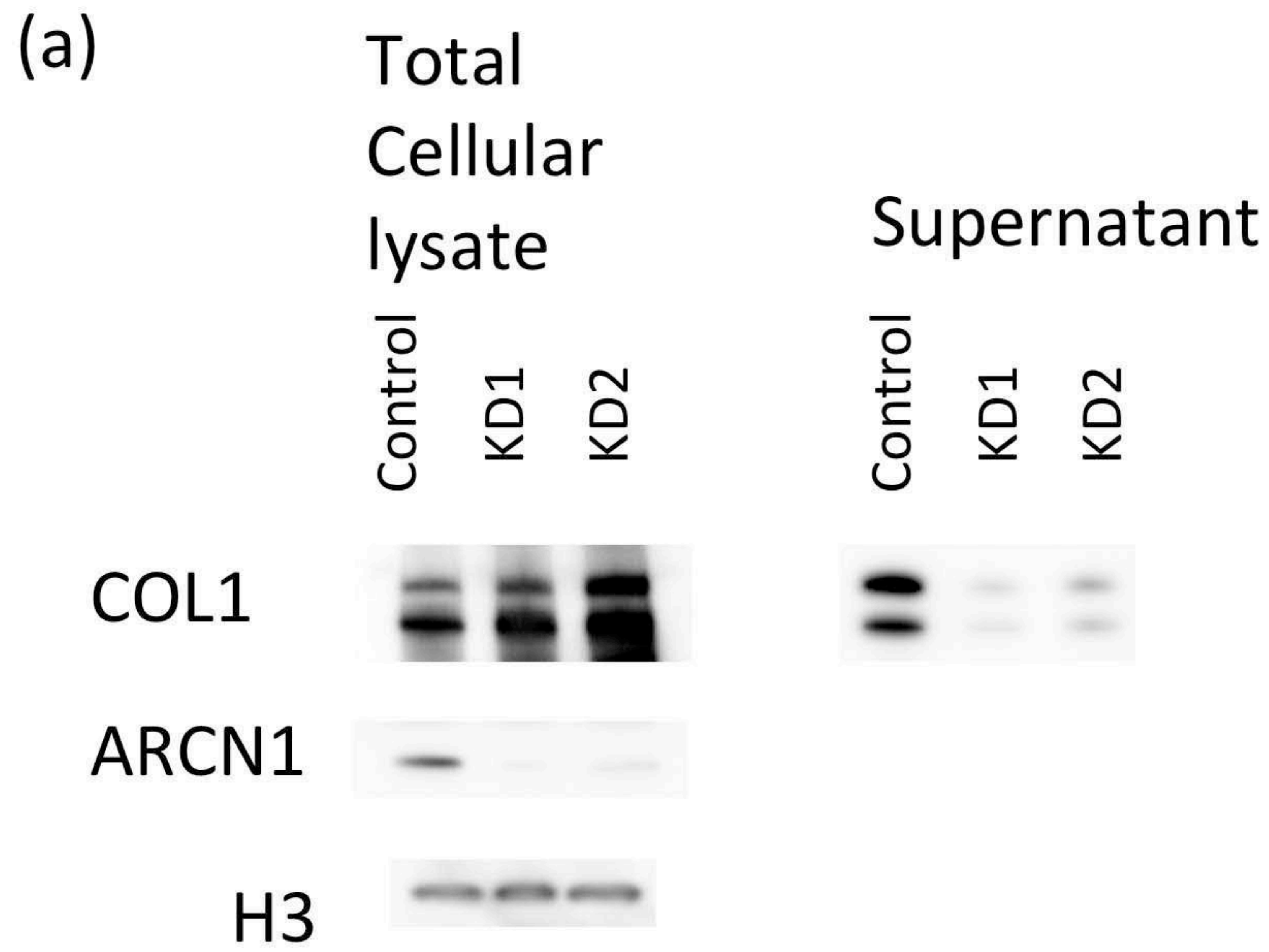
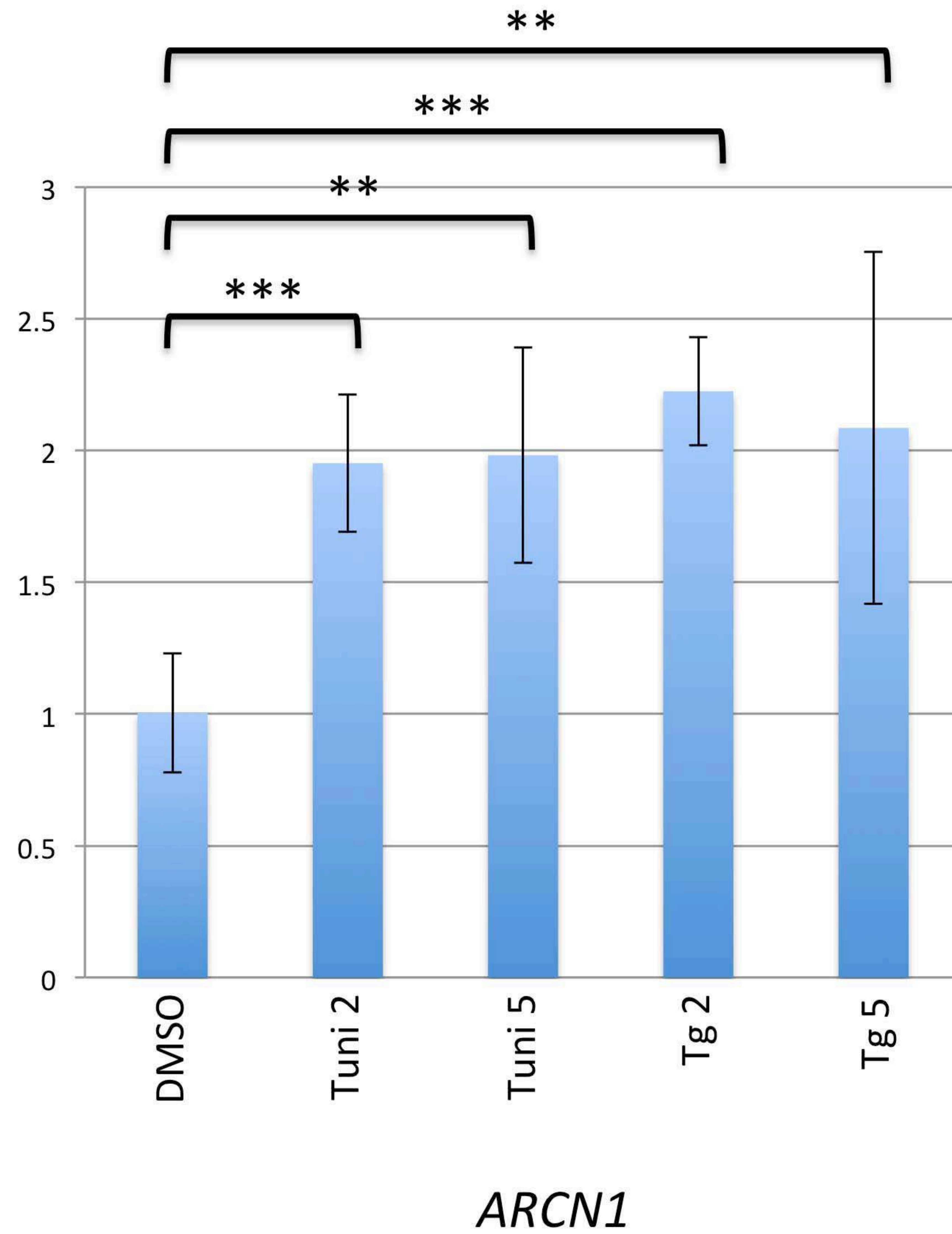


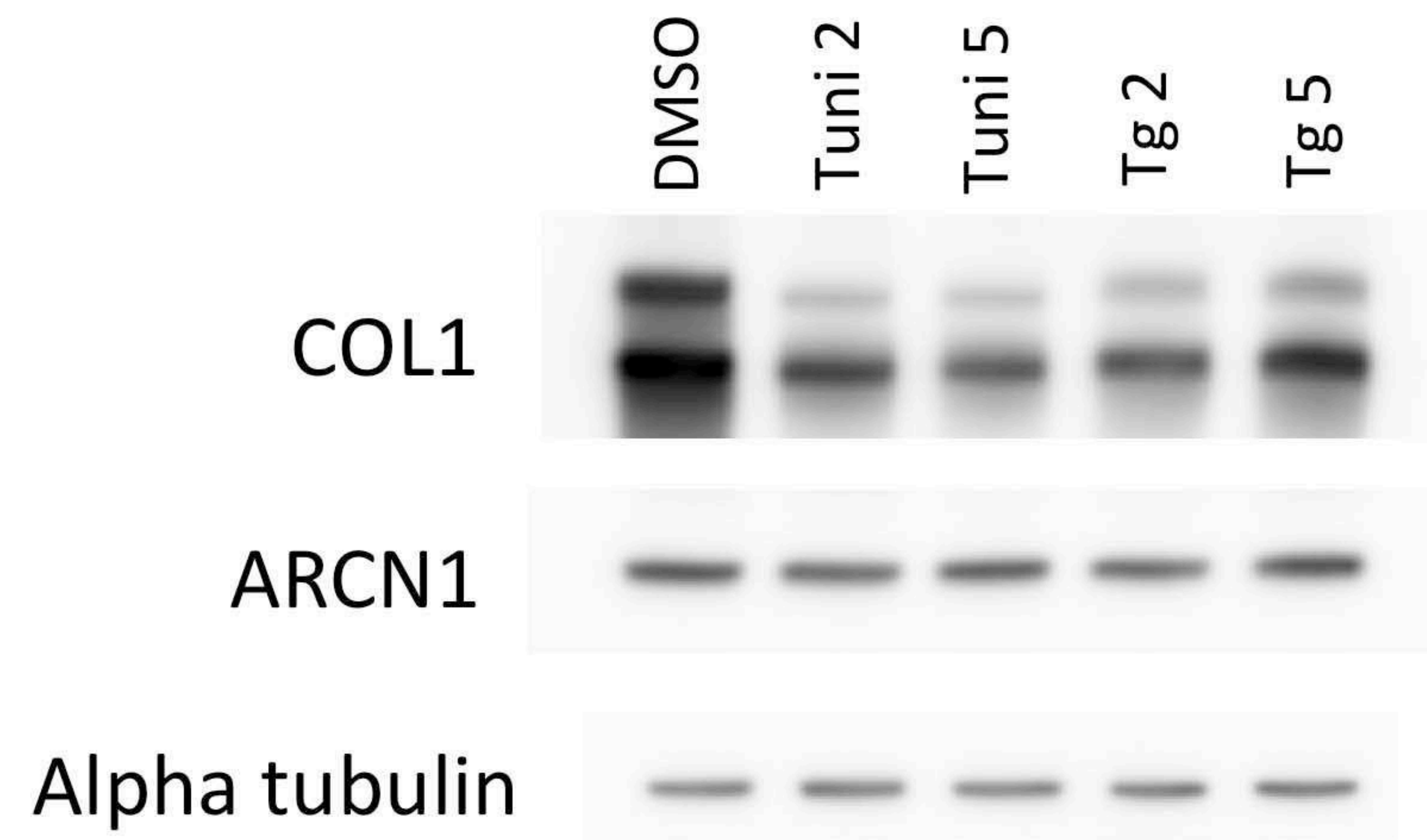


Figure 5

(a)



(b)



## Supplemental Information

### Supplemental Note: Case Reports

Subject 1 was referred to the Genetics Clinic of Nagano Children's Hospital at the age of 1 month with the chief complaints of facial dysmorphism and intrauterine growth retardation. Severe intrauterine growth retardation was noted during pregnancy. Subject 1 was born at 35 weeks gestation and was delivered by normal vaginal delivery. Her birth weight was 1100 gm (<3<sup>rd</sup> centile: -4.18SD), length was 34 cm (<3<sup>rd</sup> centile: -3.72SD), and head circumference was 26 cm (<3<sup>rd</sup> centile: -3.15SD). At birth, severe micrognathia and facial dysmorphism were noted. An echocardiogram revealed the presence of a ventricular septal defect. After birth, Subject 1 developed respiratory distress, requiring ventilator support. Severe micrognathia was considered to be the reason of respiratory distress. Therefore, when she was 6 months old, a tracheostomy was performed. The subsequent development of Subject 1 has been slightly delayed; she started standing with holding at 10 months and sitting independently at 11 months. At 1 year and 10 months, her developmental quotient (DQ) was 65. At the age of 3 years and 1 month, DQ for motor skills, cognition/adaptation, and language/social skills

were 75, 77, and 69, respectively. The overall DQ was 72. At the age of 3 years and 4 months, she recently met the developmental milestones of approximately 2 years and 6 months, since she was able to jump up, draw straight lines, and take off her clothes.

Since birth, she has continued to have short stature and has exhibited failure to thrive (Figure S1A). On physical examination, several facial dysmorphisms, including a prominent forehead, downslanted palpebral fissures, and severe micrognathia, were noted. In addition, shortening of upper arms and legs, and small joint laxity were observed (Figure 1A). Beighton score was 5/9. No skin color or hair abnormalities were identified. She has not shown any signs of cerebellar ataxia. A skeletal survey revealed widening of the metaphysis and a wide femoral neck (Figure 1B). Ophthalmological evaluation revealed astigmatism. Audiology evaluation was unremarkable. Brain MRI was also unremarkable. Currently, she is 3 years and 4 months old, and her height was 80.2cm (-4.1SD), weight was 8.8kg (-3SD), and head circumference was 43.4cm (-3.2SD). The family history of Subject 1 was unremarkable.

Subject 2, a boy with developmental delay and mild autism, was first seen in the Genetics Clinic, KK Women's & Children's Hospital, at the age of 1 year and 8

months. Antenatal scans showed intrauterine growth retardation, micrognathia, and possible ambiguous genitalia. Amniocentesis revealed a normal karyotype. Subject 2 was delivered at 34 weeks gestation via cesarean section and had a birth weight of 1360 gm (<3<sup>rd</sup> centile), birth length of 40cm (<3<sup>rd</sup> centile), and birth head circumference of 29cm (10<sup>th</sup> centile). Postnatally, it was observed that he had severe micrognathia and penoscrotal hypospadias and required tracheostomy for respiratory support during infancy (Figure S1B). He also developed severe gastroesophageal reflux, requiring fundoplication and gastrostomy insertion for the first 3 years of life. The development of Subject 2 was slightly delayed; he started walking independently at 13 months and climbing stairs at 18 months. He spoke his first words at 17 months. At 7 years of age, he has moderate-to-severe language difficulties and currently attends special education school. His medical issues include epilepsy and obstructive sleep apnea requiring continuous positive airway pressure support at night. An MRI scan of the brain of Subject 2 was normal and an electroencephalogram confirmed epileptiform activity. On physical examination, it was observed that Subject 2 had micrognathia, scaphocephaly, and hypotelorism, as well as small joint laxity (Figure 1C). Beighton score was 2/7. No

skin color or hair abnormalities identified. He has not shown any signs of cerebellar ataxia. The growth parameters of Subject 2 improved postnatally, and as of 7 years, his height is 121 cm, which is in the 10th centile. His mid-parental height is 172.5, which is 50<sup>th</sup> centile. Currently he is 10 year and 6 months old, and his current height is 136cm (10th percentile) and weight 33.2kg (25th percentile), head circumference was 51cm (6<sup>th</sup> centile (-1.53SD)). His ophthalmological issues include occasional divergent squint and myopia. He does not have hearing problems.

Subject 3 (25 years old) was born at 36 weeks gestation from consanguineous parents (cousins): weight 1.450kg (<3<sup>rd</sup> centile), length 38cm (<3<sup>rd</sup> centile), head circumference 27.6 cm (<3<sup>rd</sup> centile). Hypotelorism, micrognathia, short bowed legs, and high arched palate were noticed at birth. Bilateral posterior cataract was diagnosed when he was 6 years old. He had hypertrophic muscular appearance, rhizomelic shortness of upper and lower limbs, large hands and feet, micropenis, and hypoplastic scrotum. A skeletal survey at 7 years of age demonstrated short femoral and humeral diaphysis, thick femoral neck with coxa valga, and advanced wrist bone maturation. The Subject 3's IQ was rated at 65 based on the Wechsler Intelligence

Scale for Children (WISC). At the age of 25 years, his weight was 98kg, his length was 152cm (-3.5 SD), and his head circumference was 50 cm (-5 SD) (Figure 1D). He cannot read or write but can be understood. He is an employee in his father's company and has married a woman from another region.

Subject 4, the daughter of Subject 3, shared common features with Subject 3, which included intrauterine growth retardation followed by postnatal growth failure. Currently, she is 3 years old, and her height was 79.5 cm (-4 SD), her weight was 9.650kg (-3SD) and head circumference was 45 cm (-5 SD). She was also found to have dysmorphic features including microretrognathia, hypotelorism, shortening of upper arms and legs, and muscular hypertrophy (Figure 1E). Subject 4 was born at term: weight 1.400kg (<3<sup>rd</sup> centile), length 37cm (<3<sup>rd</sup> centile), head circumference 27.5cm (<3<sup>rd</sup> centile). She had a cleft palate, and feeding difficulties that required nutritional support until the age of 2 years. She started walking at 2 years of age with ataxia, and she does not speak yet. Her brain MRI showed microcephaly, delayed white matter myelination in T2 weight images, and mild cerebellar atrophy (Figure S1C).

Exome sequencing was performed for Subject 1 as well as her unaffected

biological parents. The G-band karyotype and SNP array results were normal. Exome capture was performed with Agilent SureSelect XT Human All Exon V5 (Agilent, Santa Clara, Calif., USA), and 100-bp or 126-bp paired-end read sequencing was performed by HiSeq 2500 (Illumina, San Diego, Calif., USA). The sequenced reads were aligned to the human genome hg19 using BWA v0.7.12 with BWA-MEM algorithm and '-M' option<sup>1</sup>. The Genome Analysis Toolkit (GATK) v3.3.0 was used for variant discovery<sup>2</sup>. After marking duplicates, local realignment around indels, and base quality score recalibration, SNVs and indels for each sample were called using the HaplotypeCaller. SNVs and indels with a GQ score above 15 were used for further analysis. The effects of variants were predicted by SnpEff v3.6b<sup>3</sup> and annotated by ANNOVAR<sup>4</sup>. Information and statistics regarding sequenced reads, mapping rate, and coverage are summarized in Table S1. Six *de novo* mutations in 4 genes (*ARCN1*, *DCAF15*, *HLA-DRB5*, *FAM58A*) were identified. However, variants in *DCAF15*, *HLA-DRB5*, and *FAM58A* were present in dbSNP, leaving *ARCN1* as the prime candidate gene to account for the subject's phenotype. The mutation in *ARCN1* resulted in a premature stop codon in exon 2 (NM\_001655:exon2:c. 260C>A:p.Ser87\*). The *ARCN1* mutation was confirmed

by Sanger sequencing (Figure 2A and Figure S2A). Sanger sequencing demonstrated that the *DCAF15* variant was inherited from her unaffected father and the *FAM58A* variants were inherited from her unaffected parents (Figure S3A and S3B).

Subject 2 was independently identified in a cohort of individuals from Singapore with undiagnosed genetic diseases. He had been previously tested and his karyotype and chromosome microarray analysis results were normal. Exome sequencing of Subject 2 and his unaffected parents was carried out by the Genome Institute of Singapore using the NimbleGen SeqCap EZ Human exome V3 kit (Roche, Basel Switzerland) and sequenced on the HiSeq 2000 platform (Illumina, San Diego, USA) using 100-bp paired-end reads. Alignment to the human genome hg19 was carried out using BWA 0.7.5a and the variants were annotated using ANNOVAR. Variants with minor allele frequency (MAF) >0.01 in dbSNP or EVS (Exome Variant Server) were excluded and rare functional variants were prioritized according to *de novo* status, homozygosity, compound heterozygosity and X chromosome-linkage. After filtration, *de novo* heterozygous variants were identified in *ARCN1* and *SYT1*. Both of these variants were confirmed by Sanger sequencing. The *ARCN1* mutation was a



frameshift variant: NM\_001655:c.633del; p.Val212Trpfs\*15 predicted to create a frameshift in exon 4 starting at codon Val212 and ending in a stop codon 14 positions downstream (Figure 2A and Figure S2B). The *SYT1* (NM\_001135805.1:c.697G>A; p.Asp233Asn) variant is a missense variant that substitutes Aspartic acid at codon 233 with Asparagine (Figure S3C). There were no other potentially pathogenic mutations.

Subjects 3 and 4 were also ascertained independently. Whole-exome sequencing was performed for Subject 3 and his healthy parents by IntegraGen Genomics. Exons of DNA samples were captured using an in-solution enrichment methodology (Agilent SureSelect All Exon V4 + UTRs) and sequenced with an Illumina HiSeq 2000 instrument. SNPs and indels were determined using the CASAVA 1.8 software. Identification of the disease-causing variants was performed with the Exome Resequencing Intelligent Sorter (ERIS, Integragen) program for the annotation and filtering of genetic variants. The following criteria were used for excluding non-pathogenic variants: (1) variants represented with an allele frequency of more than 0.5% in dbSNP 138, the NHLBI Exome Sequencing Project EVS, or the in-house database of Integragen; (2) variants in the 5' or 3' UTR; (3) variants with intronic

locations and no prediction of local splice effect; and (4) synonymous variants without prediction of local splice effect. Filtered variants occurring *de novo* were retained for further confirmation and segregation analysis by Sanger sequencing. The *ARCN1* mutation was the only probably damaging mutation that occurred *de novo* in Subject 3 and was transmitted to his affected daughter, Subject 4. The *ARCN1* mutation was NM\_001655:c.157\_158del, leading to p.Ser53Cysfs\*39 (Figure S2C).

## Supplementary Figures

**Figure S1.** Additional clinical information of the individuals with *ARCN1* mutations. (A)

Growth chart of Subject 1. Green dots represent height measurements, red dots

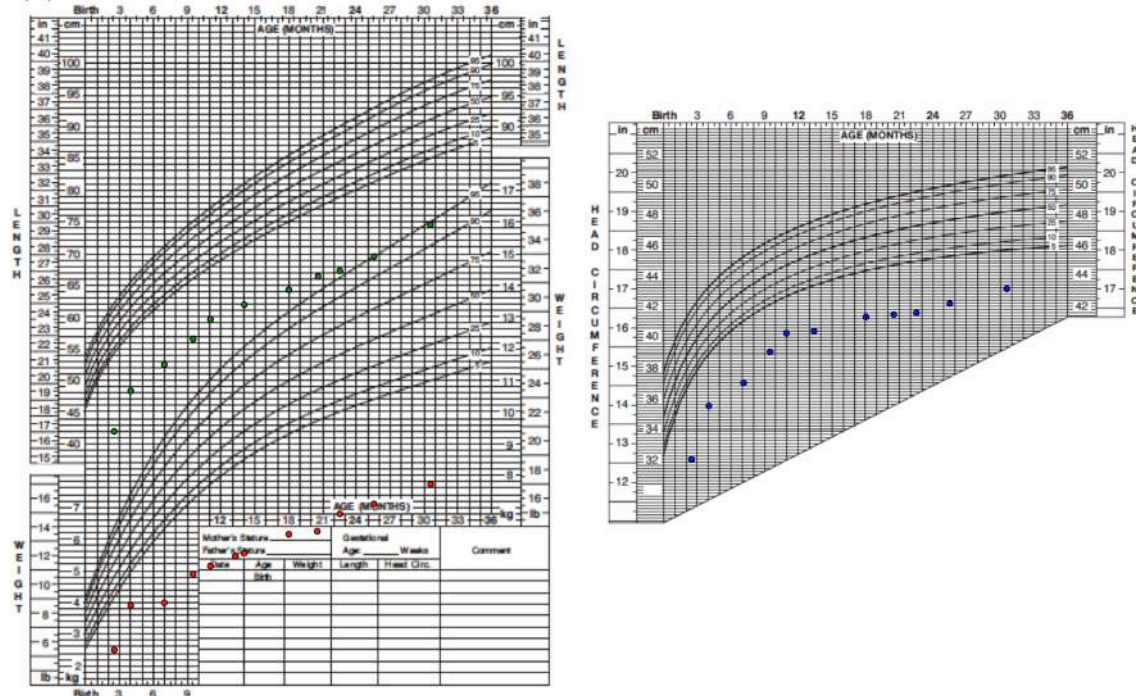
represent weight measurements, and blue dots represent head circumference

measurements. (B) Radiograph of Subject 2 demonstrating the presence of

micrognathia. (C) Brain MRI of Subject 4. Microcephaly with gyral simplification, thin

corpus callosum, delayed myelination and mild cerebellar atrophy are demonstrated.

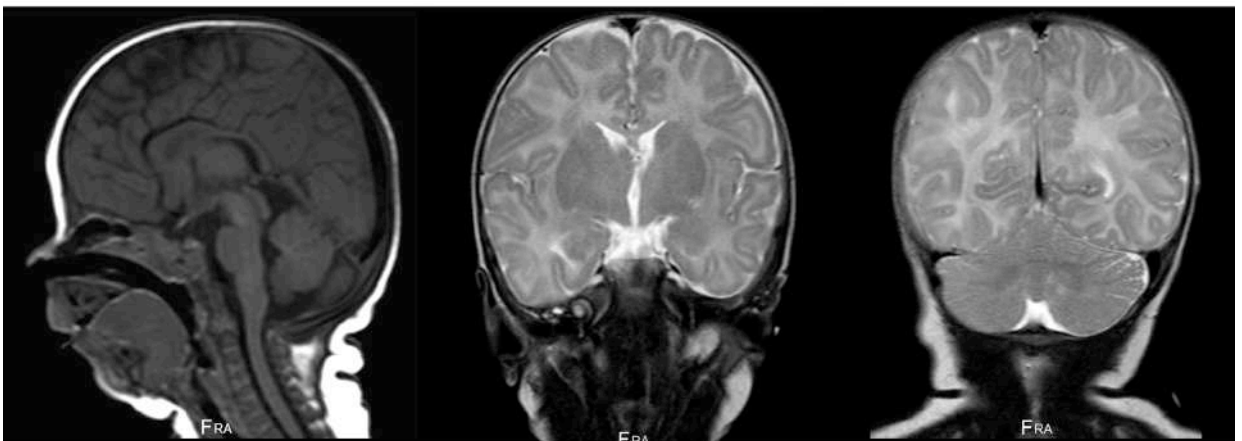
(A)



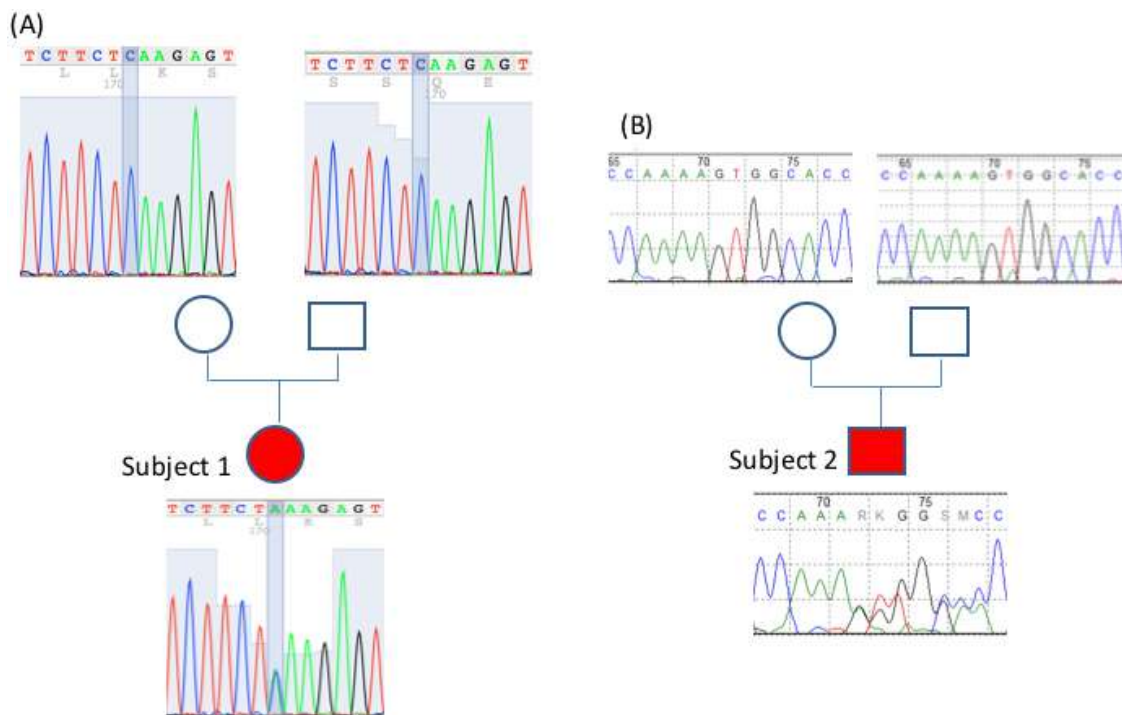
(B)



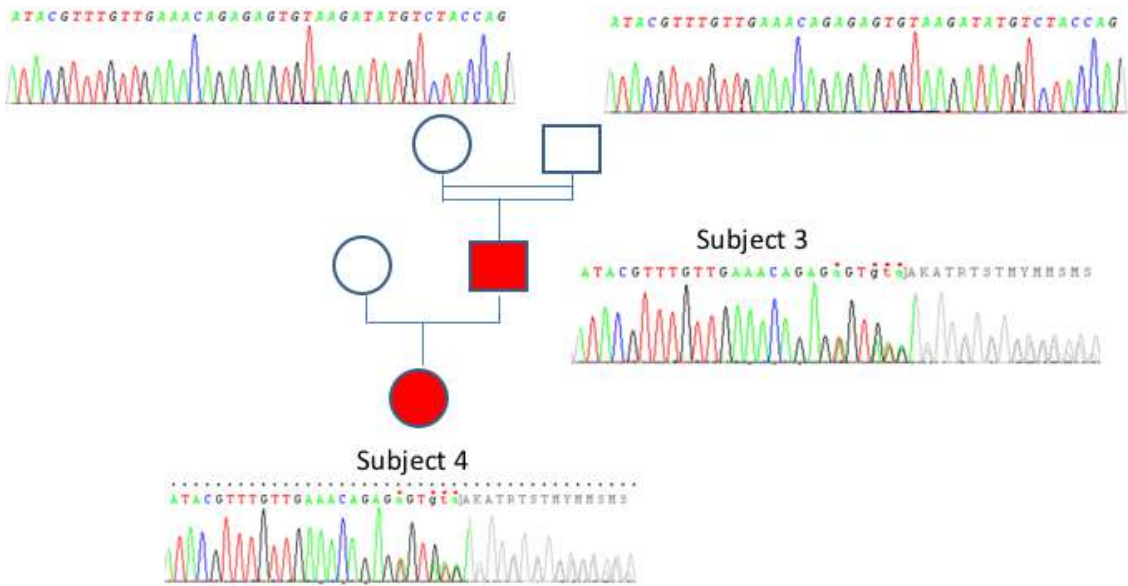
(C)



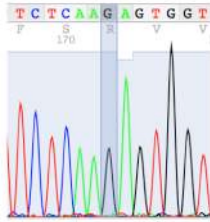
**Figure S2.** Sanger sequencing chromatogram of *ARCN1* mutations. (A) *ARCN1* mutation in Subject 1. Neither of the parents carried the same mutation. (B) *ARCN1* mutation in Subject 2. Neither of the parents carried the same mutation. (C) *ARCN1* mutation in Subjects 3 and 4. *ARCN1* mutation was not detected in the parents of Subject 3. (D) *ARCN1* mutations introduced by CRISPR/Cas9 in HCT116 cell line clones.



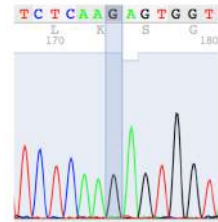
(C)



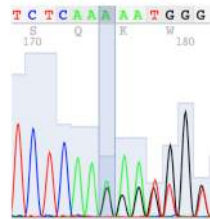
(D) WT1



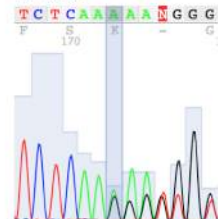
WT2



Mut1



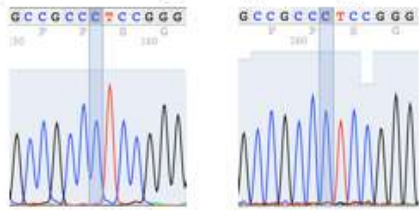
Mut2



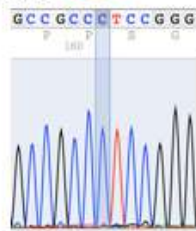
**Figure S3.** Sanger sequencing confirmation of *FAM58A*, *DCAF15* and *SYT1* variants.

(A) The *FAM58A* variant was identified not only in Subject 1, but also in both parents, arguing against a possible role of the *FAM58A* variant in explaining the clinical phenotype of Subject 1. This variant was reported in dbSNP as rs200645137, and its population frequency is between 0.987 and 1.0 in the Human Genetic Variation database (<http://www.genome.med.kyoto-u.ac.jp/SnpDB/index.html>). (B) The *DCAF15* variant was confirmed in Subject 1, but the same variant was also identified in her father, arguing against its causality. A similar insertion was reported in dbSNP as rs3217681, and its population frequency is 0.407 according to the Human Genetic Variation database. (C) The *SYT1* variant in Subject 2 was confirmed by Sanger sequencing, and neither parent was found to have this variant. This *SYT1* variant represents a novel variant, and has not been previously reported in the public SNV database.

(A) *FAM58A* Sanger sequencing

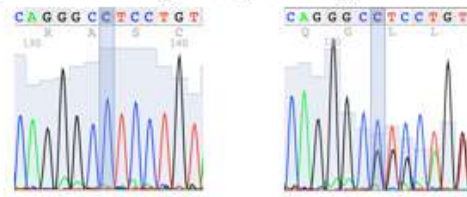


Subject 1

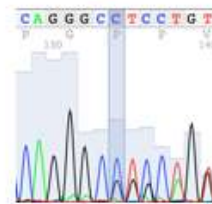


*FAM58A*: NM\_152274: c.16\_17ins G

(B) *DCAF15* Sanger sequencing

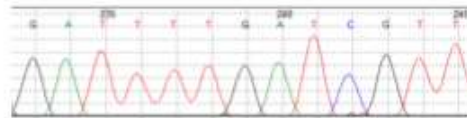
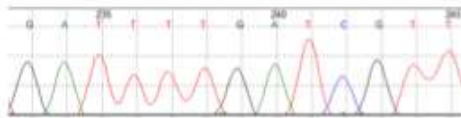


Subject 1

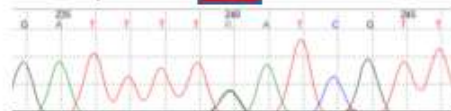


*DCAF15*: NM\_138353: c.1439\_1440insGGTGGGCCCAAGGCGGGCAG: p.E480fs

(C) *SYT1* Sanger sequencing



Subject 2

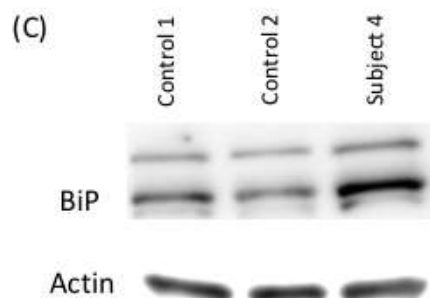
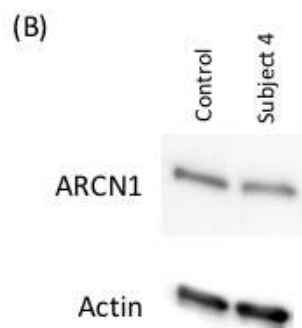
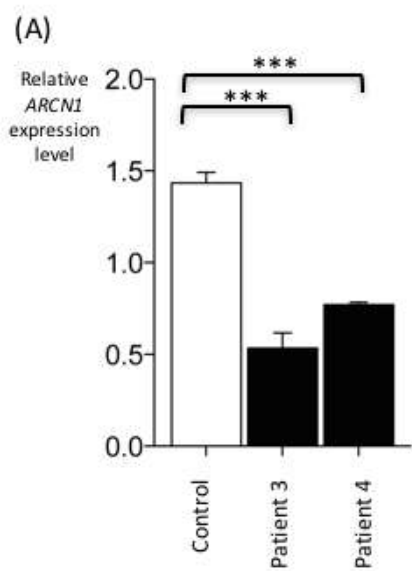


*SYT1*: NM\_001135805.1: c.697G>A

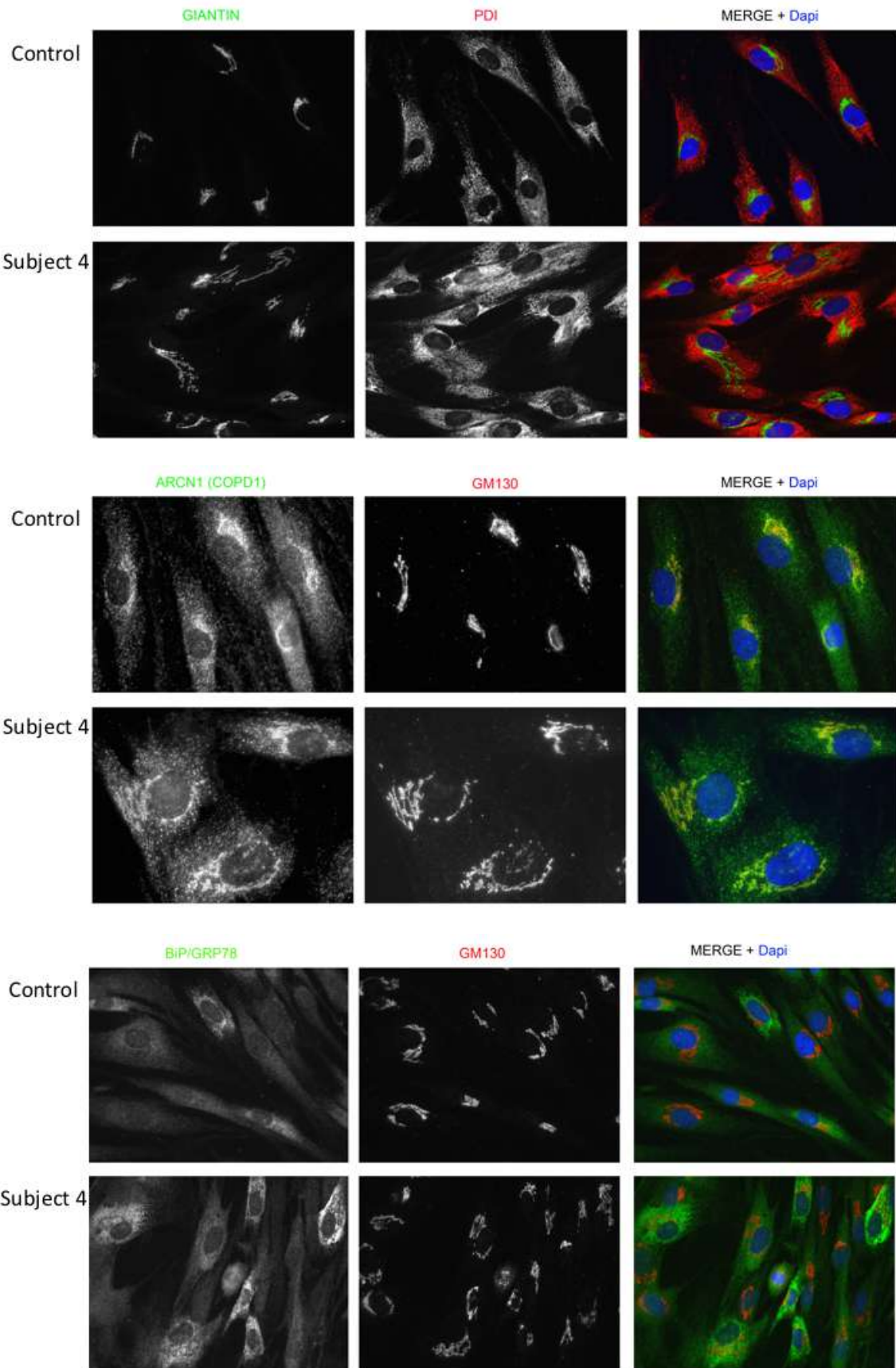


**Figure S4.** Quantitative RT-PCR and western blot using *ARCN1*-related syndrome subject-derived skin fibroblast samples. (A) Quantitative RT-PCR. Total RNA was extracted from fibroblasts with the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Courtaboeuf, France). Total RNA was subjected to reverse transcription using the iScript cDNA synthesis kit (Bio-rad, Marnes la Coquette, France). qPCR was performed in duplicate for each sample using the SybrGreen Supermix (Bio-rad). The relative expression of *ARCN1* was expressed relative to the expression of *HPRT*. The *ARCN1* expression level was significantly reduced in samples from Subjects 3 and 4 compared to that of the control sample. \*\*\*  $P < 0.001$ , one-way ANOVA followed by a Bonferroni post-test. Two replicates were performed. (B) and (C) *ARCN1* and BiP western blotting. Whole cell extracts were fractionated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. After incubation with 10% nonfat milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 1 h, membranes were incubated with antibodies against *ARCN1* (COP1D, AV54594, Sigma-Aldrich), BiP (Grp78, ab21685, Abcam) or beta-Actin (MAB1501, Millipore) overnight at 4 °C. Membranes were washed three times and incubated with horseradish

peroxidase-conjugated anti-mouse or anti-rabbit antibodies. Blots were washed with TBST and developed with the ECL system (Amersham Biosciences) according to the manufacturer's protocols. (B) The protein level of ARC1 was slightly reduced in the Subject 4 sample, compared to that of the control sample. Two replicates demonstrated consistent results. (C) Western blotting with BiP antibody yielded two bands, and lower band representing BiP based on the molecular size (about 78kDa). The BiP protein, which is an ER stress marker, was elevated in the Subject 4 sample, compared to that of the control.



**Figure S5.** Immunocytochemical analysis. Cultures were fixed for 20 min with 4% paraformaldehyde. For immunocytochemistry, the cells were permeabilized with 0.3% TritonX-100 for 15min, washed, blocked for 1 h with 10% serum in PBS and incubated with primary antibody overnight at 4°C. Primary antibodies used were anti-ARCN1/COP1D (ab96725, Abcam), anti-GM130 (610823, BD Transduction Lab), anti-GIANTIN (ab80864, Abcam), and anti-PDI (MA3-019, Thermofisher). Following washing, cells were treated with secondary antibody for 1 h in the dark at room temperature. All images were captured on a Zeiss microscope IMAGER.Z1, using the Apotome imaging system coupled to AxioCam MRm and the Axiovision Rel.4.8 software. BiP represents an ER stress marker, and GIANTIN and GM130 are Golgi markers. PDI is a marker of ER. Immunofluorescence staining of the Golgi apparatus (GIANTIN and GM130), and that of ER (PDI) as well as COPI vesicle (ARCN1) indicated the swollen appearance of the Golgi stacks in the Subject 4's fibroblast. Accumulation of BiP in the cytosol is demonstrated in the Subject 4's fibroblast compared to that of the control.



**Figure S6.** ER stress gene expression was elevated with tunicamycin and thapsigargin treatments. Data represents mean  $\pm$  2 s.d. \*\*\*  $P < 0.001$ , two-tailed  $t$  test;  $n = 3$  technical replicates per group. Quantitative PCR was run in triplicates, and mean and standard deviation was calculated. Two different concentrations of tunicamycin treatment ( $2\mu\text{g/ml}$  (Tuni2) and  $5\mu\text{g/ml}$  (Tuni5)) and thapsigargin treatment ( $2\mu\text{M}$  (Tg2) and  $5\mu\text{M}$  (Tg5)) were used. DMSO was added for control. Gene expression level was normalized against *GAPDH* expression. Relative gene expression level to the control sample is demonstrated.

