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Original Article

Sirtuin inhibitor Ex-527 causes neural tube defects, ventral edema formations, and gastrointestinal malformations in *Xenopus laevis* embryos

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Chemical reagent Ex-527 is widely used as a major inhibitor of Sirtuin enzymes, which are a family of highly conserved protein deacetylases and have been linked with caloric restriction and aging by modulating energy metabolism, genomic stability, and stress resistance. However, the extent to which Ex-527 controls early developmental events of vertebrate embryos remains to be understood. Here, we report an examination of Ex-527 effects during *Xenopus* early development, followed by a confirmation of expressions of xSirt1 and xSirt2 in embryonic stages and enhancement of acetylation by Ex-527. First, we found that reductions in size of neural plate at neurula stages were induced by Ex-527 treatment. Second, tadpoles with short body length and large edematous swellings in the ventral side were frequently observed. Moreover, Ex-527-treated embryos showed severe gastrointestinal malformations in late tadpole stages. Taken together with these results, we conclude that the Sirtuin family start functioning at early embryonic stages and is required for various developmental events.

Key words: Ex-527, Sirtuin, teratogenesis, *Xenopus*.

Introduction

The Sirtuins are highly conserved NAD+-dependent protein deacetylases and are able to extend the lifespan of several model organisms such as yeast, worm, flies, and some vertebrates (Tissenbaum & Guarente 2001; Rogina & Helfand 2004; Sommer *et al.* 2006). In mammals, the Sirtuins are composed of seven Sir2 orthologues (Sirt1-7) and have a conserved deacetylase core that uses NAD+ as a cofactor at the central region of proteins (Frye 2000). Functional specificity on Sirt1-7 is thought to be regulated by the N- or C-terminal domains. For instance, a 25 amino acids sequence at the C-terminal domain of Sirt1, called ESA (essential for Sirt1 activity), interacts with and functions as an "on switch" for the deacetylase core (Kang *et al.* 2011). Sirt1 orchestrates various biological events, including cell differentiation (Fulco *et al.* 2008), apoptosis (Luo *et al.* 2001), autophagy (Lee *et al.* 2008), cancer (Kim *et al.* 2008), metabolism (Picard *et al.* 2004; Li *et al.* 2007), and circadian rhythms (Asher *et al.* 2008). However, little is known about the role of Sirt1 on the early developmental stage in vertebrates, although Sirt1 is widely expressed during early embryogenesis (McBurney *et al.* 2003). In the study using Sirt1-deficient mice, notable developmental defects of the retina and heart are observed, and embryos of null mice become significantly smaller than normal embryos, leading to infrequent postnatal survival (Cheng *et al.* 2003).

The chemical reagent Ex-527 is a potent Sirt1 inhibitor, 200–500-fold more selectively binds to Sirt1 than to Sirt2 or Sirt3, and does not bind to Sirt4-7 (Peck *et al.* 2010). Treatment with Ex-527 can

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increase acetylation of p53 in human mammary epithelial cells and several cell lines (Solomon *et al.* 2006). Interestingly, developmental delay of cyst growth in mouse autosomal dominant polycystic kidney and apoptosis in human leukemia cells are strongly induced by treatment of Ex-527 (Cea *et al.* 2011; Zhou *et al.* 2013). In *Xenopus*, p53 activity is essential for normal development by linking to transforming growth factor (TGF)-β signaling (Wallingford *et al.* 1997; Cordenonsi *et al.* 2003; Takebayashi-Suzuki *et al.* 2003). Recently, we found that developmental delay was caused by inhibition of the TOR kinase in *Xenopus* embryos (Moriyama *et al.* 2011), which is thought to be a co-regulator of the Sirtuins for the autophagy network to prolong lifespan in several species (Medvedik *et al.* 2007; Ghosh *et al.* 2010; Zhang *et al.* 2013). These current discoveries suggest that the Sirtuins play important roles at embryonic stages of vertebrate. In this study, we first confirm expressions of the Sirtuins and acetylated-lysine level on Ex-527-treated *Xenopus* embryos, and then introduce severe phenotypical effects due to Ex-527 treatments on early *Xenopus* embryogenesis.

Materials and methods

Embryology and histology

Xenopus laevis eggs were obtained from females injected with 400 units of human chorionic gonadotropin (HCG; Fuji Pharma Co., Japan), fertilized *in vitro* with minced testis, and then cultured in 0.1× Steinberg's solution (SS). 1× SS contains 58 mmol/L NaCl, 0.67 mmol/L KCl, 0.34 mmol/L $Ca(NO_3)_2$, 0.83 mmol/L $MgSO_4$, 100 mg/L kanamycin sulfate, and 5 mmol/L Tris-HCl (pH 7.4). Frog embryos were cultured at 22°C at all times. The jelly coats were removed with thioglycolic acid solution (1% of thioglycolic acid in 0.1× SS, pH 8.0). Embryos were fixed with PBSFA for 2 h, dehydrated with ethanol, embedded in paraffin wax, sectioned at 8 μm, and stained with hematoxylin and eosin (HE).

Ex-527 treatment

Ten milligrams of Ex-527 (S1541; Selleck Chemicals, Houston, TX, USA) was dissolved in 500 μL of dimethylsulfoxide (DMSO), and each 20-μL aliquot (80 mmol/L) was stored at −20°C. In times of usage, aliquots were diluted with 0.1× SS into the proper concentration. Ex-527 (200 μmol/L), which is the maximum concentration used in this study, includes 0.25% DMSO. No toxic effects were observed in the solutions including 0.5% of DMSO. Embryos were exposed to Ex-527 solutions from the 2-cell stage to the stages designed for observation.

Nicotinamide treatment

12.5 mg of nicotinamide (N0636-100G; Sigma-Aldrich) was dissolved in 10 mL 0.1× SS (20 mmol/L). Embryos were exposed to nicotinamide solutions from the 2-cell stage to the stages designed for observation.

RT-PCR

The following primers were used for reverse transcription–polymerase chain reaction (RT–PCR):

xSirtuin1-RT-fw, 5′-CTTAATGATGGGAACGGATCCCC-3′, *xSirtuin1*-RT-rv, 5′-CGGCCTTGGATCTTTCCTGAAG-3′, *xSirtuin2*-RT-fw, 5′-TCGAGCGAACCAGCGGATCT-3′, *xSirtuin2*-RT-rv, 5′-GAAATAGTTCTCGAGCCAGTGC-3′, *xSirtuin3*-RT-fw, 5′-AGCCTTTCTTCCACCTGGCC-3′, *xSirtuin3*-RT-rv, 5′-CTTGGGAAAGTCCTGGTAAGCTT-3′, *xSirtuin4*-RT-fw, 5′-ACACACCAAGGCTGGGCAGT-3′, *xSirtuin4*-RT-rv, 5′-AGTGCAAACCGATAACCAGAATAAACT-3′, *xSirtuin5*-RT-fw, 5′-GCACTGGAAATGACACGCCC-3′, *xSirtuin5*-RT-rv, 5′-AGCCTGCCTTGCGATGTAACT-3′, *xSirtuin6*-RT-fw, 5′-ACGGGAGCCGGGATCAGTA-3′, *xSirtuin6*-RT-rv, 5′-GCCTCGCACCTTTGGCACAT-3′, *xSirtuin7*-RT-fw, 5′-GAGACGGAAGTTGCACAGCG-3′, *xSirtuin7*-RT-fw, 5′-GCTTCTCTTGGTAATCCACTCC-3′, *noggin*-RT-fw, 5′-ACATCAGACCGGCTCCTAGT-3′, *noggin*-RT-rv, 5′-CTGCGTTGACATCTCCACCT-3′, *six3*-RT-fw, 5′-GCAACTTCAGGGAGCTCTAC-3′, *six3*-RT-rv, 5′-TAGGGATCCTGCAAGTACCA-3′, *sox17*-RT-fw, 5′-AACAATGTCATGGTAGGAGAGAAC-3′, *sox17*-RT-rv, 5′-GCCATCTGTTTAGCCATCACTG-3′, *EF1alpha*-RT-fw, 5′-GCAAGTTTGCTGAGCTCAAGG-3′, *EF1alpha*-RT-rv, 5′-AATGGTCTCAAATTTGGTGACAGA-3′, *ODC*-RT-fw, 5′-GCAACTGATGCATGATATTAAAGAAC-3′, *ODC*-RT-rv, 5′-GAACTTTTATTTGTAAAACTGGTCAA-3′.

The annealing temperatures were 55°C, 30 cycles for *xSirtuin1*, *xSirtuin2*, *xSirtuin3*, *xSirtuin4*, *xSirtuin5*, *xSirtuin6*, *xSirtuin7*, *noggin, ODC,* and *EF1alpha,* and *35cycles* for *six3* and *sox17* were performed for amplifications.

Western blot analysis

Embryos were lysed in RIPA buffer containing a complete protease inhibitor cocktail (04693159001, Roche, Germany), and lipids were removed by an equal volume of trichloroethylene (208-02486, Wako, Japan). Proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels and transferred onto polyvinylidene fluoride (PVDF)

membranes. Western blots were performed using anti-acetylated-lysine (1:1000; 9441S, Cell Signaling Technology, USA) as primary antibodies, anti-Rabbit IgG as secondary antibody (1:2000; 31460, Thermo Scientific, MA, US), and Coomassie Brilliant Blue (178-0051; Wako).

Results

All Sitruin family genes were expressed from unfertilized egg to tadpole

If the Sirtuins have functions at early embryogenesis, transcripts should be expressed at the early stage of embryos. Therefore, in order to examine the expression patterns of *Sirt1-7*, RT–PCR was carried out in embryos at various developmental stages. Then, we found that high amounts of transcripts in all cases were expressed in all early embryonic stages of *Xenopus* in addition to unfertilized eggs (Fig. 1A). The expressions of *Sirt1-7* were also confirmed in the database created from RNAseq analysis that we originally performed in order to list all transcripts in gastrula embryos (data not shown). On the other hand, *noggin*, famous for its function as a dorsal factor of vertebrate (De Robertis & Kuroda 2004), was only observed zygotically as expected (Fig. 1A), supporting the fact that *Sirt1-7* are maternal expressed genes. We also carried out *in situ* hybridization to see specific expression of Sirt1. The expression of *Sirt1* was ubiquitous in all stages that we examined (Stages 1–42, data not shown).

Deacetylations in Xenopus *embryos were inhibited by Ex-527*

Acetylation is one of the major post-translational modifications for cellular proteins, and the Sirtuins are a large family of deacetylation enzymes that negatively regulate the acetylation status of several proteins such as histone and p53. Therefore, if chemical reagent Ex-527 can work for *Xenopus* embryogenesis, it should be changing the acetylation status of embryos. In order to validate activity of Ex-527 for *Xenopus* embryos, we examined acetylation level of Ex-527-treated embryos (Fig. 1B). Previous studies have shown that the acetylation of lysine 382 of p53 is effectively modulated by Sirt1 (Vaziri *et al.* 2001). As a result, Ex-527 clearly showed a concentration-dependent increase in the amount of acetylated proteins, with a maximum effect at 200 μmol/L (Fig. 1B). To eliminate the possibility that Ex-527 might influence total amount of proteins, we verified that the amount of protein was not changed by CBB staining (Fig. 1C).

Neural plate in Ex-527-treated embryos became less differentiated than normal

In order to examine whether Sirt1 was working in early embryogenesis, loss-of-functional experiment was performed. Ex-527 (100 and 200 μmol/L) was used on live *Xenopus* embryos beginning at the 2-cell stage up to stage 45. The results were not what we expected, in that the developmental delay occurred by rapamycin, which was a famous mTOR inhibitor treatment in

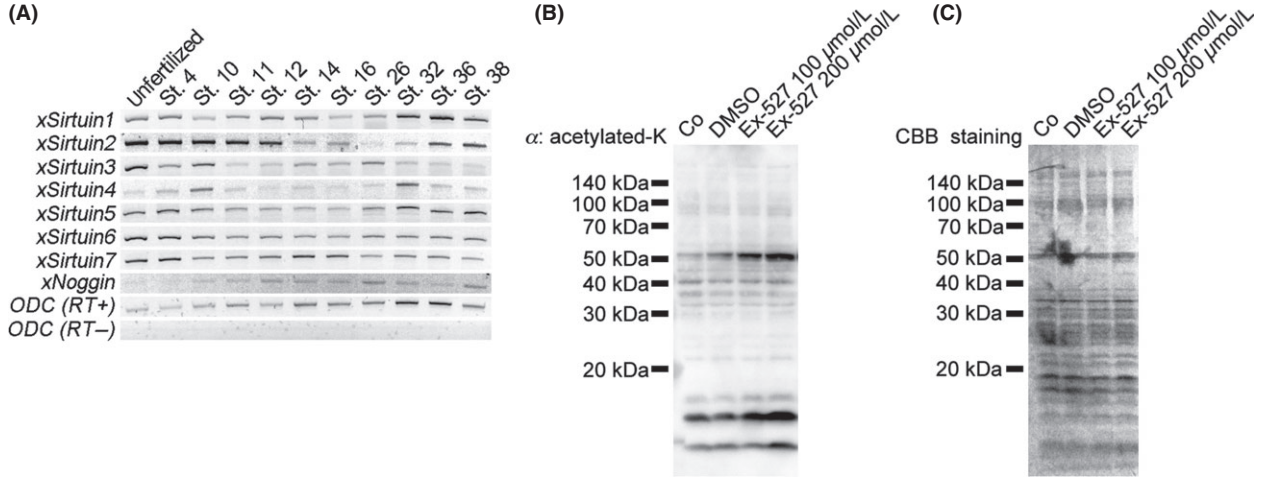

Fig. 1. Expression of *xSirtuins* and inhibition of xSirtuin1 activity by Ex-527 treatment. (A) Reverse transcription–polymerase chain reaction (RT–PCR)analyses for *xSirtuin1* to *7* mRNA expressions from unfertilized egg to tadpole stage. All *xSirtuins* are consecutively expressed in *Xenopus* embryos. (B) Western blot analysis of acetylated-lysine in Ex-527-treated embryos. Embryos were treated at the 2-cell stage with 100 and 200 μmol/L of Ex-527, and extracts were collected 12 h later. Predictable band size of p53 is 53 kDa. (C) CBB staining for SDS-PAGE gel. Total amounts of proteins were not changed by any treatments.

our previous study (Moriyama *et al.* 2011), and that Sirt1 might be working as an upstream factor of mTOR. All embryos treated with Ex-527 formed dorsal blastopore, which means stage 10, at almost the same time as control embryos, at later stages we could not observe any remarkable difference on developmental speed between treated and control embryos (data not shown). It was relevant to note that more than 400 μmol/L concentration was too high for Ex-527 treatment to observe effects because leaks of yolk occurred in all embryos under these conditions before stage 8 (data not shown). However, focusing on morphogenesis, obvious effects were recognized at neurula stages. At late neurula stages, a pigmented midline of neural plate was observed on the dorsal side of normal embryos, while a part of the anterior midline and pigmentation of presumptive cement gland region were confirmed on the ventral side (Fig. 2A). Almost the same results were obtained from DMSO-treated embryos (Fig. 2B). On the other hand, pigmented midlines on Ex-527-treated embryos were undoubtedly shorter than on control embryos and did not reach the ventral side (Fig. 2C,D). The mild blanching region, which is the feature of neural plate and observed in control and DMSO-treated embryos (Fig. 2A,B), was not detected in Ex-527-treated embryos (Fig. 2C,D). The facts suggest that Ex-527 has inhibitory activity on neural induction. On the other hand, slight swellings on the ventral side were detected in most embryos treated with 200 μmol/Lof Ex-527 (Fig. 2D). Furthermore, we performed analysis to detect the influence of inhibition of xSirtuin1 by Ex-527 for neural and endomesoderm formation. Dorsal lips treated with Ex-527 were elongated the same as control and DMSO-treated dorsal lips (Fig. 2E–G). The expression of *Sox17* in the dorsal lip, which was the marker for endoderm, was not affected by treatment of Ex-527, while the expression of neural marker in dorsal lips cut from Ex-527-treated embryos was slightly decreased (Fig. 2H). These facts suggest that Ex-527 has an inhibitory action on neural induction independent of dorsal endomesoderm formation.

Ex-527 treatment caused anterior defects, shorter body length, and large edematous swelling

At late stages, the effects of Ex-527 on morphogenesis appeared drastically. As described above, most

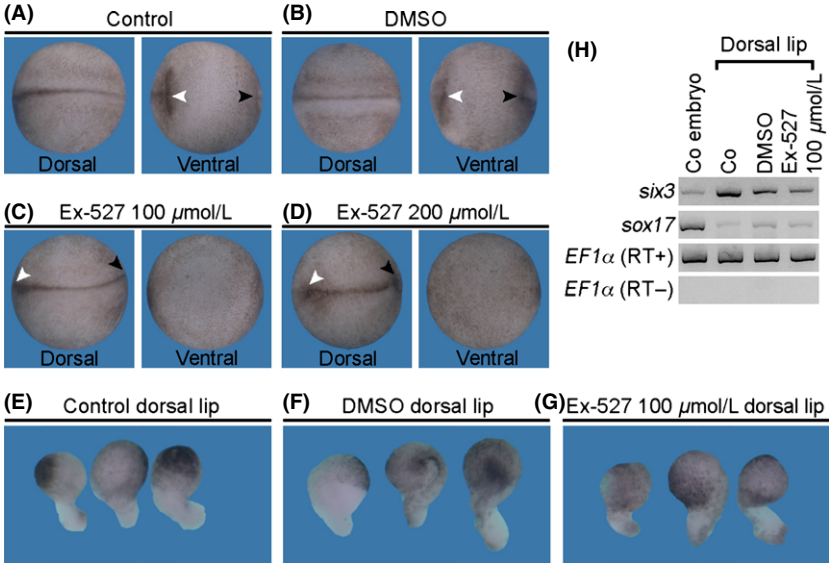

Fig. 2. Ex-527 treatments reduced the size of neural plate region. (A) Control embryo at stage 17. (B) 0.25% of dimethylsulfoxide (DMSO)-treated embryo. In both cases, the pigmented midlines of neural plate were observed from the ventral side (15/15), and no edema formation was observed. (C) Embryo treated with 100 μmol/L of Ex-527. The pigmented midlines were located at the dorsal side (15/15). No obvious edema formation was detected. (D) Embryo treated with 200 μmol/L of Ex-527. The pigmented midlines were restricted at the dorsal side (15/15). Ventral edema formations were detected (12/15). White arrowhead shows the anterior end of the pigmented midline of neural plate and black arrowhead shows the posterior end of the side. (E) Dorsal lip explants cut from control embryos were cultured in 1×SS. Elongations were detected in 18/20 of explants. (F) Dorsal lip explants cut from control embryos were treated with 0.25% of DMSO. Elongations were detected in 17/20 of explants. (G) Dorsal lip explants cut from control embryos were treated with 100 μmol/L of Ex-527. Elongations were detected in 17/20 of explants. (H) reverse transcription–polymerase chain reaction (RT–PCR) analyses for the expression of pan-neural marker *six3* and endodermal marker *sox17* in dorsal lip explants. Six3 expression was reduced by treatment of Ex-527, while sox17 expression was not.

embryos treated with 200 μmol/L of Ex-527 showed a slight edematous swelling on the ventral side at the neurula stages. Moreover, at the tailbud stages this swelling was clearly observed in all of treated embryos. Unfortunately, all embryos treated with 200 μmol/L of Ex-527 died before reaching the tailbud stage. This may be a result of explosion of these swelling structures (data not shown). Therefore, our observations after stage 38 were performed only for the embryos treated with 50 or 100 μmol/L of Ex-527. At stage 38, obvious pigmentations of the optic region were observed at the anterior region of normal and DMSO-treated embryos. Also, a body length, which is the distance from anterior to posterior end, was reached to approximately 6.0 mm (Fig. 3A,B). However, optic formations were clearly damaged in embryos treated with 50 μmol/L of Ex-527 (Fig. 3C) and almost diminished in embryos treated with 100 μmol/L (Fig. 3D). Body lengths of embryos treated with 50 and 100 μmol/L of Ex-527 became shorter than those of normal embryos, scoring around 4.6 and 3.9 mm, respectively (Fig. 3C, D). Remarkably, edematous swelling at the ventral anterior region was frequently formed in both cases (Fig. 3C,D), and in some cases of embryos treated with 100 μmol/L of Ex-527, edematous swelling was obviously detected in the posterior side (Fig. 3D). Interestingly, the same results were observed by nicotinamide treatment, which was a sirtuin inhibitor. These features observed at stage 38 became pronounced over time (Fig. 3F–I). Especially, the distance of left and right eyes in embryos treated with Ex-527 were clearly shorter than in control embryos (compare dorsal panels of Fig. 3F–I). Also, compared to stage 38, body lengths of Ex-527-treated embryos did not elongate well (Fig. 3C, D,H,I), while body lengths of control and DMSO-treated embryos reached 7.0 mm (Fig. 3F,G).

Ex-527 exerts a specific effect on the digestive tract

We additionally noticed mild phenotypic effects around yolk in Ex-527-treated embryos at stage 41, meaning that the sizes of yolk, which includes future gastrointestinal tract, were slightly larger than normal (Fig. 3F–I). At stage 45, as we expected, large effects of Ex-527 were detected at the gastrointestinal tract. Normally at this stage the intestine is forming a double-coiled structure, and both external and internal coils have two loops (Chalmers & Slack 1998). This type of structure was found in control and DMSO-treated embryos (Fig. 4A, B,E,F), while embryos treated with 50 μmol/L of Ex-527 had single-coiled and much thicker intestine (Fig. 4C, G). Moreover, in the case of 100 μmol/L, no-longer coiled structure of intestine was found (Fig. 4D,H). We next tried to determine the details of the inside structures of Ex-527-treated embryos by histological approach. All tissue and organs were actually

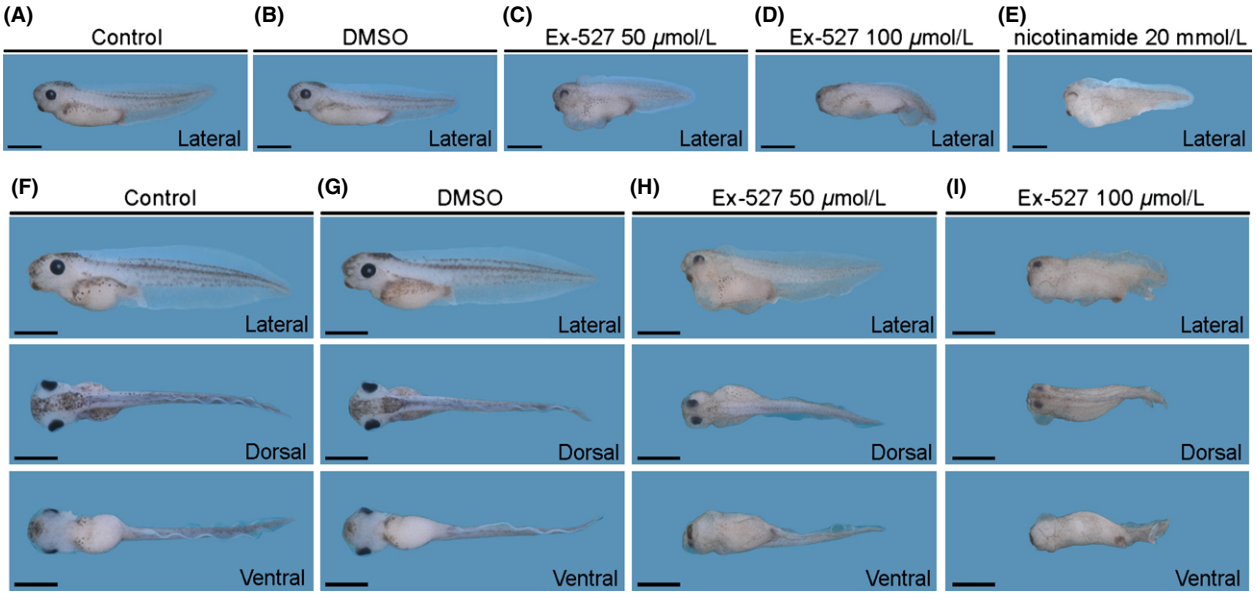

Fig. 3. Ex-527 treatment induced edematous swelling, short body axis, and small head. (A,F) Control embryos at stage 38 and 41. (B, G) Dimethylsulfoxide (DMSO)-treated embryos. (C,H) Embryos treated with 50 μmol/L of Ex-527. Ventral edema formations (11/15) and eye malformations (12/15) were detected at stage 38 and 41. (D,I) Embryos treated with 100 μmol/L of Ex-527. Ventral edema formations (12/12) and eye malformations (12/12) were detected at stage 38 and 41. (E) Embryos treated with 20 mmol/L of nicotinamide. Ventral edema formations (16/18) and eye malformations (18/18) were detected at stage 38. Average lengths of embryos were decreased in dose-dependent manner. Scale bars of A to E represent 1 mm.

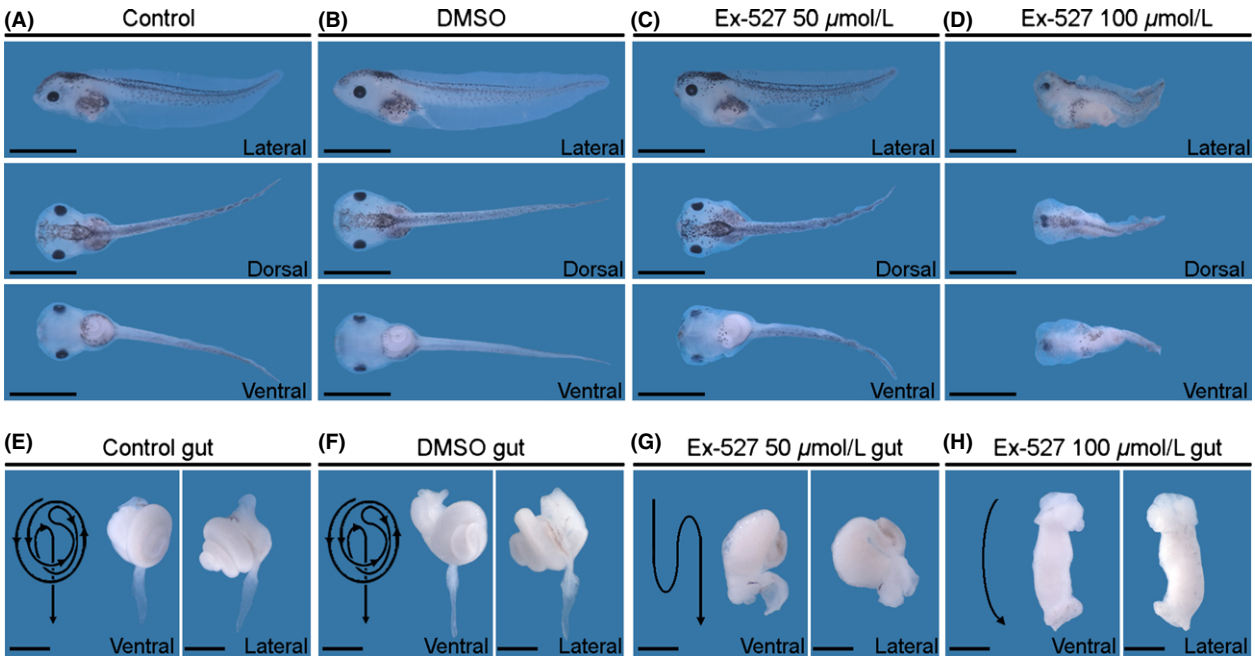

Fig. 4. Ex-527 treatment induced severe gastrointestinal malformations. (A) Control embryo at stage 45. (B) Dimethylsulfoxide (DMSO)-treated embryo. (C) Embryo treated with 50 μmol/L of Ex-527. Ventral edema formations (11/12) and eye (11/12) and gastrointestinal malformation (12/12) were detected. (D) Embryo treated with 100 μmol/L of Ex-527. Ventral edema formations (11/11) and eye (11/11) and gastrointestinal malformation (11/11) were detected. (E–H) Surgically resected guts from embryos shown in A–D. The diagram of gut structure was shown on the left. These panels respectively indicate ventral and lateral views. Secondary coiled structure of gut was not detected in Ex-527-treated embryos. Scale bars of A to D represent 2 mm, and E to H 0.5 mm.

confirmed in DMSO-treated embryos (Fig. 5A–B), while in the case of embryos treated with 50 μmol/L of Ex-527, forebrain was not detected (panel k in Fig. 5C), and in the case of 100 μmol/L no brain structure was observed (panel p in Fig. 5D). We also found that left and right eyes were fused in the case of 100 μmol/L (panel m in Fig. 5D), which resembles the phenotype called Cyclops, which resulted from interference with dorsal mesoderm (Hatta *et al.* 1991; Strähle *et al.* 1993; Goudevenou *et al.* 2011). There was no significant difference between embryos treated with 50 μmol/L and control embryos as well as DMSO-treated embryos from the aspect of the size of notochord and somite region (Fig. 5 panel c in A, panel h in B, panel m in C). However, it was obviously smaller in treatment with 100 μmol/L (Fig. 5 panel r in D). Additionally, a slight reduction in the size of pronephros was observed in embryos treated with 50 and 100 μmol/L of Ex-527 (Fig. 5 panel m in C, panel r in D). This deficiency of dorsal and paraxial structures may have induced inadequate convergent extension of dorsal mesoderm, resulting in the phenotype of Cyclops (Hatta *et al.* 1991; Strähle *et al.* 1993; Goudevenou *et al.* 2011). Focusing on gastrointestinal formations, distinct malformations or deletions of several organs had been observed in embryos treated with 100 μmol/L of Ex-527. It was impossible to distinguish between small and large intestines and find liver formation (Fig. 5 panel r in D). Moreover, reduction in the size of heart was observed in embryos treated with 50 and 100 μmol/L of Ex-527 (Fig. 5 panel l in C, panel q in D). From these results, we conclude that Sirt1 plays important roles in the early stages of *Xenopus* development.

Discussion

In this study, we have investigated the loss-of-functional effects of Sirtuin1 by using chemical reagent Ex-527 on *Xenopus* early development and confirmed that Ex-527 had a far greater influence on early embryogenesis than previously expected. Deficiencies of neural tube and anterior neural formations, frequent appearances of ectopic edematous swelling in ventral side, maldevelopment of dorsal and paraxial tissues, gastrointestinal organs, and heart formation were defined as the typical features by loss-of-functional effects of Sitruin1 on *Xenopus* early embryogenesis.

Edematous swelling induced by Ex-527 treatments

The reason why Edematous swelling appeared in sirtuin inhibitor treated *Xenopus* embryos has to be

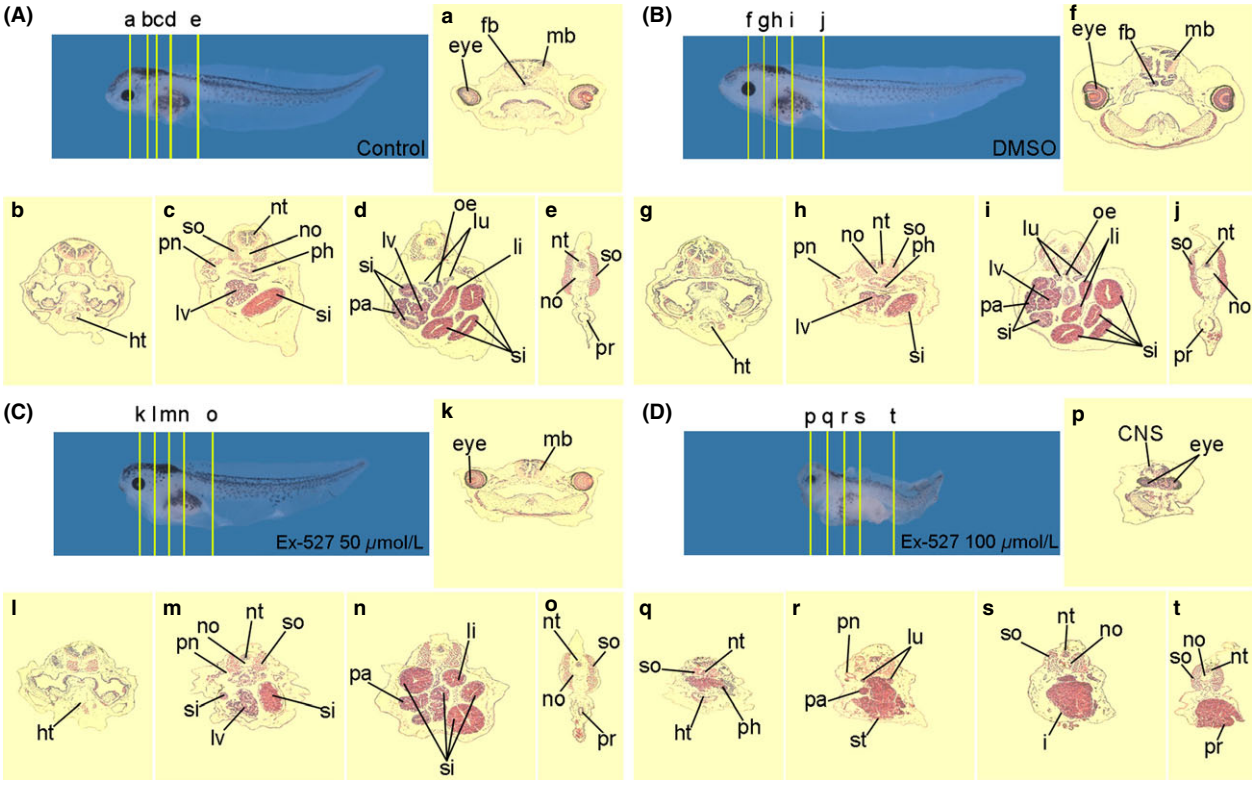

Fig. 5. Several gastrointestinal tissue, brain, and liver formations were damaged by Ex-527-treated embryos. (A) Histological section of control embryo at stage 45. (B) Dimethylsulfoxide (DMSO)-treated embryo. (C) Embryo treated with 50 μmol/L of Ex-527. The size of heart and pronephros were slightly reduced. No forebrain structure was detected. (D) Embryos treated with 100 μmol/L of Ex-527. The size of heart and pronephros were clearly reduced, while proctodeum regions were expanded. It was impossible to distinguish the large and small intestine. No brain and liver structure was detected. CNS, central nervous system; fb, forebrain; ht, heart; i, intestine; li, large intestine; lu, lung; lv, liver; mb, midbrain; no, notochord; nt, neural tube; oe, esophagus; pa, pancreas; ph, pharynx; pr, proctodeum; pn, pronephros; si, small intestine; so, somite; st, stomach.

discussed (Figs 3–5). Removal of the pronephros region from the amphibian embryos highly frequently induced the edema formation (Howland 1916). Interestingly, major pronephros markers are diminished by antisense morpholino oligomers for *golph2*, and the edema formations are frequently observed in these morphants (Li *et al.* 2012). In our study even though pronephros formed in Ex-527-treated embryos, the size of pronephros was certainly reduced (Fig. 5), suggesting that edematous swelling observed in our study was caused by nonfunctional or less-functional pronephros. Edematous swelling is also observed in embryos treated with SU5402, which is generally used as an inhibitor for fibroblast growth factor (FGF) receptor 1 (Doherty *et al.* 2010). SU5402-treated *Xenopus* embryos also have short axis formations and reduction of heart formation identical to Ex-527-treated embryos (Doherty *et al.* 2010; Deimling & Drysdale 2011). Therefore, crosstalk of Sirtuin1 and FGF signals can be an alternative possibility.

The roles of Sirtuins in early embryogensis

Studies on expression of the Sirtuin family revealed that *Sirt1-7* existed both maternally and zygotically (Fig. 1A). The Sirtuin family is originally recognized as anti-aging genes in various organisms such as yeast, *Caenorhabditis elegans*, *Drosophila melanogaster*, mouse, and human (Guarente 2007; Tang 2011). However, our study clearly revealed additional understanding towards the function of the Sirtuin family. In *Sirt1* null mouse, lethal or sublethal effects were caused (Li *et al.* 2008; Boily *et al.* 2009). TOR kinase, which is another famous anti-aging factor, is known to function under regulations of the Sirtuin family (Tucci 2012; Lionaki *et al.* 2013) and starts working at early developmental stages (Murakami *et al.* 2004; Guertin *et al.* 2006; Moriyama *et al.* 2011). Interestingly, the chemical reagent rapamycin is a potent TOR inhibitor, which causes malformations in *Xenopus* early development (Moriyama *et al.* 2011). In these phenotypes,

short body length, edematous swelling, and gastrointestinal malformation were observed identically to Ex-527-treated-embryos (Fig. 5C,D), suggesting that the Sirtuin signal may have a crosstalk with the TOR signal. Moreover, we showed that Ex-527 inhibited deacetylation activity of xSirtuin1 for p53 and the other target proteins (Fig. 1B). p53 regulates cellular differentiation by modulating the signaling of TGF-β family during *Xenopus* embryogenesis (Cordenonsi *et al.* 2003; Takebayashi-Suzuki *et al.* 2003). However, Ex-527 does not seem to regulate mesoderm formation. The activation of p53 requires phosphorylation of serine 392 in addition to acetylation of lysine 382 (Shahar *et al.* 2013), so only inhibition of deacetylation by Ex-527 might not be enough to lead activation of p53. These reports and our present findings strikingly suggest that the Sirtuin family is not only the key factor required for adult stage, but also has fundamental roles from the beginning of the life cycle.

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