dXNP, a *Drosophila* homolog of XNP/ATRX, induces apoptosis via Jun-N-terminal kinase activation

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Received 14 February 2007; revised 27 April 2007; accepted 3 May 2007

Available online 11 May 2007

Edited by Francesc Posas

Abstract **XNP/ATRX**, a causative gene of X-linked α-thalassemia/mental retardation syndrome, encodes an SNF2 family ATPase/helicase protein. To better understand the role of XNP/ATRX in development, we isolated and characterized a *Drosophila* XNP/ATRX homolog, dXNP, which contains highly conserved SNF2 and helicase domains. Ectopically expressed dXNP induced strong apoptosis in the developing eye and wing, but did not affect cell cycle progression or the expression of wingless and engrailed, essential regulators of development. The dXNP-induced apoptosis was strongly suppressed by DJNK1*hemipterous* mutation, and dXNP increased JNK activity. Taken together, these results suggest that dXNP regulates apoptosis via JNK activation.

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Keywords: Apoptosis; ATRX; *Drosophila*; dXNP; JNK; XNP

1. Introduction

Mutations in **XNP/ATRX** cause several X-linked mental retardation syndromes that also feature facial dysmorphism, urogenital defects, and α-thalassemia [1,2]. In males, somatic mutations of this gene also cause α-thalassemia myelodysplasia syndrome (OMIM 300448) [3]. Female carriers, however, are physically and intellectually normal [1].

XNP/ATRX, which was first isolated as a gene potentially coding for a nuclear protein [4], encodes a member of the SNF2 family proteins with ATPase and helicase domains that are most similar to those of RAD54 [5,6]. XNP/ATRX is also known to associate with nuclear matrix and chromatin [7,8]. Recently, it was shown that XNP/ATRX forms a complex, which displays ATP-dependent chromatin remodeling activities including triple-helix DNA displacement and alteration of mononucleosome disruption patterns [9], and was found to be a part of the NuRD complex [10].

A series of studies have shown that XNP/ATRX is involved in diverse cellular processes, including DNA methylation, transcription, cell cycle, and apoptosis [11–14]. The methylation pattern of several highly repeated sequences changes in the genomic DNA of ATR-X syndrome patients’ lymphocytes [11]. The fusion protein of XNP/ATRX and GAL4 DNA binding domain strongly inhibits the expression of thymidine kinase promoter driven luciferase [12]. BrdU incorporation and histone H3 phosphorylation, which are cell cycle markers for S phase and G2/M phase, respectively, are altered in transgenic XNP/ATRX embryos [13]. In addition, loss of XNP/ATRX protein by conditional gene-targeting in mice causes a 12-fold increase in neuronal apoptosis during early stages of corticogenesis, and resulted in hypopcellularity in the mutant brain [14].

Despite the XNP/ATRX-related cellular processes have been revealed, their molecular mechanisms and the role in development are still remain to be determined. Here, to uncover the function of XNP/ATRX in developing tissues, we isolated and characterized *Drosophila* XNP/ATRX homologue (dXNP). The ectopically expressed dXNP by UAS-GAL4 system in developing tissues induced apoptosis in a cell autonomous manner. Genetic and biochemical analysis showed that the dXNP-induced apoptosis is mediated by activation of JNK.

2. Materials and methods

2.1. *Drosophila* strains

Glass multimer reporter (GMR)-GAL4, patched (ptc)-GAL4, aporterous (ap)-GAL4, UAS-DIAPI, UAS-puckered, UAS-lacZ, wingless (wg)-lacZ, engrailed (en)-lacZ, dXNPp (EP635), and dXNp2 (U33132) were obtained from the Bloomington *Drosophila* Stock Center. MS1096-GAL4 and *hemipterous* were gifts from Dr. M. Freeman (MRC Laboratory of Molecular Biology, UK) and Dr. S. Noselli (CNRS, France), respectively.

2.2. Ectopic gene expression using UAS-GAL4 system

To ectopically overexpress dXNP, we used the modular misexpression system based on a P-element vector carrying a GAL4-regulated promoter [15–17]. In dXNPp and dXNp2 lines, the P-elements are inserted in the flanking region of dXNP so that GAL4-dependent transcription begins within the P-element and extends out into the
dXNP, GMR-GAL4, MS1096-GAL4, ptc-GAL4, and ap-GAL4 directed dXNP expression in the developing eye, the whole developing wing (strongly in the dorsal part), the border of anterior/posterior compartment of wing, and the dorsal compartment of wing, respectively.

External eye morphologies were observed by scanning electron microscopy (Korea Basic Science Institute, Korea).

2.3. Acridine orange (AO) staining and BrdU labeling experiment

Eye and wing imaginal discs of stage L3 larvae were dissected in phosphate-buffered saline (PBS). The discs were then incubated for 5 min in 1.6 × 10^{-6} M solution of acridine orange (Aldrich, USA), and rinsed briefly in PBS. The samples were examined under an Axioshot2 fluorescent microscope (Carl Zeiss, Germany).

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Fig. 1. Drosophila XNP and its mutants. (A) Comparison of Drosophila XNP with its human counterpart. Protein domains conserved between Drosophila and Human XNP. Abbreviations: SNF2 N-terminal domain (SNF2_N) and helicase superfamily C-terminal domain (HELICc). (B) Northern blot analysis of dXNP transcripts during Drosophila development. 18S rRNA was used as a loading control (lower panel). E, embryo; L, third instar larva; P, pupa; M, adult male; F, adult female. (C) Real time RT-PCR analysis of dXNP transcripts in the body and head of Drosophila. Upper panel shows the relative transcript levels determined by real time RT-PCR analysis, normalized with Actin, and expressed as mean ± S.E. % of the body (**P < 0.01 vs. body by paired Student’s t-test; n = 3). Lower panel shows the representative picture for the RT-PCR products of dXNP. RT-PCR products were resolved on 1% agarose gels and visualized by ethidium bromide staining. (D) Genomic structures of dXNP. Exons are drawn as boxes and coding regions are highlighted in black. The P-element insertion sites of dXNP^1 and dXNP^2 are indicated. (E–G) Ectopic expression of dXNP using UAS-GAL4 system. (E) Determination of expression levels of dXNP transcript in the eye imaginal discs of GAL4 driven dXNP alleles using RT-PCR method. Actin was used as an internal control (lower panel). The genotypes of the samples are GMR-GAL4 (GMR-GAL4/+), GMR > dXNP^1 (GMR-GAL4/+; dXNP^1/+), and GMR > dXNP^2 (GMR-GAL4/+; dXNP^2/+). (F,G) RNA in situ hybridization shows that the induction of dXNP mRNA expression in the eye imaginal discs of GMR-GAL4 driven dXNP alleles. Samples were prepared from control (GMR-GAL4/+), F) or GMR > dXNP (GMR-GAL4/+; dXNP^1/+, G).
5-Bromo-2′-deoxyuridine (BrdU) labeling experiment of wing imaginal discs were performed using the in situ cell proliferation kit (Roche, Germany) according to the manufacturer's protocol.

2.4. RT-PCR, RNA in situ hybridizations, and Northern blot analysis

Total RNAs from flies were isolated by TRIZOL-bromo-chloroform protocol. For RT-PCR, one microgram of RNA templates were reverse-transcribed with 200 U of Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, WI) using manufacturer's instructions. RT-PCR was performed using the following primer pairs: dXNP (5′ATGGGAAAGAAAAACCCCAACG3′ for 5′ and 5′TTGGCGACCTTGGCAGACGCG3′ for 3′), Actin (5′GATTGCAGAAGTGCGACAAA3′ for 5′ and 5′GGCTTCTTGGACAGCTTGAG3′ for 3′), hep (5′GATTGCAGAAGTGCGACAAA3′ for 5′ and 5′GGCTTCTTGGACAGCTTGAG3′ for 3′), bsk (5′AAATGCCTGCCACTTTGAGT3′ for 5′ and 5′TGGCTGTAACCGTTGACATAA3′ for 3′), DJun (5′ACACCCGATTGGAGAAG3′ for 5′ and 5′ATTGTAGATTTGCCAGCC3′ for 3′), and Dfos (5′CAGGACACGACCGATACTT3′ for 5′ and 5′ATTGTAGATTTGCCAGCC3′ for 3′). RT products were amplified 28 times by PCR.

In situ hybridization and Northern blot analysis was performed using standard method using the probe made from the PCR product that corresponds to the 3′ region of the dXNP amplified with the primers (5′TTCTCATGGCTCCTCCAGCC3′ for 5′ and 5′TCATCCTCGGTTTCGTC3′ for 3′).

2.5. Real time RT-PCR

A 2 µl aliquot of each RT sample of head and body was subjected to real time PCR in a 20 µl reaction mixture containing 4 mM MgCl₂, 10 pmol of upstream and downstream primers and 2 µl of 10X LightCycler FastStart® DNA Master SYBR Green 1 (Roche, Germany). Data were analyzed with LightCycler Software version 3.5. Same primers were used as conventional RT-PCR.

2.6. Immunohistochemistry and X-gal staining

Drosophila eye or wing imaginal discs were dissected and fixed in 4% paraformaldehyde in PBS and then washed with PBT (PBS + 0.1% Tween 20) and blocked in PBT with 2% Normal Goat Serum. The discs were incubated overnight with rabbit anti-active-Drice antibody (1:200) [18], rabbit anti-phospho-JNK antibody (Promega, 1:200) and then washed in PBT and subsequently further incubated for 1 h at room temperature in FITC or rhodamine-labeled goat anti-rabbit IgG (H + L) secondary antibody (1:200 in PBT). The samples were observed by a Axioptot2 fluorescent microscope (Carl Zeiss, Germany).

For 5-bromo-4-chloro-3-indoly]-b-D-galactopyranoside (X-Gal) staining, eye and wing imaginal discs were fixed in 4% formaldehyde in PBS for 4 min, washed and then incubated in standard X-Gal staining solution [4.9 mM X-Gal, 3.1 mM K₄Fe(CN)₆, 3.1 mM K₃Fe(CN)₆, 1 mM MgCl₂, 150 mM NaCl, 10 mM Na₂HPO₄, 10 mM NaH₂PO₄, 0.3% Triton X-100] for 30 min at 37°C before observation.

3. Results

3.1. Drosophila XNP and its mutants

The dXNP gene, which corresponds to the Berkeley Drosophila Genome Project annotated sequence CG4548, was isolated by PCR-based cloning. The conceptually translated dXNP protein (1311 amino acids) contains the characteristic

Fig. 2. The effect of dXNP overexpression in developing tissues. Scanning electron micrographs of the external eyes (A–C) and the phenotypes of wing (D–L) resulted from overexpression of dXNP gene are shown. Asterisks in (K,L) indicate reduced distance between L3 and L4 veins. The genotypes of the samples are (A) GMR-GAL4/+, (B) GMR-GAL4/+; dXNP1/dXNP1, (C) GMR-GAL4/++; dXNP1/dXNP1, (D,G) MS1096-GAL4/Y, (E,H) MS1096-GAL4/Y; dXNP1/+; (F,I) MS1096-GAL4/Y; dXNP2/+; (J) ptc-GAL4/+, (K) ptc-GAL4/++; dXNP1/+; and (L) ptc-GAL4/++; dXNP2/+.
motifs, a SNF2 family N-terminal domain (SNF2_N) and helicase superfamily C-terminal domain (HELICc) (Fig. 1A). The C-terminal region of dXNP, which contains these domains, shows 66% homology (48% identity) to the human counterpart. As these domains have been known to be important in chromatin remodeling, it is very likely that dXNP may function as a chromatin remodeling factor just as mammalian XNP/ATRX. Consistent with the sequence results, we detected a single ~4.0 kb-transcript of dXNP in all developmental stages from Northern blot analysis (Fig. 1B). Real time RT-PCR showed that dXNP is expressed in the whole body, and its expression is elevated approximately 2-fold in the head compared with that of the body (220 ± 10.9% of the body, p < 0.01) (Fig. 1C), implying that dXNP may play an important role in the head.

With the help of the Berkeley Drosophila Genome Project, we found two P-element insertion lines, dXNP1 and dXNP2, which contain P-elements in either the 5′ upstream or first exon of the dXNP gene (Fig. 1D). The directions of the P-elements are oriented to induce gene expression, implying that these mutants can be used to study the gain-of-function of dXNP [16]. As expected, strong expression of dXNP was observed in the
eye imaginal discs of dXNP\(^1\) and dXNP\(^2\) lines carrying the GMR-GAL4 driver by RT-PCR (Fig. 1E) and RNA in situ hybridization (Fig. 1F and G).

3.2. dXNP induces apoptosis

To verify the function of dXNP in Drosophila development, we examined the effect of overexpression of dXNP on developing tissues using the UAS-GAL4 system. Overexpression of dXNP in the developing eye under the control of GMR-GAL4 destroyed all ommatidia (Fig. 2A–C). In addition, we examined the effect of overexpression of dXNP in a specific compartment of the wing. When dXNP is ectopically overexpressed in the entire wing pouch with MS1096-GAL4 driver, the wings were rumpled and diminished (Fig. 2D–I). And, the ectopically expressed dXNP in the border of anterior/posterior compartment of wing by ptc-GAL4 resulted in a reduction of the distance between L3 and L4 veins (Fig. 2J–L). These results suggest that dXNP is essential for proper tissue growth during development.

As dXNP overexpression reduces the compartment size of wing, we tested whether overexpression of dXNP causes apoptosis by acridine orange (AO) staining. As expected, we found strong staining signals in all dXNP overexpressed tissues (Fig. 3A–C, E–G, and I–K). The AO staining patterns are found in the region showing GAL4-driven gene expression, which is reported by lacZ expression (Fig. 3D, H, and L), suggesting that dXNP induced apoptosis in a cell autonomous manner. Furthermore, dXNP-induced destruction of the eye was almost completely inhibited by coexpression of Drosophila inhibitor of apoptosis protein 1 (DIAP1) (Fig. 3 M–O), suggesting that the apoptosis induced by dXNP is mediated by caspase activation. Supporting this idea, we found strong activation of Drice, an essential caspase of Drosophila [18], in the eye imaginal disc of dXNP over-expressed fly (Fig. 3Q) but not in the control fly (Fig. 3P).

3.3. dXNP does not affect the cell differentiation and cycle

The reduction of tissue compartment also can be a result of defective cell cycle or gene expression. Therefore, we tested whether overexpressed dXNP cause impairment of these cellular processes. However, there was no difference between the control and dXNP overexpressed tissues in antibody staining for phospho-Histone H3 (Fig. 4A and B), a marker for mitosis, or incorporation of BrdU, a marker for S phase (Fig. 4C and D). These results imply that cell cycle is not affected by dXNP overexpression. Next, we examined whether the expression of genes which are essential for development is altered by elevated dXNP level. As shown in Fig. 4 E–H, we did not find any difference in the expression level of these genes in dXNP-overexpressed tissues, implying that dXNP overexpression do not affect these gene expression as well as cell cycle.

3.4. dXNP induces apoptosis through JNK signaling pathway

As activation of the JNK signaling pathway is frequently correlated with induction of apoptosis, and chromatin remodeling proteins has been implicated in the JNK-induced apoptosis [19], we tested whether JNK signaling pathway mediates dXNP-induced apoptosis. First, we examined the genetic inter-

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**Fig. 4.** dXNP does not affect cell cycle and expression of developmental regulators. Cell cycle was determined by anti-phospho-histone H3 antibody staining (A,B) or BrdU incorporation analysis (C,D), and transcriptional level of compartment determining gene was determined by X-gal staining (E–H). (A,C) MS1096-GAL4/Y, (B) MS1096-GAL4/Y; dXNP\(^1\)/+, (D) MS1096-GAL4/Y; dXNP\(^2\)/+, (E) MS1096-GAL4/Y; wg-lacZ/+, (F) MS1096-GAL4/Y; wg-lacZ/+, dXNP\(^1\)/+, (G) MS1096-GAL4/Y; en-lacZ/+, (H) MS1096-GAL4/Y; en-lacZ/+, dXNP\(^1\)/+. 

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Fig. 5. dXNP induces apoptosis through the activation of JNK signaling pathway. Scanning electron micrographs of the external eyes (A–F), and AO staining (G–I) and anti-phospho-JNK antibody staining (J,K) of the eye imaginal discs are shown. (A, D, and G) hep^{1/Y} (B, E, and H) GMR-GAL4/+, dXNP^{1+}, (C, F, and I) hep^{1/Y}; GMR-GAL4^{1+}; dXNP^{1+}, (J) GMR-GAL4/+, (K) GMR-GAL4/+, dXNP^{1+}dXNP^{2}. (L) Real time RT-PCR analysis of Chic and Puc transcripts in the heads of GMR-GAL4^{1+} (GMR) and GMR-GAL4^{1+}; dXNP^{1+} (X^2) flies. Upper panel shows the relative transcript levels determined by real time RT-PCR analysis, normalized with Actin, and expressed as means ± S.E.% of the heads of GMR-GAL4^{1+} (*P < 0.05 and **P < 0.01 vs. body by paired Student’s t-test; n = 3). Lower panel shows the representative picture for the RT-PCR products of Chic, Puc and Actin. RT-PCR products were resolved on 1% agarose gels and visualized by ethidium bromide staining. (M) Determination of expression levels of the components of JNK signaling pathway in the eye imaginal discs using RT-PCR. The genotypes of the samples are GMR-GAL4 (GMR-GAL4^{1+}), GMR > dXNP^{1} (GMR-GAL4^{1+}; dXNP^{1+}), and GMR > dXNP^{2} (GMR-GAL4^{1+}; dXNP^{2+}). Actin was used as an internal control (lower panels).
of the compound eye was strongly suppressed by the hep^1 mutation (Fig. 5C and F). Furthermore, as shown in Fig. 5G-I, the dXNP-induced apoptosis was also inhibited by hep^1, suggesting that JNK signaling pathway acts as a vital downstream effector of dXNP in the regulation of apoptosis. Consistent with these results, we also observed that puckered, a negative regulator of JNK, suppressed the dXNP-induced wing defect (Fig. S1). In addition, we found that overexpression of dXNP increases phosphorylation of JNK in both eye (Fig. 5J and K) and wing (Fig. S2) imaginal discs, and the expression of direct targets of JNK signaling pathway such as chickadee (Chic) and puckered (Puc) (Fig. 5L). The expressions of Chic and Puc were increased 1.8-fold (179 ± 29.8% of the WT, p < 0.05, n = 3) and 2.2-fold (224 ± 14.6% of the WT, p < 0.01, n = 3), respectively, in the dXNP overexpressed samples. These results suggest that dXNP regulates the JNK signaling pathway.

Because XNP has been known as a chromatin remodeling factor, we tested whether dXNP increases the activity of JNK signaling pathway by controlling transcription of its components. To do this, the transcript levels of bsk, hep, Dfos, and DJun were measured by quantitative RT-PCR in the eye discs of the GMR-GAL4 controls and dXNP overexpression flies. However, as shown in Fig. 5M, their transcript levels were not altered by overexpression of dXNP, implying that activation of the JNK signaling pathway is not resulted from an increase in the transcriptional level of signaling components but probably by the post-transcriptional regulation, such as phosphorylation.

4. Discussion

In the present study, we have isolated and characterized Drosophila XNP (dXNP). Overexpression of dXNP induced strong apoptosis without affecting cell cycle and the expression level of essential regulators of development. Co-expression of DIAPI suppressed dXNP-induced rough eye phenotype (Fig. 3M–O). As it has been reported that DIAPI degrades two proteins, Drosophila caspase DRONC [20] and TRAF1 [21] as its E3 substrate, our result suggest that the caspase cascade is implicated in the apoptosis induced by dXNP. Supporting this idea, Drice, a downstream caspase of DRONC [22], was activated in dXNP-overexpressing tissues (Fig. 3Q). Despite the detailed mechanism by which XNP/ATRX involved in apoptosis is unclear, our study clearly showed that the apoptosis induced by dXNP is mediated by caspase cascade. It was interesting to note that XNP/ATRX is localized in promyelocytic leukemia (PML) nuclear bodies [23], which have been implicated in apoptosis and senescence [24]. Moreover, the accumulated XNP/ATRX at PML nuclear bodies interacts with Daxx, a multifunctional apoptosis-related protein [23,25], and strong apoptosis was observed in the XNP/ATRX-null mice [14], implying that XNP/ATRX is a potent regulator of apoptosis. Since, as a chromatin remodeling factor, XNP has been associated with DNA methylation and transcription [11,13], it is possible that dXNP regulates apoptosis through direct control of transcription of proapoptotic factors. However, our study argues that dXNP induces apoptosis by activating JNK signaling pathway, dXNP-induced apoptosis was almost completely suppressed by hep/DJNKK mutation, suggesting that the apoptosis might be resulted from the activation of JNK signaling pathway. Moreover, we also found that overexpressed dXNP increases phosphorylation of JNK (Fig. 5K) and the expression of direct targets of JNK pathway (Fig. 5L). These results demonstrate that dXNP regulates apoptosis via regulating the JNK signaling pathway rather than via direct regulation of the transcription of apoptotic factors.

Then, how dXNP can activate JNK? One possible scenario is that dXNP activates JNK by controlling the transcription of upstream regulator of JNK. Although we showed that the transcriptional level of hep/DJNKK is not altered by dXNP overexpression, we can not exclude the possibility that the transcription of other upstream regulator(s) of JNK signaling pathway is changed. Further study to find dXNP targets will determine this possibility. Alternatively, other interesting scenario is that Daxx can mediate the dXNP-induced JNK activation and apoptosis. Recently, it has been reported that Ras-association domain family 1C (RASSF1C), a binding partner of Daxx, is released from the nucleus to cytoplasm and activates JNK signaling pathway, when Daxx is degraded in response to DNA damage [26]. Because Daxx associates with XNP or RASSF1C in PML nuclear bodies [9,26], overexpression of XNP may abrogate the balance between Daxx-XNP and Daxx-RASSF1C complexes and increase free RASSF1C which, in turn, activate the JNK signaling pathway. Since Drosophila RASSF homolog has been identified and characterized recently [27], it may be interesting to test whether Daxx and RASSF are involved in the dXNP-induced JNK activation and apoptosis.

In summary, we have provided genetic and histochemical evidences that dXNP controls apoptosis in a JNK-dependent manner, which may explain the underlying mechanism of some symptoms of ATR-X syndrome, such as mental retardation. In addition, we here provide evidence for the first time that dXNP, which was previously known as a chromatin remodeler, could also play a role as a signal transducer. Elucidating the molecular mechanism through which dXNP regulates JNK signaling and apoptosis might be helpful to understand the developmental role of XNP and this complex disorder.

Acknowledgements: This work was supported by the faculty research fund of Konkuk University in 2005. We thank Jeehye Park (KAIST, Korea) for critical reading of this manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.05.005.

References


