Fig. 6. Suppression of the expression of several genes in R80S embryos (A–C) and *ESCO2*-knockdown morphants (D,E). The expression of neurogenesis markers, *notch1a* and *notch1b* (A), neural progenitor markers, *ascl1a* and *ascl1b* (B) and vascular differentiation markers, *GATA-1* and *notch3* (C) in wild-type (WT), heterozygous (*ESCO2$^{R80S/WT}$*, hetero) and homozygous (*ESCO2$^{R80S/R80S}$*, R80S) mutants (2.0 days postfertilization [dpf]) was investigated by quantitative reverse transcription polymerase chain reaction. Normal and abnormal indicate apparently normal mutants and developmentally abnormal mutants, respectively. The expression of *notch1a* and *notch1b* (D) and *GATA-1* and *notch3* (E) in morphants (2.0 dpf) with the injection of 5mis mo (control) or ATG mo was examined. NS, *, ** and *** indicate $P \geq 0.05$, $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, compared to WT or control embryos; ANOVA and Tukey's test, $n = 13$–14 (A–C); Student's t-test, $n = 13$ (D,E).

genetic approach. Morpholino antisense oligonucleotide is a very useful tool when using medaka or zebrafish as an animal model to efficiently reduce the expression of a gene of interest. However, it is sometimes difficult to ascertain the cause–effect relationship because morpholinos are often complicated by off-target effect. It should be especially difficult to analyze the morphants when the phenotypes vary as in RBS/SC. The mutant has a great advantage over morphants in that the gene alteration is fixed in the genome and the phenotypes can be analyzed in the uniform genetic background. Compared to other classical gene disruption methods such as homologous recombination and transposon/retrovirus insertion,

TILLING has the great advantage of missense mutant recovery. Amino acid (a.a.) substitutions reveal the unexpected role of a protein, which cannot be obtained by knockout (KO) and knockdown methods. Missense mutants are particularly useful in the analysis of essential genes because simple disruption of the gene would cause lethality at the cellular or animal level. To our knowledge, *ESCO2*-KO mice have not been reported to date. Fish such as medaka are suited to investigation of the gene function during embryonic development as they develop *ex vivo*. The development of organs in fish share common molecular pathways with mammals. The developmental abnormality and death during development can be closely monitored in fish while

many of the lethal phenotypes may be overlooked in humans. Taken together, our system by using medaka could provide a powerful tool for investigating unidentified missense mutations in autosomal recessive disorders *in vivo*, even in embryonic lethal mutants.

The R80S mutant was created by ethyl nitroso urea (ENU) mutagenesis, which randomly introduces point mutations throughout the genome. In order to examine whether the observed phenotype was related to the R80S mutation, and to exclude the possibility that it was not derived from other mutation(s), we took two approaches: *in vivo* morpholino injections into the fertilized medaka eggs and linebreeding. The *ESCO2* morphants exhibited developmental abnormality and reduction of *notch1a* and *notch3* expression similar to that observed in the mutants. Moreover, the developmental and cytological abnormalities observed in mutants with a K-Cab (southern population) genetic background could be transferred to K-Kaga (northern population) genetic background by crossing original mutants with WT K-Kaga. Therefore, we suggest that the R80S mutation is responsible for the observed phenotypes.

Clinical feature in RBS/SC model medaka

The variety of phenotypes among individuals and the lack of a clear correlation between genotype and phenotype are some of the clinical features of RBS/SC. It has been shown that the same mutation results in different phenotypes in RBS/SC (Schüle *et al.* 2005;

Vega *et al.* 2005, 2010; Gordillo *et al.* 2008). Recently, Mönnich *et al.* (2011) demonstrated that *ESCO2*-KD zebrafish showed craniofacial and fin abnormality. We summarized the phenotype of RBS/SC, R80S mutants, medaka *ESCO2* morphants (present study) and zebrafish *ESCO2* morphants (Mönnich *et al.* 2011) (Table 3). We also observed phenotypic diversity in our mutant medaka: craniofacial abnormalities, cardiac defect, growth retardation and PCS. RBS/SC is characterized by craniofacial abnormalities including bilateral cleft lip and/or palate, micrognathia, hypertelorism, exophthalmos, downslanting palpebral fissures, malar hypoplasia, hypoplastic nasal alae and ear malformation (Gordillo *et al.* 2006). R80S mutants exhibited a severe phenotype in the head region and were deficient in all head elements except for the eyes, even which showed developmental delay and incorrect structure. Thus, the morphological phenotype of the R80S mutant was very similar to RBS/SC and zebrafish *ESCO2* morphants except for limb abnormalities. The upper and lower limbs of humans and the pectoral and pelvic fins of medaka are evolutionarily derived from the same origins (Hinchliffe 2002). However, long bone growth in the stylopod and zeugopod is absent in the fins. Moreover, the fins have no part that is homologous to the autopod, which constitutes the distal part of mammalian limbs (Hinchliffe 2002). Therefore, phocomelia, long bone hypoplasia, ectrodactylia and brachydactylia cannot be detected in fish. In zebrafish *ESCO2* morphants, shorter and smaller fins were observed (Mönnich *et al.* 2011). Our R80S

Table 3. Summary of phenotypes of Roberts syndrome/SC phocomelia (RBS/SC) and medaka and zebrafish models

RBS/SC (human)	R80S mutants (medaka)	Morphants (medaka)	Morphants[†] (zebrafish)
Autosomal recessive	Autosomal recessive		
Intra uterine fetal death	Embryonic lethal	Survive	Embryonic lethal
Pre- and postnatal growth retardation	Developmental delay	Developmental delay	
	Small size	Small size	Small size
	Body axis abnormality	Body axis abnormality	Body axis abnormality
Craniofacial abnormality			
Microcephaly	Microcephaly	Normal	Smaller heads
Hyper telorism	Smaller and abnormal eye	Normal	Smaller eye
Hypoplastic nasal alae	Lack of nasal part	Normal	ND
Malar hypoplasia	Cartilage defect	ND	ND
Cleft lip and palate	Lack of mouth part	Normal	Lack of jaw
Limb abnormality			
Phocomelia	ND	ND	ND
Ulnar defects	ND	ND	ND
Radial defects	ND	ND	ND
Thumb defects	Normal (delayed)	Normal	Shorter and smaller fin
Renal abnormality	ND	ND	ND
Heart defect	Heart malformation	Heart malformation	Cardiac edema
Mental retardation	ND	ND	ND
Premature centromere separation	Premature centomere separation	ND	ND

[†]After Mönnich *et al.* 2011. ND, not determined.

mutants had normal pectoral fins. However, some of them exhibited developmental delay. Therefore, shorter and smaller fins of zebrafish morphants may correspond to the mutant fins developmentally delay. Medaka *ESCO2* morphants also had normal pectoral fins. The effect of morpholino was weaker in medaka than in zebrafish. This may be due to the time frame of morpholino action as medaka develops much more slowly than zebrafish and the effect of morpholino may be reduced by the time these organs are formed.

R80S mutants also exhibited developmental arrest by 2 dpf with incomplete penetrance (Table 1). Most of the escapers exhibited heart malformation and/or body axis abnormalities. This may be pertinent to the phenotypic variation observed in humans. Concerning congenital cardiac abnormality, atrial septal defect, ventricular septal defect and patent ducts arteriosus were reported (Gordillo *et al.* 2006). The escapers of R80S mutant and some of *ESCO2* morphants showed looping defect. Disturbance in cardiac looping is thought to cause congenital cardiac malformations (Männer 2009).

ESCO2-KD zebrafish showed cell cycle arrest and apoptosis with caspase activation (Mönnich *et al.* 2011). In R80S mutant of medaka, we could not clearly detect significant cell cycle arrest. This disagreement may be attributable to the difference of species or to the difference of knockdown (zebrafish) and missense mutant (medaka). We also detected apoptosis throughout the body in R80S mutants. This apoptosis may be associated with caspase activation, as in zebrafish. Moreover, microarray analysis revealed the gene regulated by ESCO2 in zebrafish and some gene regulation overlaps with cohesin-dependent transcription (Rhodes *et al.* 2010; Mönnich *et al.* 2011).

R80S mutation is responsible for embryonic death, and deficiency of Eco1 in yeast causes cellular lethality. On the contrary, RBS/SC patients can give birth. It was reported that the related molecule, ESCO1 in humans, and suggested that ESCO1 and ESCO2, play distinct roles in cell cycle progression (Hou & Zou 2005). Although ESCO1 and ESCO2 acetylate SMC3, the knocking down of *ESCO1* is more effective on the SMC acetylation than that of *ESCO2* in humans (Zhang *et al.* 2008). The different effect of functional deficiency in ESCO2 between RBS/SC patients and R80S mutants may be explainable by the difference of functionalization of ESCO1 and ESCO2. The function of ESCO1 in medaka needs to be elucidated. On the other hand, the mutant medaka showed more severe phenotypes than RBS/SC and morphants of medaka and zebrafish. Only mutant medaka is embryonically lethal. RBS/SC patients are rare and RBS/SC is characterized by intrauterine fetal death. A few R80S

mutants can live up to adult fish. Thus, RBS/SC patients may be escapers as in R80S mutants. The escapers of R80S mutants will be analyzed in a future study.

R80S mutation

We have found that the mutation in the conserved amino acid (a.a.) in the N-terminal portion of ESCO2, R80 (in human K), disrupts the establishment of cohesion function of ESCO2, demonstrating the importance of this previously undescribed region of this protein. This is the first case in which a missense mutation outside the acetyltransferase domain causes the loss-of-function phenotype. The region (73–84 a.a.) including R80 is well conserved from fish to humans, although the mutation in this region has not been reported in RBS/SC. ECO1 lacks this region. Although Eso1 (Tanaka *et al.* 2010), budding yeast homologue, deco (Williams *et al.* 2003), *Drosophila* homologue and ESCO1 (Hou & Zou 2005) of humans contain extradomain at the N-termius, no motif or similarity have been found in the N-terminal portion of ESCO2. The molecular function of this conserved region is of special interest.

Variety of clinical features in RBS/SC

The *ESCO2* expression in the proliferating region and the chromosomal phenotype, namely, PCS, caused by the R80S mutation indicate that ESCO2 plays an important role in cell division in medaka. In other species, ESCO2 functions in the establishment of sister chromatid cohesion via acetylation of the cohesin complex (Rolef Ben-Shahar *et al.* 2008; Unal *et al.* 2008; Zhang *et al.* 2008). Therefore, the R80S mutation of ESCO2 may cause a reduction of cohesin complex acetylation, resulting in an anti-establishment state in sister chromatid cohesion during S-phase similar to that in other species including humans. On the other hand, RBC/SC has a variety of clinical features (Schüle *et al.* 2005; Vega *et al.* 2005, 2010; Gordillo *et al.* 2008). The same mutation of ESCO2 causes different phenotypes. R80S mutants also have a variety of clinical phenotypes. Pathological features in the establishment of sister chromatid cohesion are attributable to a lack of ESCO2 function in cell division. However, this is not sufficient to explain the variety in the clinical features. The cohesin complex, the target of ESCO2, causes Cornelia de Lange syndrome (CdLS) (OMIM 122470, 300590 and 610759). CdLS also has a variety of clinical features (Dorsett 2007; Liu & Krantz 2009). The clinical features of CdLS are distinct from RBS/SC, but with some overlap (Liu & Krantz 2009). Recently, cohesion-mediated gene regulation was

demonstrated in *Drosophila*, zebrafish, mice and humans. Homeobox gene expression is required for Nipped-B, a cohesin regulator in *Drosophila* (Dorsett 2009). The gene expressions of *runx1* and *runx3* (transcription factors) are required for Rad21 and Smc3 (components of the cohesin complex) in zebrafish (Horsfield *et al.* 2007). Genome-wide chromatin immunoprecipitation experiments in human and mouse cells have revealed co-localization of cohesin and CTCF, a zinc-finger protein with enhancer blocking and barrier activities (Parelho *et al.* 2008; Stedman *et al.* 2008; Wendt *et al.* 2008). CTCF is thought to have a potential function in directly regulating gene transcription both positively and negatively (Ishihara *et al.* 2006; Chernukhin *et al.* 2007). ESCO2 functions in establishment of sister chromatid cohesion by acetylation of the cohesin complex. Therefore, mutation of ESCO2 may cause lack of functional cohesin. Consequently, it is suggested that mutation of ESCO2 affects the expression of several genes. In this study, we demonstrated that R80S mutation actually suppresses *notch1a*, its downstream genes and *notch3* expression. Downregulation of *notch1a* and its downstream gene expression result from the mutation of the cohesin component in zebrafish (Horsfield *et al.* 2007). Therefore, we propose that the neural and heart malformations may involve downregulation of these genes in R80S mutant medaka and RBS/SC. The clinical feature of cardiac defects in R80S mutants is similar to the medaka knocking-down of congenital heart disease 5 (*mCHD5*) gene function (Murata *et al.* 2009). *Notch3* suppressed in ESCO2 R80S mutant may be involved in *mCHD5* expression. Recently, cohesin-independent regulation of gene expression by Eco1 was reported (Choi *et al.* 2010). Eco1 suppresses transcription via association with histone demethylase, LSD1, to convert chromatin to an inactive state. Thus, ESCO2 regulates the expression of several genes. Research on the genes regulated by ESCO2 is an interesting area for further study. The functional analyses of ESCO2 have only been performed at cellular level so far. This mutant medaka enables analysis at a whole-body level, particularly, in the early embryonic development. The mechanisms producing the pathological variety of RBS/SC will be elucidated.

In conclusion, we isolated *ESCO2* mutant medaka from the TILLING library and characterized it using a reverse genetic approach. The $ESCO2^{R80S/R80S}$ homozygous mutants had phenotypic features reminiscent of RBS/SC. From the gene expression analysis, some gene expression was downregulated in mutants. Thus *ESCO2* mutant medaka is the animal model for RBS/SC and a valuable resource for future research.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Alignment of deduced amino acid sequences of ESCO2 in medaka (*Oryzias latipes*), human (*Homo sapiens*, accession no. NP_001017420), mouse (*Mus musculus*, accession no. NP_082315) and zebrafish (*Danio rerio*, accession no. Q5SPR8).

Figure S2. Expression of *ESCO2* in early development of medaka embryo.

Figure S3. Developmental profile of the expression alleles of *ESCO2*.

Figure S4. Construction of morpholinos.

Figure S5. Pectoral width of WT (empty bars) and R80S (solid bars) embryos (2.0 dpf, A), and morphants (2.0 dpf) with 300 nmol/L of 5mis mo (empty bars), ATG mo (solid bars) or E2I2 mo (shaded bars) injected just after fertilization (B). dpf, days postfertilization; WT, wild type.

Figure S6. Expression of marker gene in R80S (uppermost) and wild-type embryos.

Table S1. Primer using for cloning, genotyping and real-time polymerase chain reaction.

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