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Title	Functional analysis of duck, goose, and ostrich 2 '-5 '-oligoadenylate synthetase
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Citation	Infection, Genetics and Evolution, 62, 220-232 https://doi.org/10.1016/j.meegid.2018.04.036
Issue Date	2018-08
Doc URL	http://hdl.handle.net/2115/75092
Rights(URL)	http://creativecommons.org/licenses/by-nc-nd/4.0/
Туре	article (author version)
File Information	Infection, Genetics and Evolution_62_220_232.pdf



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#### 1 ABSTRACT

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3 Up-to-date the flavivirus infection in avian taxa is not clearly defined. Several reports have demonstrated that many viruses belonging to *Flaviviridae* may cause diseases in poultry species; 4 however, the susceptibility of other avian species is variable and still unclear. In human and mice, the  $\mathbf{5}$ 2'-5'-oligoadenylate synthetase (OAS) proteins are associated with resistance to the flavivirus 6 $\overline{7}$ infection as well as other virus infections. However, the avian OAS proteins are rarely studied. In our previous studies, we confirmed that the chicken OAS-like protein (chOASL) expressed OAS-8 enzymatic activity (the classical OAS/RNase L-dependent pathway) as well as the anti-flavivirus 9 activity (the putative OAS/RNase L-independent pathway). Therefore, the current study aimed at 10functional analysis of avian OAS proteins from duck, goose, and ostrich. The duOASL, goOASL, 11 and osOAS1 proteins expressed enzymatic activity as well as chOASL, whereas osOASL expressed 1213little enzymatic activity. On the other hand, duOASL, goOASL, and osOASL possessed significant antiviral activity against West Nile virus (WNV)-replicon replication as well as chOASL, whereas 14osOAS1 did not. In addition, similar to chOASL, their antiviral activity was independent of RNase L 1516 activation. These results suggest that OASL is the only OAS protein in the duck and goose as well as chicken and possesses both OAS-enzymatic and anti-flavivirus activities, whereas the ostrich 17possesses both OAS1 and OASL proteins with sharing the functional activities, OAS-enzymatic and 18anti-flavivirus activities, respectively. It is of interest that the ostrich undergoes differential process 19in OAS gene evolution from other poultries and thus possesses different molecular mechanism in 2021antiviral activity.

#### 1 **1. Introduction**

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Several members of flavivirus are zoonotically important due to their pathogenesis to both wild 3 4 and domestic avian species as well as their global distribution in variable environmental conditions. Besides the wide range of the host diversity, flaviviruses could cause diseases varying from mild viral  $\mathbf{5}$ 6 infection to severe and fatal diseases. In avian hosts, viruses including Tembusu virus (TMUV),  $\mathbf{7}$ turkey meningoencephalitis virus (TMEV), West Nile virus (WNV), etc. are reported to cause diseases in some avian species (Davidson, 2015). WNV is maintained in an enzootic mosquito-bird 8 cycle and detected in around 150 avian species and several mammalian species. Reptiles are 9 10 suggested to be the potential amplifying hosts, because they develop long-lasting detectable viremia duration lasting overwinter in some cases (Thomas and Eklund, 1960). In contrast, avian species 11 suffer from infectious viremia around 1 to 4 days after infection, and then they develop a life-long 12immunity. Unlike avian species, humans and horses as well as most other mammals are incidental 13"dead-end" hosts (Dauphin et al., 2004). 14

Wild and domestic avian species express wide range of WNV-resistant or susceptible phenotypes. 15The most susceptible species belong to the Charadriiformes and Passeriformes orders and they 16develop severe neurological signs and high mortality rates such as the American crow with 100% 17mortality (Hofmeister et al., 2015; Nemeth et al., 2011). However, in other birds, they express more 18restrictive symptoms and death is rarely observed (Van der Meulen et al., 2005). In contrast, little 19information is known about the susceptibility of WNV infection on aquatic birds except for few 20reports demonstrating that WNV may cause diseases and mortality in geese (Austin et al., 2004; 2122Glávits et al., 2005; Swayne et al., 2001) and ducks (Hofmeister et al., 2015; Shirafuji et al., 2009) as 23well as giant flightless birds such as ostrich (Allwright et al., 1995).

Pérez-Ramírez et al. (2014) indicated that the avian species could be categorized into three classes; highly competent birds (susceptible) such as *Passeriformes* (*Corvidae* and *Passeridae*) and *Charadriiformes*, which develop high viremia level more than 10<sup>6</sup> pfu/ml, moderately competent birds including reservoir birds such as several species belonging to *Anseriformes* and *Passeriformes*orders with viremia level of 10<sup>4</sup> - 10<sup>6</sup> pfu/ml, and incompetent (resistant) birds with viremia level less
than 10<sup>4</sup> pfu/ml such as *Galliformes*. Goose and duck are considered as low-moderate viremia birds.
However, up till now the underlying reasons for this variation are still unclear.

The balance between innate and adaptive immunity, T-cell-mediated immunity, and WNV  $\mathbf{5}$ pathogenesis are very important factors to coordinate and achieve the effective control of WNV 6 infection (Samuel and Diamond, 2006). Mandl et al. (2015) have proposed several hypotheses that  $\mathbf{7}$ explain why viruses cause diseases in humans and other animals but not in reservoir hosts. For 8 example, the reservoir host immune response controls the viral replication more effectively than that 9 10 of humans and other animals. However, how the reservoir host immune system controls the virus is still unclear. Likewise, the avian innate immune response against WNV infection is still not 11 12completely understood (Gamino and Höfle, 2013).

The viral infection initiates type I interferon production and sensing the viral nucleic acids in the infected cells, which in turn evokes and elevates a cascade expression of interferon-stimulated proteins that interacts with the infectious pathogens through various pathways. Among these interferon-stimulated proteins, the Mx-GTPase, 2'-5'-oligoadenylate synthetase (OAS), and protein kinase RNA-activated (PKR) are well recognized as antiviral proteins (Der et al., 1998; Sadler and Williams, 2008; Santhakumar et al., 2017).

The OAS proteins belong to the nucleotidyltransferase superfamily involved in diverse 19biological functions. The four major types of OAS gene families, OAS1, OAS2, OAS3, and OASL, are 20conserved in almost all studied mammalian species, whereas some of the OAS genes have been lost, 2122transformed to pseudo-genes, or undergone duplication during evolution. In Rodentia (mouse and rat), 23the Oas1 gene shows interesting multiplication of eight paralogues (ex. Oas1a to Oas1h in mice) (Justesen et al., 2000; Kjaer et al., 2009; Perelygin et al., 2006). However, in avian species, the only 24well characterized OAS gene is the chicken OASL, a single copy gene located on chromosome 12 2526encoding two alternatively spliced alleles, *chOASL-A* and *chOASL-B* (Tatsumi et al., 2002 and 2003;

1 Yamamoto et al., 1998).

Activation of the classical OAS/RNase L pathway results in degradation of both viral and  $\mathbf{2}$ cellular RNA including ribosomal RNA of infected cells. Thereafter, the protein translation is 3 4 inhibited in these cells and viral replication is effectively prevented (Silverman, 2007; Tkachuk, 2013). Similarly, the chOASL/RNase L pathway might be involved in the response to avian infectious  $\mathbf{5}$ bronchitis virus (IBV) infection (Cong et al., 2013). However, it has been recently confirmed that  $\mathbf{6}$  $\mathbf{7}$ several members of the OAS protein family such as human OASL/a (huOASL/a) and murine Oas1b (mOas1b) can prevent viral replication through OAS/RNase L-independent pathway, in which these 8 proteins associate with WNV resistance without showing OAS enzymatic activity (Kajaste-Rudnitski 9 10 et al., 2006: Kristiansen et al., 2011: Marques et al., 2008: Moritoh et al., 2009: Perelygin et al., 2002: Scherbik et al., 2007; Zhu et al., 2014). Recently, we reported that the chOASL protein possessed 11 antiviral activity against WNV and this antiviral activity was independent of its enzymatic activity 12(Tag-EL-Din-Hassan et al., 2012 and 2017). In addition, Li et al. (2007) found that the relative 13expression of *chOASL* mRNA significantly increased in an IFN-independent manner in chicken 14embryo cells infected with infectious bursal diseases virus (IBDV), where the expression level of IFN 15mRNA was rarely upregulated, indicating that the chOASL might play a role in viral sensing in 16chicken (Lee et al., 2014). Furthermore, the chOASL/RNase L pathway plays a role in regulating the 17oviduct weight, ovalbumin, and estrogen-induced mRNA of egg white proteins, indicating that the 18chOASL protein is a multifunctional factor (Cohrs et al., 1988). 19

20Therefore, this research aims at functional analysis of avian OAS families from duck, goose, and ostrich by comparing that of chOASL. 21

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#### 1 **2. Materials and methods**

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#### 3 2.1. Blood samples

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Blood samples were collected from adult mallard ducks and white Chinese geese (reared in  $\mathbf{5}$ Sapporo Maruyama Zoo, Sapporo, Hokkaido, Japan) in heparinized blood collection tubes to prevent 6  $\mathbf{7}$ blood coagulation. Blood samples were centrifuged at 400 Xg for 15 min at room temperature (RT), pellets were suspended in 10 ml of phosphate-buffered saline (PBS), and then, carefully layered on 8 3.0 ml of HISTOPAQUE-1077 (Sigma-Aldrich, St. Louis, MO, USA) followed by centrifugation at 9 10 400 Xg for 30 min at RT. The opaque interface layer containing leukocytes was collected and transferred into a new tube, washed twice with 10 ml of PBS, and then centrifuged at 250 Xg for 10 11 12min at RT. Thereafter, the leukocytes were suspended and cultured in 10 ml of RPMI 1640 medium (Gibco/Invitrogen, Carlsbad, CA, USA) containing 1% penicillin/streptomycin/glutamine 13(Gibco/Invitrogen), 10% fetal bovine serum (FBS) (Atlas Biologicals, Inc. Fort Collins, CO, USA), 141µg/ml phytohaemagglutinin P (Sigma-Aldrich), and 10 µg/ml pokeweed mitogen (Sigma-Aldrich) 15in a 5% CO<sub>2</sub> incubator at 37 °C for 48 h. 16

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#### 18 2.2. Cell culture

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BHK-21, 293FT, and primary culture cells of ostrich renal epithelial cells (kindly gifted from
Prof. Mayumi Ishizuka, Laboratory of Toxicology, Faculty of Veterinary Medicine, Hokkaido
University, Japan) were cultured in DMEM (Gibco/Invitrogen) supplemented with 10% FBS (Atlas
Biologicals) and 1% penicillin/streptomycin/glutamine (Gibco/Invitrogen). Cells were cultured in a
5% CO<sub>2</sub> incubator at 37 °C and passaged every two days.

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#### 26 2.3. Interferon treatment

Duck and goose leukocytes cultured with mitogens for 48 h and ostrich renal epithelial cells were  $\mathbf{2}$ treated with 3,000 IU/ml of IFNα-2β (INTRON<sup>®</sup> A, MSD K.K., Tokyo, Japan) for 6 h, and then, total 3 RNA was extracted. In addition, both BHK-21 and 293FT cells were treated with 3,000 IU/ml of 4  $\mathbf{5}$ IFN $\alpha$ -2 $\beta$  for 24 h, and then, the whole cell lysates were extracted and stored at -80 °C until used. 6  $\mathbf{7}$ 2.4. RNA extraction and cDNA synthetase 8 Total RNA was extracted using TRIZOL reagent (Gibco/Invitrogen) according to the 9 10 manufacturer's protocol and concentration of RNA was determined using SmartSpec<sup>TM</sup> (BioRad, Hercules, CA, USA). Purified total RNA (1 µg) was used to generate cDNA using ReverTra Ace® 11 12(Toyobo Co., Ltd. Osaka, Japan) according to manufacturer's protocol. The condition for reverse

transcription was 42 °C for 60 min followed by 5 min at 99 °C. The synthesized cDNA was stored at
-20 °C until used.

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#### 16 2.5. Cloning of duOASL, goOASL, osOASL, and osOAS1

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18 Total RNA samples extracted from the duck and goose leukocytes and ostrich renal epithelial 19 cells were used for the cloning and sequencing of full-length mRNA of *duOASL*, *goOASL*, *osOASL*, 20 and *osOAS1*.

At first, since full length sequence of *duOASL* and *goOASL* were not available in database, 5'and 3'-UTR ends of *duOASL* and *goOASL* were cloned using the 5'/3' RACE Kit, 2nd generation (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. Table 1 shows the primers used in this study. Then, the full-length cDNAs of *goOASL* and *duOASL* were amplified using specific primers for duck and goose *OASL* (Table 1) using TaKaRa Ex Taq<sup>®</sup> polymerase (Takara Bio. Inc., Otsu, Shiga, Japan). The PCR conditions are as follows; denature at 95 °C for 5 min, followed by 35 cycles of denature at 95 °C for 30 sec, annealing at 60 °C for 30 sec,
and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 2 min. The amplified
fragments were extracted, ligated into pGEM-T-Easy vector, and then transfected into DH5α *E. coli*competent cells. Plasmids were then extracted and sequenced.

The open reading frames (ORF) of *duOASL*, *goOASL*, *osOASL*, and *osOAS1* were amplified  $\mathbf{5}$ using specific primers (Table 1) to conjugate the FLAG-TAG sequence at the 3'-end as well as the  $\mathbf{6}$  $\mathbf{7}$ NotI restriction site at both 5'- and 3'-ends using the KOD FX NEO polymerase (Toyobo Co., Ltd.) according to the manufacturer's protocol. To amplify *duOASL* and *goOASL*, pGEM-T-Easy plasmids 8 described above were used as template. To amplify osOASL and osOAS1, cDNA prepared from total 9 RNA of ostrich renal cells was used as template. A chOASL-A clone conjugated with FLAG-TAG and 10 NotI restriction site was previously prepared (Tag-EL-Din-Hassan et al., 2012 and 2017). The 11 amplified fragments were extracted and digested with NotI restriction enzyme (Toyobo Co., Ltd.), 12and then purified and cloned into the NotI restriction site of the pIRES-EGFP (the mammalian 13expression vector, Clontech Bio. Inc., Shiga, Japan). Similarly, the ORFs of duRNASEL and 14osRNASEL were amplified using specific primers (Table 1), extracted and cloned to pGEM-T-Easy 15vector, digested with NotI restriction enzyme, and then cloned into pIRES-EGFP. In this study, all 16DNA fragment extraction from agarose gel was performed using FastGene Gel/PCR Extraction Kit 17(Nippon Genetics Co., Ltd, Tokyo, Japan). All cloning reactions were performed using 2X Ligation-18Convenience Kit (Wako Nippon Gene Co., Ltd., Toyama, Japan) according to the manufacturer's 19protocol. All transfections were performed using DH5a E. coli competent cells. All plasmid DNA 20extractions were performed using FastGene Plasmid Mini Kit (Wako Nippon Gene Co., Ltd.). The 21large-scaled plasmid purification was performed using CsCL<sub>2</sub>-ethidium bromide gradient purification 2223method as described previously (Sambrook and Russell, 2001).

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#### 25 2.6. Alignment analysis and neighboring tree

1 The multiple sequence alignment was performed with the ClustalW program  $\mathbf{2}$ (http://www.genome.jp/tools-bin/clustalw) using default parameters to compare the OAS proteins from the following species; Gallus gallus (chOASL, BAB19016.1), Anser cygnoides (goOASL, 3 4 ANW12075), Anas platyrhynchos (duOASL, ANW12076), Struthio camelus australis (osOASL, XP\_009671383) and ostrich OAS3, XP\_009667960 "named osOAS1 in this study"). The output was  $\mathbf{5}$ embellished using BOXSHADE 3.21 software (www.ch.embnet.org/software/BOX\_form.html).  $\mathbf{6}$  $\mathbf{7}$ Conserved analysis protein performed domain in the was using motif search (http://www.genome.jp/tools/motif/). The evolutionary history was inferred using the neighbor-8 joining method. The percentage of replicate trees, in which the associated taxa clustered together in 9 10 the bootstrap test (500 replicates), is shown next to the branches. The tree is drawn to scale with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to 11 12infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and were expressed as the units of the number of amino acid substitutions per site. 13Evolutionary analyses were conducted using MEGA7 software (Kumar et al., 2016) and the following 14protein sequences: Gallus gallus (chOASL, BAB19016.1), Anser cygnoides (goOASL, ANW12075), 15Anas platyrhynchos (duOASL, ANW12076), Struthio camelus australis (osOASL, XP\_009671383 16and osOAS3, XP\_009667960 "named osOAS1 in this study"), Columba livia (pigeon OASL, 17XP 005508920), Meleagris gallopavo (turkey OASL, XP 019475496), Corvus brachvrhvnchos 18(crow OASL, KFO58001), Coturnix japonica (Japanese quail OASL, XM\_015874647), Amazona 19aestiva (turquoise-fronted parrot OASL, KQK84582.1), Taeniopygia guttata (zebra-finch OASL, 20XP\_004176346), Mus musculus (mouse Oasl1, NP\_660210 and mouse Oasl2, NP\_035984), Rattus 2122norvegicus (rat Oasl1, NP\_001009681 and rat Oasl2, NP\_001009682), Homo sapiens (human 23OASL/a, NP\_003724 and human OAS1, NP\_058132), Canis lupus familiaris (dog OASL1, AAX58775, dog OASL2, AAX58776, and dog OAS1, AAX56077), Sus scrofa (pig OAS1x, 24NP\_999468, pig OAS1y, AAT34965, and pig OASL, AAT44895), Bos taurus (cattle OAS1x, 2526NP 835209, cattle OAS1y, AAP69995, cattle OAS1z, NP 001025017, and cattle OASL, AAP94112),

1	Equus caballus (horse OAS1, ABF82432 and horse OASL, AAS44555), and Pan troglodytes
2	(chimpanzee OAS1, ACJ13106 and chimpanzee OASL, NP_001267398).

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4 2.7. Preparation of cell lysate

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Both 293FT and BHK-21 cells were transfected with 10 µg of pIRES-EGFP (empty vector as 6  $\overline{7}$ a control), pduOASL-FL-EGFP, pgoOASL-FL-EGFP, posOASL-FL-EGFP, posOAS1-FL-EGFP, and pchOASL-A-FL-EGFP using Lipofectamine 2000® (Gibco/Invitrogen) according to the 8 manufacturer's protocol. pduRNASEL-EGFP and posRNASEL-EGFP were also transfected into 9 BHK-21 cells with the same method. Transfected cells were seeded with the density of  $1.5 \times 10^6$ 1011 cells/100-mm dish and cultured for 24 h. Then, EGFP expression was monitored for all transgenes using a NIKON ECLIPSE TE2000E microscope. Then, both 293FT and BHK-21 cells were 1213continued for culture until 48 h and 72 h post-transfection, washed twice with PBS, scratched with 1 14ml of PBS, and centrifuged at 780 xg for 5 min. The supernatant was discarded and the cells were suspended in Flag-Tag lysis buffer as described previously (Sarkar et al., 2005). Both cell lysates 1516were kept at -80 °C until used. The 293FT cell lysates were used for the Western blotting and enzymatic activity analysis, whereas the BHK-21 cell lysates were used for RNase L activity assay. 17

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#### 19 2.8. Western blotting

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Western blotting was performed as described previously (Tag-EL-Din-Hassan et al., 2012 and 2017). Briefly, 12 µg proteins of each sample were separated in 10% SDS-polyacrylamide gel electrophoresis on 100 V for 1 h and blotted onto PVDF membrane. Blotted membrane was blocked with 3% non-fatty milk in PBS containing 0.1% Tween-20 (PBS-Tween-20) for 1 h at RT. Then, the membrane was incubated with diluted (1/2,000) mouse anti-FLAG M2 antibody (Sigma-Aldrich) in PBS-Tween-20 at RT for 2 h, washed 3 times with PBS-Tween-20 for 10 min, incubated with diluted (1/5,000) horse radish peroxidase-conjugated goat anti-mouse antibody in PBS-Tween-20 at RT for
1 h, and washed 3 times with PBS-Tween-20 for 10 min each. The immune-reactive bands were
detected by ECL<sup>TM</sup> Western Blotting Detection Reagents (GE Healthcare UK Limited, Little Chalfont,
UK) according to manufacturer's protocol.

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#### 6 2.9. Assay of enzymatic activity

 $\mathbf{7}$ The enzymatic activity was measured as described previously (Sarkar et al., 2005) with a slight modification. An aliquot of 2.5 µl of whole cell lysates of 293FT cells expressing duOASL, goOASL, 8 9 osOASL, osOAS1, and chOASL-A was incubated in incubation buffer containing 20 mM Tris-HCl 10 at pH 7.4, 20 mM magnesium acetate, 2.5 mM dithiothreitol, 5 mM ATP, 50 ug/ml poly(I):(C), and 5  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P] ATP (3,000 Ci/mmol) in a final volume of 10  $\mu$ l at 30 °C for 18 h. The reaction was 11 stopped by boiling at 95 °C for 5 min, followed by adding loading buffer containing 25% formamide, 120.5% bromophenol blue, and 0.5% xylene cyanol. The synthetized 2'-5'-adenylates (2'-5'A) products 13were run in a 20% polyacrylamide-urea (7 M) gel. The electrophoresis was performed on 200 V for 144 h, and then products were visualized by autoradiography with a BAS2500 Bio-Imaging analyzer 1516(FUJIFILM, Tokyo, Japan).

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#### 18 2.10. Inhibitory activity on WNV replicon replication

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WNV genomic RNA harboring the secreted alkaline phosphatase (SEAP) reporter gene instead of viral structural genes, WNV/SEAP-replicon RNA was propagated as described previously (Moritoh, et al., 2011; Tag-EL-Din-Hassan, et al., 2017). BHK-21 cells were cultured in a 100-mm plate at the density of  $1.5 \times 10^6$  cells/plate for 24 h and then, 10 µg of pIRES-EGFP (empty vector as the control), pduOASL-FL-EGFP, pgoOASL-FL-EGFP, posOASL-FL-EGFP, posOAS1-FL-EGFP, and pchOASL-A-FL-EGFP were transfected using Lipofectamine 2000<sup>®</sup> (Invitrogen) according to the manufacturer's protocol. The EGFP expression was analyzed at 24 h post-transfection using a

NIKON ECLIPSE TE2000E microscope. Afterwards, all transfected cells were harvested and  $5 \times 10^6$ 1 cells of non-transfected (mock transfection) and all transfectants were suspended in 500 µl of PBS on  $\mathbf{2}$ ice with 10 µg of WNV/SEAP-replicon RNA in 4 mm cuvette (Nepa Gene Co., Ltd. Chiba, Japan). 3 4 The WNV/SEAP-replicon RNA was electroporated using a Gene-Pulser X Cell (Bio-Rad) under the condition of two pulses on 1.5 kV at 25 µF of infinite resistance. The supernatant was collected after  $\mathbf{5}$ 6 culturing the cells for 24 and 72 h post-electroporation. The SEAP activity was measured in the 7supernatant using a Great EscAPe<sup>™</sup> SEAP Chemiluminescence Kit 2.0 (Clontech Bio. Inc.) and a Infinite M200 PRO plate reader (TECAN Japan Co., Ltd., Kawasaki, Japan) according to the 8 9 manufacturer's protocol.

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#### 11 2.11. RNase L activation assay

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The florescence resonance energy transfer (FRET) assay was performed as described previously 13(Thakur, et al., 2005) with a slight modification as follows; an aliquot of 2 µg of whole cell lysates of 14BHK-21 cells transfected with pIRES-EGFP (empty vector as the control), pduOASL-FL-EGFP, 15pgoOASL-FL-EGFP, posOASL-FL-EGFP, posOAS1-FL-EGFP, and pchOASL-A-FL-EGFP was 16incubated in 1x incubation buffer containing 25 mM Tris-HCl at pH 7.4, 0.1 M KCl, 10 mM MgCl<sub>2</sub>, 1750 μM ATP, 7 mM β-mercaptoethanol, and 180 nM FRET probe in a volume of 50 μl for 0, 5, 10, 15, 1830, 45, 60, 90 and 120 min at 21 °C. As a negative control, lysates of BHK-21 and 293FT non-19transfected cells were used, whereas for the positive control, lysates of BHK-21 and 293FT non-20transfected cells treated with 3,000 IU/ml IFNα-2β (INTRON® A, MSD K.K.) for 24 h were used as 21described previously (Tag-EL-Din-Hassan et al., 2017). The fluorescence was determined using a 2223Infinite M200 PRO plate reader (TECAN Japan Co., Ltd.).

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25 2.12. Localization of the OAS proteins

BHK-21 cells were transfected with 0.1 µg of pIRES-EGFP (empty vector as the control). 1 pgoOASL-FL-EGFP, posOASL-FL-EGFP, posOAS1-FL-EGFP,  $\mathbf{2}$ pduOASL-FL-EGFP, and pchOASL-A-FL-EGFP using 0.4 µl of Lipofectamine 2000® (Gibco/Invitrogen) according to 3 4 manufacturer's protocol at 24 h after culturing in 96 wells plate ( $2 \times 10^4$  cells/well) and then, after 48 h culture, the cells were fixed with 4% paraformaldehyde in PBS for 15 min at RT. Cells were washed  $\mathbf{5}$ twice with 1x washing buffer (PBS-Tween-20), permeabilized with 0.1% Triton X-100 in PBS for 5  $\mathbf{6}$ min at RT, followed by washing twice with 1X washing buffer and blocking by 10% horse serum in  $\mathbf{7}$ PBS for 30 min at RT. Cells were incubated with mouse anti-FLAG M2 primary antibody (Sigma-8 Aldrich) diluted to 1:1,000 in the blocking buffer for 1 h at RT, followed by washing three times (5 9 min each) with 1X washing buffer. Cells were then incubated with the secondary antibody (Alexa 10 Fluor 568-conjugated goat anti-mouse IgG, Gibco/Invitrogen) diluted in PBS for 1 h at RT, followed 11 by washing three times (5 min each) with 1X washing buffer. Finally, cells were incubated with DAPI 12in PBS for 5 min at RT for the nuclei counterstaining, followed by washing two times (5 min each) 13with 1X washing buffer and then, the fluorescence images were captured using a BZ-X700 All-in-14One Florescence Microscope (KEYENCE, Osaka, Japan). 15

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17 2.13. Statistical analysis

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All experiments were performed independently three times. Collected data were subjected to the one-way analysis of variance (ANOVA) using a SPSS 16.0 software. All values were represented as means  $\pm$  SD. Statistical differences were analyzed using Tukey's HSD (honest significant difference) test. Values of *p* <0.05 and *p* <0.01 were considered to be significant.

- 1 **3. Results**
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#### 3 3.1. Bioinformatics information

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The sequence results demonstrated that the full length of *duOASL* (KU569292), *goOASL*  $\mathbf{5}$ (KU569293) and osOASL (XM\_009673088) mRNA were 1,689, 1,649, and 1,640 bp with open 6  $\overline{7}$ reading frame of 1,512, 1,527, and 1,590 bp, encoding 503, 508, and 529 amino acids with predicted 8 molecular weight of 57.44, 58.09, and 59.4 kDa, respectively. The sequence alignment showed that duOASL, goOASL, and osOASL mRNA exhibited 76%, 76%, and 73% homology to chOASL-A 9 mRNA, respectively. Moreover, duOASL, goOASL, and osOASL proteins exhibited 64%, 64%, and 1063% homology to chOASL-A protein, respectively. The sequence alignment between duOASL and 11 12goOASL mRNA showed 88% homology as well as both mRNA showed 75% homology to osOASL mRNA. In addition, the protein sequence alignment between duOASL and goOASL showed 80% 13homology as well as both proteins showed 64% homology to osOASL. Domain structure analysis of 1415duOASL, goOASL, and osOASL showed that these proteins were OAS-like proteins conserving three main domains, nucleotidyltransferase, OAS1\_C, and two ubiquitin-like domains, UBL1 and UBL2, 16whereas the osOAS1 possessed only the nucleotidyltransferase and OAS1\_C domains similar to 17OAS1 protein family. 18

The phylogenetic tree was constructed by using neighbor-joining analyses based on various 19species OAS proteins (Fig. 1). Both avian and mammalian OASL families descend from the same 20ancestor B. All avian OASL families descend from the same ancestor F and show high homology 21each other than the mammalian OASL families which descend from the ancestor E. The goOASL and 2223duOASL show high homology each other than the other avian OASL proteins. On the other hand, chOASL protein is more closely related to the quail and turkey OASL proteins than duOASL and 24goOASL. Moreover, osOASL protein shows higher homology and evolutionarily more closely related 2526to pigeon, parrot, crows, and zebra finish OASL proteins. The avian and mammalian OAS1 family is

1	considered to be a different member from the OASL family, where mammalian OAS1 family makes
2	cluster D and makes further cluster C with osOAS1. These results are consistent with the evolution
3	theory proposed by Perelygin et al. (2006).

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5 *3.2. The enzymatic activity* 

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OAS enzymatic activity was assessed using 293FT cells transfected with pIRES-EGFP empty vector as a control, pduOASL-EGFP, pgoOASL-EGFP, posOASL-EGFP, posOAS1-EGFP, and pchOASL-A-EGFP. The expression of all genes was confirmed by RT-PCR and Western blotting (Fig. 2A). Fig. 2B shows that duOASL, goOASL, osOAS1, and chOASL-A proteins possess the enzymatic activity to convert the ATP to 2'-5' A up to tetramer, whereas only the osOASL protein expresses very weak enzymatic activity.

13

#### 14 3.3. RNase L activation assay

15

The results of the RNase L activation assay showed that lysates from BHK-21 cells transfected with duOASL, goOASL, osOASL, osOAS1, and chOASL-A emitted fluorescence as little as did the negative control (non-transfected and pIRES-EGFP-transfected BHK-21 cells) in all time points, indicating that ectopically expressed proteins were all unable to activate RNase L (Fig. 3). On the other hand, when BHK-21 cells were stimulated with the IFN, the RNase L activity was significantly up-regulated.

However, when exogenous RNase L proteins (duRNase L and/or osRNase L) were added to the reaction mixture, duOASL, goOASL, osOAS1, and chOASL-A proteins significantly emitted fluorescence with almost similar extent as did the positive control (293FT and BHK-21 cells treated with IFN). However, the osOASL emitted very low extent of florescence, approximately 30% of the positive control (Fig. 4A-E). These results indicate that the osOASL protein expresses very low 1 enzymatic activity in comparison with osOAS1, duOASL, goOASL and chOASL.

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# *3.4. Inhibitory activity on WNV replicon replication*

5	Inhibitory activity on WNV replicon replication was measured using BHK-21 cells transfected
6	with pduOASL-EGFP, pgoOASL-EGFP, posOASL-EGFP, posOAS1-EGFP, and pchOASL-A-EGFP
7	genes as well as mock- and pIRES-EGFP-transfected cells as a negative control. Fig. 5A shows the
8	SEAP expression level at 24 and 72 h post-electroporation of WNV/SEAP-replicon RNA. The
9	duOASL, goOASL, osOASL, and chOASL-A proteins significantly ( $p$ <0.01) inhibited the
10	WNV/SEAP replicon RNA replication in comparison with the control groups, whereas only osOAS1
11	did not. Expression of all genes was confirmed at 24 h after the transfection by the expression of
12	EGFP fluorescence (data not shown) and by RT-PCR before and 72 h after the WNV/SEAP replicon
13	RNA electroporation (Fig. 5B).

#### 1 **4. Discussion**

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The 2'-5'-OAS gene, one of the most important type I IFN-stimulated genes, is initiated and 3 4 triggered inside the cell upon the stimulation by IFN secreted from the virus-infected cells (Der et al., 1998; Sadler and Williams, 2008; Santhakumar et al., 2017). Four major types of OAS genes (OAS1,  $\mathbf{5}$ 6 OAS2, OAS3, and OASL) are almost conserved in all mammals (e.g. human, mouse, rat, cattle, pig,  $\mathbf{7}$ horse, etc.); however, some of the OAS genes have been lost, transformed to pseudo-genes, or undergone duplication during mammalian evolution (Justesen et al., 2000; Kjaer et al., 2009; 8 Perelygin et al., 2006). On the other hand, in aves up-to-date, the only well characterized OAS gene 9 10 is chOASL (Tatsumi et al., 2002 and 2003; Yamamoto et al., 1998). Recently, we have reported that the chOASL protein possesses antiviral activity against WNV independent of its enzymatic activity 11 12(Tag-EL-Din-Hassan et al., 2012 and 2017); however, the other avian OAS proteins are rarely studied. Therefore, this study aims at the functional analysis of avian OAS families by comparing that of 13chOASL-A. 14

We demonstrated for the first time the full-length sequences of duOASL (KU569293) and 15goOASL mRNA (KU569292), with open reading frame of 1,512 and 1,527 bp, encoding 503 and 508 16amino acids with predicted molecular weight of 57.44 and 58.09 kDa, respectively. Domain structure 17analysis of duck and goose OAS proteins showed that both proteins have three conserved domains; 18nucleotidyltransferase and OAS1\_C domains as well as ubiquitin-like domains (two repetitive 1920ubiquitin-like domains, UBL1 and UBL2), indicating that both proteins are OAS-like proteins (Player and Torrence, 1998). Moreover, although OAS gene families in mammals include four types of OAS 2122genes (OAS1, OAS2, OAS3, and OASL), the avian taxa possess decreasing or limiting OAS genes 23during the evolution process. In all known avian genome database there is no evidence for the presence of OAS1, OAS2, and OAS3 genes (Perelygin et al., 2006). Magor et al. (2013) discussed this 2425theory for other missing genes in avian taxa such as TLR8 and ISG15 genes. Furthermore, based on 26database for OAS proteins of almost all avian species except for the ostrich, we can find only one OAS protein, OASL that possesses both enzymatic and antiviral activities. On the other hand in ostrich, we can find two different OAS proteins, osOASL (XP\_009671383) and osOAS1 (XP\_009667960), indicating the duplication of the small OAS proteins into osOAS1 and osOASL, resulting in a unique evolutional process from the other avian species. Moreover, the osOAS proteins show development of a semi-functional specification not seen in the other avian species; osOAS1 possesses enzymatic activity but not antiviral activity, whereas osOASL possesses antiviral activity but little enzymatic activity (Fig. 2B; Fig. 4D and E and Fig.5).

8 During infection, activation of OAS proteins result in OAS-pathogen interaction through direct or indirect pathways. The classical antiviral OAS/RNase L pathway or the enzymatic activity results 9 10 in viral and cellular RNA degradation including ribosomal RNA within infected cells, subsequently effectively inhibits the viral replication (Silverman, 2007). In the current study, we investigated the 11 classical OAS/RNase L activity. We demonstrated that duOASL, goOASL and osOAS1 proteins 12showed enzymatic activity resembling chOASL-A to convert the ATP into mono, di, tri, and tetra 2'-135'(A)s, except for the osOASL protein which expressed very low enzymatic activity (Fig. 2B and 4A-14E). Similarly, Sokawa et al. (1984) conducted one of the most earlier studies with avian species, where 1516they investigated the 2'-5'-oligoadenylate synthetase activity in erythrocytes. They found that all studied avian species (chicken, goose, and pigeon) expressed 2-5A synthetase in erythroid cells. In 17the current study, the conserved domain analysis in the OAS unit of duOASL, goOASL, osOASL as 18well as osOAS1 showed high homology to those of chOASL-A (Fig. 7 A and B). However, the LxxxP 1920domain that is important for the enzymatic activity of OAS proteins showed one amino acid substitution in osOASL protein in position 8 from proline to threonine (P8T). This amino acid 2122substitution may be the underlying reason for the partial deactivation of osOASL enzymatic activity 23(Fig. 2B and 4E). Similarly, Ghosh et al. (1997) indicated that the generated mutation P7T in LxxxP domain of mouse 9-2 OAS protein resulted in almost 60% deactivation of the enzymatic activity 2425compared with the unmutated protein. However, this mutation seems to have more stronger effects 26on the OASL proteins, where it is resulted in a severe deactivation of osOASL enzymatic activity.

1 In respect of the alternative OAS/RNase L-independent pathway, where the OAS proteins may interact directly with the pathogens, previous reports indicated that huOASL/a, mOas1b and chOASL  $\mathbf{2}$ display antiviral activity against WNV. Moreover, this viral inhibitory function was independent of 3 4 the RNase L activity (Kajaste-Rudnitski et al., 2006; Kristiansen et al., 2011; Marques et al., 2008; Moritoh et al., 2009; Perelygin et al., 2002; Scherbik et al., 2007; Tag-EL-Din-Hassan et al., 2012  $\mathbf{5}$ 6 and 2017; Zhu et al., 2014). Results obtained in the current study showed that duOASL, goOASL,  $\mathbf{7}$ and osOASL express the antiviral activity against WNV-replicon replication to the similar extent to 8 chOASL-A, except for the osOAS1 which was similar to control groups (Fig. 5A). Moreover, the 9 antiviral activity was found to be independent of the enzymatic activity, where all proteins were 10 unable to activate the RNase L (Fig. 3), and all proteins were localized into cytoplasm (Fig. 6) similar to both chOASL and mOas1b proteins (Tag-EL-Din-Hassan et al., 2012 and 2017). 11

12However, few previous reports have indicated that duck and goose as well as ostrich show susceptibility to WNV infection. However, almost all these studies conducted infection experiments 13using juveniles after hatch, where the immune system was not completely developed. Furthermore, 14all of these studies illustrated the roles of avian ages that consider to be one of the most important 1516undeniable factors during WNV infection and have a great significant effect on the variability of WNV pathogenesis outcome (James and Mertyn, 2008). Generally, nestling and juvenile birds 17showed more susceptibility to mosquito-borne viral infections, where they were not fully feathered 18and could not show the defensive behavior, thus, it could be more susceptible to mosquito bites which 1920increased viral infection load and pathogenesis (Pérez-Ramírez et al., 2014), in parallel with the fact that WNV highly replicated in the feather pulps (Docherty et al., 2004). Moreover, Lowenthal et al. 2122(1994) have indicated that although the initiation of development of avian immune system occurs 23during the embryogenesis, it is not completely developed until weeks after hatch depending on the avian species (up to 14 wks). As well, the temporary susceptibility of young duckling (Hofmeister et 2425al., 2015; Komar et al., 2003 Shirafuji; et al., 2009), gosling (Austin et al., 2004; Komar et al., 2003; 26Swayne et al., 2001), ostriches (Allwright et al., 1995), and chicks had declined by age and these

birds became resistant to WNV infection. Similar to WNV infection in ducks, Ramadori and Armbrust
(2001) and Song et al. (2014) clearly illustrated the age-related susceptibility to duck hepatitis virus1 (DHV-1). Moreover, the behavioral and managemental problems such as feather picking and
cannibalism (Banet-Noach et al., 2003) as well as pathogen co-infection (Glávits et al., 2005) have a
great impact on WNV susceptibility in avian species.

6 In addition, Li et al. (2007) and Lee et al. (2014) found that chOASL was involved in the 7antiviral response to IBDV infection and might play a role in viral sensing in chicken regardless of the IFN pathway. As well, Cong et al. (2013) reported that the chOASL/RNase L pathway might be 8 involved in anti-avian-IBV infection. Sokawa et al. (1984) indicated that chOASL might be involved 9 10 in other regulatory functions other than the immune activity. Moreover, Song et al. (2014) found that the duOASL might play a role in the resistance to DHV-1 infection in duck. As well, Yang et al. 11 (2016) recently indicated that the goOASL significantly increased antiviral activity against gosling 12plaque virus, duck-TMUV and H9N2 avian influenza virus. Furthermore, the expression of goOASL 13in the goose embryonic fibroblast showed significant inhibitory effect on the Newcastle diseases virus 14replication in comparison with the control group. These facts indicate that the avian OAS proteins are 1516very important and playing crucial roles in the avian immune response.

In summary, the function of the avian OAS proteins is rarely studied and incompletely 17understood. Recently, we have reported that the chOASL protein possesses antiviral activity against 18WNV independent of its enzymatic activity (Tag-EL-Din-Hassan et al., 2012 and 2017). In the current 1920study we have demonstrated that duOASL and goOASL are dual-function proteins similar to the chOASL, where they express antiviral activity through both the OAS/RNase L-dependent and 2122independent pathways. However, we have demonstrated that ostrich OAS proteins undergo a 23duplication to produce two OAS proteins (osOAS1 and osOASL) during the evolution process. It is of interest that, unlike chicken, duck, and goose OASs, the function of ostrich OAS family are split, 2425where the osOAS1 expresses the OAS-enzymatic activity to stimulate RNase L, whereas the osOASL 26expresses the anti-flavivirus activity independent of OAS-enzymatic activity. The obtained results in

this study spotlight the importance and reconsideration of the avian OAS proteins as an antiviral factor.
Further investigation is required to uncover the mechanism underlying the antiviral properties of avian OAS proteins against flavivirus infection, which will contribute to the understanding and control of the spread flavivirus infection in avian species.

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## 1 Acknowledgments

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We thank Dr. Minooka, Sapporo Maruyama Zoo for bloods of duck and goose, and Prof. Ishizuka, Faculty of Veterinary Medicine, Hokkaido University for primary culture cells of ostrich renal epithelial cells.

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### 1 Figure legends

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3	Fig. 1. Thirty-five OAS proteins were subjected to the neighborhood tree analysis using the MEGA
4	7 software. Blue rectangles, chOASL; green rectangles, duOASL; purple rectangles, goOASL;
5	orange rectangles, osOASL; red rectangles, osOAS1. A, D, C, D, E, and F indicate the common
6	ancestor for specific descendants. Numbers shown on branches represent the percentage of replicate
7	trees, in which the associated taxa clustered together in the bootstrap test. Numbers below branches
8	represent the evolutionary distances. The evolutionary history was inferred using the Neighbor-
9	Joining method. The evolutionary distances were computed using the Poisson correction method.
10	
11	Fig. 2. Enzymatic activity of duOASL, goOASL, oSOASL, osOAS1, and chOASL-A. A) The RT-
12	PCR and western blot of RNA and protein samples extracted at 48 h post transfection from 293FT
13	cells transfected with pIRES-EGFP, pduOASL-FL-EGFP, pgoOASL-FL-EGFP, posOASL-FL-EGFP,
14	posOAS1-FL-EGFP, and pchOASL-A-FL-EGFP. B) The measurement of the enzymatic activity of
15	duOASL, goOASL, osOAS1, and chOASL-A proteins.
16	
17	Fig. 3. RNase L activation assay of duOASL, goOASL, osOASL, osOAS1, and chOASL-A proteins
18	using FRET assay. The samples were incubated in cleavage buffer with 180 ng of FRET probe for
19	periods of 0, 5, 10, 15, 30, 45, 60, 90, and 120 min at 21 °C. The background fluorescence activity
20	was subtracted from all reads before statistical analysis. ** and * indicate statistical difference from
21	other samples in the same incubation period with P<0.01 and P<0.05, respectively.
22	
23	Fig. 4. RNase L activation assay of duOASL, goOASL, osOASL, osOAS1, and chOASL-A proteins

using FRET assay. A), duOASL; B), GoOASL; C), chOASL; D), osOAS1; and E), osOASL using

- FRET probe for periods of 0, 60, and 120 min at 21 °C. The background fluorescence activity was
- subtracted from all reads before statistical analysis. \*\* and \* indicate statistical difference from other

1 samples in the same incubation period with P<0.01 and P<0.05, respectively.

 $\mathbf{2}$ 

Fig. 5. Antiviral activity assay of duOASL, goOASL, osOASL, osOAS1, and chOASL-A. A) RTPCR of RNA extracted from the mock-transfected BHK-21 cells, pIRES-EGFP as well as all
transfectants before and 72 h post WNV-replicon RNA electroporation. B) Amplification of WNVreplicon replication as determined by the SEAP expression level at 24 and 72 h post WNV-replicon
RNA electroporation in all transfectants (mean ± SD).

8

Fig. 6. Cellular localization of duOASL, goOASL, osOASL, osOAS1, and chOASL-A proteins. The
immunofluorescence analysis was performed at 48 h after BHK-21 cells transfected with pIRESEGFP as well as all transfectants using mouse α-FLAG M2 antibody and Alexa Fluor 568-conjugated
goat anti-mouse IgG to detect the protein expression. DAPI was used for the nucleus counterstaining.
The white bars indicate the length of 100 µm.

14

Fig. 7. Alignment for protein sequences and conserved domains. A) The important conserved 15domains are as follows; the LxxxP (yellow box), P-loop (brown box), D-D box (red box) and K-R 16rich reign (blue box) as well as high homology in the UBL1 (green arrow) and UBL2 (red arrow) 17units in OASL proteins. B) Schematic diagram of the OAS protein. The amino acids important for 18the enzymatic activity in the LxxxP domain are shown as the yellow, green, and light blue highlights. 19Amino acids with the purple highlight show the amino acid substitution, P8T in the LxxxP domain. 20The grey highlight shows the conserved sequence in P-LooP. The red highlight shows the conserved 21amino acids in D-D box. The pink highlight shows the conserved amino acid in K-R rich region. 22

Fig. 1

Α





**Fig. 2** 







pppA(pA)4 pppA(pA)3

pppA(pA)2 pppApA ATP

Fig. 3

# **Incubation period**















**Hours post-electroporation** 





Fig.	7
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U	
	(
<b>(A)</b>	,

		LXXXP
goOASL	1	<mark>mb</mark> lrdvp <mark>a</mark> kQldgwvastlQpstaftlavkntvrQICdflkbrCfEdgsfe
duOASL	1	<mark>ME</mark> LWNVS <mark>I</mark> SE <mark>LDGWVA</mark> ATLQPST <mark>DFTAAVK</mark> KTVRQICDFLKEECFED
chOASL-A	1	MGLESVS 3RQLEGWVAAHLQPSTEFSTAVKQTVKDICDFLKERCFEG
OSOASL	1	MDGLETVI <mark>A</mark> RDLDAWLARTLEPSTAFRIQVKQTVKQICDFLKEQCEQG
os0AS1	1	MLSQLLSCLMB <mark>L</mark> YNTP <mark>BEQLD</mark> KFLYEVLQPDRTELEQLRCAVHTICEFIRDNCFAG-APP
		P-LooP D-D Box
GOOASL	52	D×IRVFKTVKGGSAGKGTALRNNSDADVVLFLSYFTSYLDORQEHPKIRLLIEKRLLECM
duOASL	48	-KTRVFKTVKGGSAGKGTALRNNSDADVVTFLSCFSNYRQQREEHPTILQLLEKRLTECM
chOASL-A	48	IISVLKTVKGGSAGKGNAURNNSDADVVIJE NOESSYQ QEARREHILA LIERRINEC
OSOASL	49	IRVHKTVKGGSAGKGHALQNNSDADVVHFLSOFSSYQDQTQRRAEHLAHLBERHECCR
OSOAS1	60	PRTRULKVWKGGSAGKGTALKKSSDAD, VVFLSCREDWKDOEKNRAET, RETOKRIVECO
GOOAST.	112	
duOASI.	107	
	106	
OROASI.	107	OKLETTVNI SPORIKCSGED SPSI SLETIOSKSCAFSTDVDI LFATGALGOVIODA ADA AD
OSOAS1	120	OKHERVEFEVSPUSNDRVLSFOLSSKILHESTSFDVDVHATDALGOVIGD AFAAD
OBOADI	120	
		K-R Rich Region
GOOASL	165	VYVELLKAHSSPGEFSTCFTELOKKFVKRCPAKIKDHRIVKHWYKOULKPOVPRAD
duOASL	160	VYVDLLDVNSNPGEFSTCFTELOKKFVKRCPAKLKNLLRLVKHWYKOTLKEOYPGAE
chOASL-A	166	VYVKLLATRCGPGDESPSETELOKNEV
OSOASL	167	VYVRLLAAPSGPGEESPCETELOKKEV CRCPAKLKNLLRLVKHWYKELLKPRYPDGD
osOAS1	176	VY OLFOOCSRGGEFSTCFTELORDFILSRPTKVKSLIRLVKHWYKTYLCPHKWALRGGE
goOASL	222	-LPPKYALELLTIYAWEQGTGSKEAFSLAEGFCTVL <mark>KLL</mark> CQYRDICVYWEKYYSLQH <mark>EQ</mark> I
duOASL	217	-LPPKYALELLTIYTWEQGANSNEAFNMAEGFCTVLKLLGQYQEICIYWERYYSLQHHRI
chOASL-A	223	-LPPKYALELLTIYAWE <mark>GGTG-RE</mark> DFSMAEGFCTVLELL <mark>GQYR</mark> DICIYWEKYYSLEDEWV
OSOASL	224	-LPPKYALELLTIYAWEQGTG <mark>AKEGFNMAEGF</mark> RTVLELLCRYQET <mark>VIYWEKYYSLQH</mark> AEV
os0AS1	236	TLPPQYALELLTVYAWEQGSG-ETNFSVAKAERTVLELLQHYQQLCVYWTVNYDFRDATL
GOOASL	281	GTHLKOLLERKPRPLILLDPADPTGILLGODROWDLVABE/AARCCASLPCLTGVNPWNV
duOASL	276	GAHLKOLLRMPCPIIILDPADPTGILGODKRWDLVAKEAARCCASMRCITGVQPWNV
ChOASL-A	281	GAYLKKOLROPRPVILDPADPTGILGOCKNWDLVAKEAAESRVSLPCVSDVCSWDV
OSUASL	283	
USUADI	295	SCHISSQIISKS <u>KIVIIIDIADII</u> NI (C <mark>KGSKWDIVIKS)</mark> BKCCSQRCC®ISKGVP <mark>VQPWD</mark> V
		UBL1
GOOASL	337	<u> OPAKPVTVEVKGLOGARIRTTVSPSTTIWOMKEMIEREMRISRYOORISOOP-AGTPLII</u>
duOASL	332	QPAKPVTIEVRGLQGDRIRITVSPSTTIWOLKEEISKNWGIPPYQQRISQQP-AGTPLIL
ChOASL-A	337	
OSUASL	338	<u>QPAQPVTVBIKRIVETSITKTISEDTPILTIKRBIMQQWGTPLHQQRIGQQEPEQTLRTI</u>
OSUASI	300	SPEQIR
goOASL	396	QENESLASYGIYYDTYLVHLQTQPRQMDIFVKDIKNQTMTYTVQPTDTVLQLKTKINTRQ
duOASL	391	HNDKSLASYGTYYDTTLVLLRTEPQEMEIFVKDIKNQTMTYSVRPTDTVLQLKKKINSRQ
chOASL-A	397	RDGDSLAAH LF IV L C EVLVKDSN-KTTVYTVRPTDTVKQLKQQIYARQ
OSOASL	398	RDSETLATYNT FYSTTLVLLHTETQEMDVLVKDDKNRTSTYTMQPTDTVRQLKEQTCNRL
OSOAS1		
		UBL2
CODA ST	456	
duOASI.	451	
	456	
OSOASI.	458	CDDVD OOPL TH CSKELED RHTLAHVCTONKSTTYLLLRLRCCACEP ADDED CSLALLCTA
os0AS1	100	OF THE REAL PROPERTY OF THE REAL PROPERTY OF THE PROPERTY OF T
		UBL2
goOASL		
duOASL		
chOASL-A		

OSOASL 518 QASDYTAVPMYH OSOAS1 ----- **Fig. 7** 

