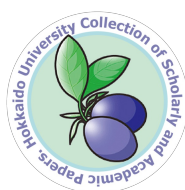




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Functional analysis of duck, goose, and ostrich 2'-5'-oligoadenylate synthetase

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1 **ABSTRACT**

2

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

22

1. Introduction

Several members of flavivirus are zoonotically important due to their pathogenesis to both wild 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 infection to severe and fatal diseases. In avian hosts, viruses including Tembusu virus (TMUV), 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 cycle and detected in around 150 avian species and several mammalian species. Reptiles are 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 suffer from infectious viremia around 1 to 4 days after infection, and then they develop a life-long immunity. Unlike avian species, humans and horses as well as most other mammals are incidental “dead-end” hosts (Dauphin et al., 2004).

Wild and domestic avian species express wide range of WNV-resistant or susceptible phenotypes. The most susceptible species belong to the *Charadriiformes* and *Passeriformes* orders and they develop severe neurological signs and high mortality rates such as the American crow with 100% mortality (Hofmeister et al., 2015; Nemeth et al., 2011). However, in other birds, they express more restrictive symptoms and death is rarely observed (Van der Meulen et al., 2005). In contrast, little information is known about the susceptibility of WNV infection on aquatic birds except for few reports demonstrating that WNV may cause diseases and mortality in geese (Austin et al., 2004; Glávits et al., 2005; Swayne et al., 2001) and ducks (Hofmeister et al., 2015; Shirafuji et al., 2009) as well 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 10^6 pfu/ml, moderately competent

1 birds including reservoir birds such as several species belonging to *Anseriformes* and *Passeriformes*
2 orders with viremia level of 10^4 - 10^6 pfu/ml, and incompetent (resistant) birds with viremia level less
3 than 10^4 pfu/ml such as *Galliformes*. Goose and duck are considered as low-moderate viremia birds.
4 However, up till now the underlying reasons for this variation are still unclear.

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

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

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

1 Yamamoto et al., 1998).

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

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

2. Materials and methods

2.1. Blood samples

Blood samples were collected from adult mallard ducks and white Chinese geese (reared in Sapporo Maruyama Zoo, Sapporo, Hokkaido, Japan) in heparinized blood collection tubes to prevent 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 3.0 ml of HISTOPAQUE-1077 (Sigma-Aldrich, St. Louis, MO, USA) followed by centrifugation at 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 min 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 (Gibco/Invitrogen), 10% fetal bovine serum (FBS) (Atlas Biologicals, Inc. Fort Collins, CO, USA), 1 µg/ml phytohaemagglutinin P (Sigma-Aldrich), and 10 µg/ml pokeweed mitogen (Sigma-Aldrich) in a 5% CO₂ incubator at 37 °C for 48 h.

2.2. Cell culture

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% CO₂ incubator at 37 °C and passaged every two days.

2.3. Interferon treatment

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Duck and goose leukocytes cultured with mitogens for 48 h and ostrich renal epithelial cells were treated with 3,000 IU/ml of IFN α -2 β (INTRON[®] A, MSD K.K., Tokyo, Japan) for 6 h, and then, total RNA was extracted. In addition, both BHK-21 and 293FT cells were treated with 3,000 IU/ml of IFN α -2 β for 24 h, and then, the whole cell lysates were extracted and stored at -80 °C until used.

2.4. RNA extraction and cDNA synthetase

Total RNA was extracted using TRIZOL reagent (Gibco/Invitrogen) according to the manufacturer's protocol and concentration of RNA was determined using SmartSpec[™] (BioRad, Hercules, CA, USA). Purified total RNA (1 μ g) was used to generate cDNA using ReverTra Ace[®] (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.

2.5. Cloning of *duOASL*, *goOASL*, *osOASL*, and *osOAS1*

Total RNA samples extracted from the duck and goose leukocytes and ostrich renal epithelial cells were used for the cloning and sequencing of full-length mRNA of *duOASL*, *goOASL*, *osOASL*, 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[®] 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 using specific primers (Table 1) to conjugate the FLAG-TAG sequence at the 3'-end as well as the 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 described above were used as template. To amplify *osOASL* and *osOAS1*, cDNA prepared from total RNA of ostrich renal cells was used as template. A *chOASL-A* clone conjugated with FLAG-TAG and NotI restriction site was previously prepared (Tag-EL-Din-Hassan et al., 2012 and 2017). The amplified fragments were extracted and digested with NotI restriction enzyme (Toyobo Co., Ltd.), and then purified and cloned into the NotI restriction site of the pIRES-EGFP (the mammalian expression vector, Clontech Bio. Inc., Shiga, Japan). Similarly, the ORFs of *duRNASEL* and *osRNASEL* were amplified using specific primers (Table 1), extracted and cloned to pGEM-T-Easy vector, digested with NotI restriction enzyme, and then cloned into pIRES-EGFP. In this study, all DNA fragment extraction from agarose gel was performed using FastGene Gel/PCR Extraction Kit (Nippon Genetics Co., Ltd, Tokyo, Japan). All cloning reactions were performed using 2X Ligation-Convenience Kit (Wako Nippon Gene Co., Ltd., Toyama, Japan) according to the manufacturer's protocol. All transfections were performed using DH5α *E. coli* competent cells. All plasmid DNA extractions were performed using FastGene Plasmid Mini Kit (Wako Nippon Gene Co., Ltd.). The large-scaled plasmid purification was performed using CsCL₂-ethidium bromide gradient purification method as described previously (Sambrook and Russell, 2001).

2.6. Alignment analysis and neighboring tree

1 The multiple sequence alignment was performed with the ClustalW program
2 (<http://www.genome.jp/tools-bin/clustalw>) using default parameters to compare the OAS proteins
3 from the following species; *Gallus gallus* (chOASL, BAB19016.1), *Anser cygnoides* (goOASL,
4 ANW12075), *Anas platyrhynchos* (duOASL, ANW12076), *Struthio camelus australis* (osOASL,
5 XP_009671383) and ostrich OAS3, XP_009667960 “named osOAS1 in this study”). The output was
6 embellished using BOXSHADE 3.21 software (www.ch.embnet.org/software/BOX_form.html).
7 Conserved domain analysis in the protein was performed using motif search
8 (<http://www.genome.jp/tools/motif/>). The evolutionary history was inferred using the neighbor-
9 joining method. The percentage of replicate trees, in which the associated taxa clustered together in
10 the bootstrap test (500 replicates), is shown next to the branches. The tree is drawn to scale with
11 branch lengths (next to the branches) in the same units as those of the evolutionary distances used to
12 infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction
13 method and were expressed as the units of the number of amino acid substitutions per site.
14 Evolutionary analyses were conducted using MEGA7 software ([Kumar et al., 2016](#)) and the following
15 protein sequences: *Gallus gallus* (chOASL, BAB19016.1), *Anser cygnoides* (goOASL, ANW12075),
16 *Anas platyrhynchos* (duOASL, ANW12076), *Struthio camelus australis* (osOASL, XP_009671383
17 and osOAS3, XP_009667960 “named osOAS1 in this study”), *Columba livia* (pigeon OASL,
18 XP_005508920), *Meleagris gallopavo* (turkey OASL, XP_019475496), *Corvus brachyrhynchos*
19 (crow OASL, KFO58001), *Coturnix japonica* (Japanese quail OASL, XM_015874647), *Amazona*
20 *aestiva* (turquoise-fronted parrot OASL, KQK84582.1), *Taeniopygia guttata* (zebra-finch OASL,
21 XP_004176346), *Mus musculus* (mouse Oasl1, NP_660210 and mouse Oasl2, NP_035984), *Rattus*
22 *norvegicus* (rat Oasl1, NP_001009681 and rat Oasl2, NP_001009682), *Homo sapiens* (human
23 OASL/a, NP_003724 and human OAS1, NP_058132), *Canis lupus familiaris* (dog OASL1,
24 AAX58775, dog OASL2, AAX58776, and dog OAS1, AAX56077), *Sus scrofa* (pig OAS1x,
25 NP_999468, pig OAS1y, AAT34965, and pig OASL, AAT44895), *Bos taurus* (cattle OAS1x,
26 NP_835209, cattle OAS1y, AAP69995, cattle OAS1z, NP_001025017, and cattle OASL, AAP94112),

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

2.7. Preparation of cell lysate

Both 293FT and BHK-21 cells were transfected with 10 µg of *pIRES-EGFP* (empty vector as 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 manufacturer's protocol. *pduRNASEL-EGFP* and *posRNASEL-EGFP* were also transfected into BHK-21 cells with the same method. Transfected cells were seeded with the density of 1.5×10^6 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 continued for culture until 48 h and 72 h post-transfection, washed twice with PBS, scratched with 1 ml 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 were 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.

2.8. Western blotting

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™ Western Blotting Detection Reagents (GE Healthcare UK Limited, Little Chalfont, UK) according to manufacturer's protocol.

2.9. Assay of enzymatic activity

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, osOASL, osOAS1, and chOASL-A was incubated in incubation buffer containing 20 mM Tris-HCl at pH 7.4, 20 mM magnesium acetate, 2.5 mM dithiothreitol, 5 mM ATP, 50 µg/ml poly(I):(C), and 5 µCi of [α -³²P] ATP (3,000 Ci/mmol) in a final volume of 10 µl at 30 °C for 18 h. The reaction was stopped by boiling at 95 °C for 5 min, followed by adding loading buffer containing 25% formamide, 0.5% bromophenol blue, and 0.5% xylene cyanol. The synthesized 2'-5'-adenylates (2'-5'A) products were run in a 20% polyacrylamide-urea (7 M) gel. The electrophoresis was performed on 200 V for 4 h, and then products were visualized by autoradiography with a BAS2500 Bio-Imaging analyzer (FUJIFILM, Tokyo, Japan).

2.10. Inhibitory activity on WNV replicon replication

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 1.5×10^6 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® (Invitrogen) according to the manufacturer's protocol. The EGFP expression was analyzed at 24 h post-transfection using a

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

11 2.11. RNase L activation assay

13 The fluorescence resonance energy transfer (FRET) assay was performed as described previously
14 (Thakur, et al., 2005) with a slight modification as follows; an aliquot of 2 μ g of whole cell lysates of
15 BHK-21 cells transfected with pIRES-EGFP (empty vector as the control), pduOASL-FL-EGFP,
16 pgoOASL-FL-EGFP, posOASL-FL-EGFP, posOAS1-FL-EGFP, and pchOASL-A-FL-EGFP was
17 incubated in 1x incubation buffer containing 25 mM Tris-HCl at pH 7.4, 0.1 M KCl, 10 mM MgCl₂,
18 50 μ M ATP, 7 mM β -mercaptoethanol, and 180 nM FRET probe in a volume of 50 μ l for 0, 5, 10, 15,
19 30, 45, 60, 90 and 120 min at 21 °C. As a negative control, lysates of BHK-21 and 293FT non-
20 transfected cells were used, whereas for the positive control, lysates of BHK-21 and 293FT non-
21 transfected cells treated with 3,000 IU/ml IFN α -2 β (INTRON® A, MSD K.K.) for 24 h were used as
22 described previously (Tag-EL-Din-Hassan et al., 2017). The fluorescence was determined using a
23 Infinite M200 PRO plate reader (TECAN Japan Co., Ltd.).

25 2.12. Localization of the OAS proteins

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

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17 2.13. Statistical analysis

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

23

3. Results

3.1. Bioinformatics information

The sequence results demonstrated that the full length of *duOASL* (KU569292), *goOASL* (KU569293) and *osOASL* (XM_009673088) mRNA were 1,689, 1,649, and 1,640 bp with open reading frame of 1,512, 1,527, and 1,590 bp, encoding 503, 508, and 529 amino acids with predicted 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* mRNA, respectively. Moreover, *duOASL*, *goOASL*, and *osOASL* proteins exhibited 64%, 64%, and 63% homology to *chOASL-A* protein, respectively. The sequence alignment between *duOASL* and *goOASL* 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% homology as well as both proteins showed 64% homology to *osOASL*. Domain structure analysis of *duOASL*, *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, whereas the *osOAS1* possessed only the nucleotidyltransferase and OAS1_C domains similar to OAS1 protein family.

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

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

3.2. *The enzymatic activity*

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.

3.3. *RNase L activation assay*

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 fluorescence, approximately 30% of the positive control (Fig. 4A-E). These results indicate that the osOASL protein expresses very low

enzymatic activity in comparison with osOAS1, duOASL, goOASL and chOASL.

3.4. Inhibitory activity on WNV replicon replication

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

4. Discussion

The 2'-5'-*OAS* gene, one of the most important type I IFN-stimulated genes, is initiated and 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*, *OAS2*, *OAS3*, and *OASL*) are almost conserved in all mammals (e.g. human, mouse, rat, cattle, pig, 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; Pereygin et al., 2006). On the other hand, in aves up-to-date, the only well characterized *OAS* gene 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 (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 *chOASL*-A.

We demonstrated for the first time the full-length sequences of *duOASL* (KU569293) and *goOASL* mRNA (KU569292), with open reading frame of 1,512 and 1,527 bp, encoding 503 and 508 amino acids with predicted molecular weight of 57.44 and 58.09 kDa, respectively. Domain structure analysis of duck and goose *OAS* proteins showed that both proteins have three conserved domains; nucleotidyltransferase and *OAS1_C* domains as well as ubiquitin-like domains (two repetitive ubiquitin-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* genes (*OAS1*, *OAS2*, *OAS3*, and *OASL*), the avian taxa possess decreasing or limiting *OAS* genes during the evolution process. In all known avian genome database there is no evidence for the presence of *OAS1*, *OAS2*, and *OAS3* genes (Pereygin et al., 2006). Magor et al. (2013) discussed this theory for other missing genes in avian taxa such as *TLR8* and *ISG15* genes. Furthermore, based on database 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 evolutionary 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).

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 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 classical OAS/RNase L activity. We demonstrated that duOASL, goOASL and osOAS1 proteins showed enzymatic activity resembling chOASL-A to convert the ATP into mono, di, tri, and tetra 2'-5'(A)s, except for the osOASL protein which expressed very low enzymatic activity (Fig. 2B and 4A-E). Similarly, [Sokawa et al. \(1984\)](#) conducted one of the most earlier studies with avian species, where they 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 the current study, the conserved domain analysis in the OAS unit of duOASL, goOASL, osOASL as well as osOAS1 showed high homology to those of chOASL-A (Fig. 7 A and B). However, the LxxxP domain 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 substitution may be the underlying reason for the partial deactivation of osOASL enzymatic activity (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 compared with the unmutated protein. However, this mutation seems to have more stronger effects on 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
2 interact directly with the pathogens, previous reports indicated that huOASL/a, mOas1b and chOASL
3 display antiviral activity against WNV. Moreover, this viral inhibitory function was independent of
4 the RNase L activity (Kajaste-Rudnitski et al., 2006; Kristiansen et al., 2011; Marques et al., 2008;
5 Moritoh et al., 2009; Perelygin et al., 2002; Scherbik et al., 2007; Tag-EL-Din-Hassan et al., 2012
6 and 2017; Zhu et al., 2014). Results obtained in the current study showed that duOASL, goOASL,
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
11 to both chOASL and mOas1b proteins (Tag-EL-Din-Hassan et al., 2012 and 2017).

12 However, few previous reports have indicated that duck and goose as well as ostrich show
13 susceptibility to WNV infection. However, almost all these studies conducted infection experiments
14 using juveniles after hatch, where the immune system was not completely developed. Furthermore,
15 all of these studies illustrated the roles of avian ages that consider to be one of the most important
16 undeniable factors during WNV infection and have a great significant effect on the variability of
17 WNV pathogenesis outcome (James and Mertyn, 2008). Generally, nestling and juvenile birds
18 showed more susceptibility to mosquito-borne viral infections, where they were not fully feathered
19 and could not show the defensive behavior, thus, it could be more susceptible to mosquito bites which
20 increased viral infection load and pathogenesis (Pérez-Ramírez et al., 2014), in parallel with the fact
21 that WNV highly replicated in the feather pulps (Docherty et al., 2004). Moreover, Lowenthal et al.
22 (1994) have indicated that although the initiation of development of avian immune system occurs
23 during the embryogenesis, it is not completely developed until weeks after hatch depending on the
24 avian species (up to 14 wks). As well, the temporary susceptibility of young duckling (Hofmeister et
25 al., 2015; Komar et al., 2003 Shirafuji; et al., 2009), gosling (Austin et al., 2004; Komar et al., 2003;
26 Swayne 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 virus-1 (DHV-1). Moreover, the behavioral and managerial 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.

In addition, Li et al. (2007) and Lee et al. (2014) found that chOASL was involved in the antiviral 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 involved in anti-avian-IBV infection. Sokawa et al. (1984) indicated that chOASL might be involved 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. (2016) recently indicated that the goOASL significantly increased antiviral activity against gosling plaque virus, duck-TMUV and H9N2 avian influenza virus. Furthermore, the expression of goOASL in the goose embryonic fibroblast showed significant inhibitory effect on the Newcastle diseases virus replication in comparison with the control group. These facts indicate that the avian OAS proteins are very important and playing crucial roles in the avian immune response.

In summary, the function of the avian OAS proteins is rarely studied and incompletely understood. Recently, we have reported that the chOASL protein possesses antiviral activity against WNV independent of its enzymatic activity (Tag-EL-Din-Hassan et al., 2012 and 2017). In the current study 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 independent pathways. However, we have demonstrated that ostrich OAS proteins undergo a duplication 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, where the osOAS1 expresses the OAS-enzymatic activity to stimulate RNase L, whereas the osOASL expresses the anti-flavivirus activity independent of OAS-enzymatic activity. The obtained results in

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

5

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2

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References

- Allwright, D.M., Geyer, A., Burger, W.P., Williams, R., Gerdes, G.H., Barnard, B.J., 1995. Isolation of Wesselsbron virus from ostriches. *Vet. Rec.* 136, 99.
- Austin, R.J., Whiting, T.L., Anderson, R.A., Drebot, M.A., 2004. An outbreak of West Nile virus-associated disease in domestic geese (*Anser anser domesticus*) upon initial introduction to a geographic region, with evidence of bird to bird transmission. *Can. Vet. J.* 45, 117-123.
- Banet-Noach, C., Simanov, L., Malkinson, M., 2003. Direct (non-vector) transmission of West Nile virus in geese. *Avian Pathol.* 32, 489-494.
- Cohrs, R.J., Goswami, B.B., Sharma, O.K., 1988. Occurrence of 2-5A and RNA degradation in the chick oviduct during rapid estrogen withdrawal. *Biochemistry* 27, 3246-3252.
- Cong, F., Liu, X., Han, Z., Shao, Y., Kong, X., Liu, S., 2013. Transcriptome analysis of chicken kidney tissues following coronavirus avian infectious bronchitis virus infection. *BMC Genomics* 14, 743-755.
- Dauphin, G., Zientara, S., Zeller, H., Murgue, B., 2004. West Nile: worldwide current situation in animals and humans. *Comp. Immunol. Microbiol. Infect. Dis.* 27, 343-355.
- Davidson, I., 2015. A New Look at Avian *Flaviviruses*. *Israel Journal of Veterinary Medicine* 70, 3-8.
- Der, S.D., Zhou, A., Williams, B.R., Silverman, R.H., 1998. Identification of genes differentially regulated by interferon α , β , or γ using oligonucleotide arrays. *Proc. Natl. Acad. Sci. USA* 95, 15623-16528.
- Docherty, D.E., Long, R.R., Griffin, K.M., Saito E.K., 2004. Corvidae feather pulp and West Nile virus detection. *Emerg. Infect. Dis.* 10, 907-909.
- Fair, J.M., Nemeth, N.M., Taylor-McCabe, K.J., Shou, Y., Marrone, B.L., 2011. Clinical and acquired immunologic responses to West Nile virus infection of domestic chickens (*Gallus gallus domesticus*). *Poult. Sci.* 90, 328-336.

1 Gamino, V., Höfle, U., 2013. Pathology and tissue tropism of natural West Nile virus infection in
2 birds: a review. *Vet. Res.* 44, 39.

3 Ghosh, A., Desai, S.Y., Sarkar, S.N., Ramaraj, P., Ghosh, S.K., Bandyopadhyay, S., Sen, G.C., 1997.
4 Effects of mutating specific residues present near the amino terminus of 2'-5'-oligoadenylate
5 synthetase. *J. Biol. Chem.* 272, 15452-15458.

6 Glávits, R., Ferenczi, E., Ivanics, E., Bakonyi, T., Mató, T., Zarka, P., Palya, V., 2005. Co-occurrence
7 of West Nile Fever and circovirus infection in a goose flock in Hungary. *Avian Pathol.* 34, 408-
8 414.

9 Hofmeister, E., Porter, R.E., Franson, J.C., 2015. Experimental susceptibility of wood ducks (*AIX*
10 *SPONSA*) for West Nile virus. *J. Wildl. Dis.* 51, 411-418.

11 James, S., Mertyn, M., 2008. Arbovirus Infections. *Diseases of poultry* 12th edition, section I. pp. 414-
12 425.

13 Justesen, J., Hartmann, R., Kjeldgaard, N.O., 2000. Gene structure and function of the 2'-5'-
14 oligoadenylate synthetase family. *Cell. Mol. Life Sci.* 57, 1593-1612.

15 Kajaste-Rudnitski, A., Mashimo, T., Frenkiel, M.P., Guénet, J.L., Lucas, M., Desprès, P., 2006. The
16 2'-5'-oligoadenylate synthetase 1b is a potent inhibitor of West Nile virus replication inside
17 infected cells. *J. Biol. Chem.* 281, 4624-4637.

18 Kjaer, K.H., Poulsen, J.B., Reintamm, T., Saby, E., Martensen, P.M., Kelve, M., Justesen, J., 2009.
19 Evolution of the 2'-5' oligoadenylate synthetase family in eukaryotes and bacteria. *J. Mol. Evol.*
20 69, 612-624.

21 Komar, N., Langevin, S., Hinten, S., Nemeth, N., Edwards, E., Hettler, D., Davis, B., Bowen, R.,
22 Bunning, M., 2003. Experimental infection of North American birds with the New York 1999
23 strain of West Nile virus. *Emerg. Infect. Dis.* 9, 311-322.

24 Kristiansen, H., Gad, H.H., Eskildsen-Larsen, S., Despres, P., Hartmann, R., 2011. The oligoadenylate
25 synthetase family: an ancient protein family with multiple antiviral activities. *J. Interferon*
26 *Cytokine Res.* 31, 41-47.

1 Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular evolutionary genetics analysis version
2 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870-1874.

3 Langevin, S.A., Bunning, M., Davis, B., Komar, N., 2001. Experimental infection of chickens as
4 candidate sentinels for West Nile virus. *Emerg. Infect. Dis.* 7, 726-729.

5 Lee, C.C., Wu, C.C., Lin, T.L., 2014. Chicken melanoma differentiation-associated gene 5 (MDA5)
6 recognizes infectious bursal disease virus infection and triggers MDA5-related innate immunity.
7 *Arch. Virol.* 159, 1671-1686.

8 Li, Y.P., Handberg, K.J., Juul-Madsen, H.R., Zhang, M.F., Jørgensen, P.H., 2007. Transcriptional
9 profiles of chicken embryo cell cultures following infection with infectious bursal disease virus.
10 *Arch. Virol.* 152, 463-478.

11 Lowenthal, J.W., Connick, T.T., McWaters, P.G., York, J.J., 1994. Development of T cell immune
12 responsiveness in the chicken. *Immunol. Cell Biol.* 72, 115-122.

13 Magor, K.E., Miranzo-Navarro, D., Barber, M.R., Petkau, K., Fleming-Canepa, X., Blyth, G.A.,
14 Blaine, A.H., 2013. Defense genes missing from the flight division. *Dev. Comp. Immunol.* 41,
15 377-388.

16 Mandl, J.N., Ahmed, R., Barreiro, L.B., Daszak, P., Epstein, J.H., Virgin, H.W., Feinberg, M.B., 2015.
17 Reservoir host immune responses to emerging zoonotic viruses. *Cell* 160, 20-35.

18 Marques, J., Anwar, J., Eskildsen-Larsen, S., Rebouillat, D., Paludan, S.R., Sen, G., Williams, B.R.,
19 Hartmann, R., 2008. The p59 oligoadenylate synthetase-like protein possesses antiviral activity
20 that requires the C-terminal ubiquitin-like domain. *J. Gen. Virol.* 89, 2767-2772.

21 Moritoh, K., Maeda, A., Nishino, T., Sasaki, N., Agui, T., 2011. Development and application of West
22 Nile virus subgenomic replicon RNA expressing secreted alkaline phosphatase. *J. Vet. Med.*
23 *Sci.* 73, 683-686.

24 Moritoh, K., Yamauchi, H., Asano, A., Yoshii, K., Kariwa, H., Takashima, I., Isoda, N., Sakoda, Y.,
25 Kida, H., Sasaki, N., Agui, T., 2009. Generation of congenic mouse strains by introducing the
26 virus-resistant genes, Mx1 and Oas1b, of feral mouse-derived inbred strain MSM/Ms into the

1 common strain C57BL/6J. Jpn. J. Vet. Res. 57, 89-99.

2 Nemeth, N.M., Bowen, R.A., 2007. Dynamics of passive immunity to West Nile virus in domestic
3 chickens (*Gallus gallus domesticus*). Am. J. Trop. Med. Hyg. 76, 310-317.

4 Nemeth, N.M., Thomsen, B.V., Spraker, T.R., Benson, J.M., Bosco-Lauth, A.M., Oesterle, P.T., Bright,
5 J.M., Muth, J.P., Campbell, T.W., Gidlewski, T.L., Bowen, R.A., 2011. Clinical and pathologic
6 responses of American crows (*Corvus brachyrhynchos*) and fish crows (*C. ossifragus*) to
7 experimental West Nile virus infection. Vet. Pathol. 48, 1061-1074.

8 Perelygin, A.A., Scherbik, S.V., Zhulin, I.B., Stockman, B.M., Li, Y., Brinton, M.A., 2002. Positional
9 cloning of the murine *Flavivirus* resistance gene. Proc. Natl. Acad. Sci. USA 99, 9322-9327.

10 Perelygin, A.A., Zharkikh, A.A., Scherbik, S.V., Brinton, M.A., 2006. The mammalian 2'-5'
11 oligoadenylate synthetase gene family: evidence for concerted evolution of paralogous Oas1
12 genes in Rodentia and Artiodactyla. J. Mol. Evol. 63, 562-576.

13 Pérez-Ramírez, E., Llorente, F., Jiménez-Clavero, M.Á., 2014. Experimental infections of wild birds
14 with West Nile virus. Viruses 6, 752-781.

15 Player, M.R. Torrence, P.F., 1998. The 2-5A system: modulation of viral and cellular processes
16 through acceleration of RNA degradation. Pharmacol. Ther. 78, 55-113.

17 Ramadori, G., Armbrust, T., 2001. Cytokines in the liver. Eur. J. Gastroenterol Hepatol. 13, 777-784.

18 Sadler, A.J., Williams, B.R., 2008. Interferon-inducible antiviral effectors. Nat. Rev. Immunol. 8, 559-
19 568.

20 Sambrook, J. Russell, D.W., 2001. Molecular cloning: A laboratory manual, 3rd edition, volume 1. pp.
21 65-68.

22 Samuel, M.A., Diamond, M.S., 2006. Pathogenesis of West Nile Virus infection: a balance between
23 virulence, innate and adaptive immunity, and viral evasion. J. Virol. 80, 9349-9360.

24 Santhakumar, D., Rubbenstroth, D., Martinez-Sobrido, L., Munir, M., 2017. Avian Interferons and
25 Their Antiviral Effectors. Front. Immunol. 8, 49.

26 Sarkar, S.N., Pandey, M., Sen, G.C., 2005. Assays for the interferon-induced enzyme 2'-5'

oligoadenylate synthetases. Methods Mol. Med. 116, 81-101.

Scherbik, S.V., Kluetzman, K., Perelygin, A.A., Brinton, M.A., 2007. Knock-in of the *Oas1b^(r)* allele into a *Flavivirus*-induced disease susceptible mouse generates the resistant phenotype. Virology 368, 232-237.

Shirafuji, H., Kanehira, K., Kubo, M., Shibahara, T., Kamio, T., 2009. Experimental West Nile virus infection in aigamo ducks, a cross between wild ducks (*Anas platyrhynchos*) and domestic ducks (*Anas platyrhynchos* var. *domesticus*). Avian Dis. 53, 239-244.

Silverman, R.H., 2007. Viral encounters with 2'-5' oligoadenylate synthetase and RNase L during the interferon antiviral response. J. Virol. 81, 12720-12729.

Sokawa, J., Shimizu, N., Sokawa, Y., 1984. Presence of (2'-5') Oligoadenylate Synthetase in Avian Erythrocytes. J. Biochem, 96, 215-222.

Song, C., Yu, S., Duan, Y., Hu, Y., Qiu, X., Tan, L., Sun, Y., Wang, M., Cheng, A., Ding, C., 2014. Effect of age on the pathogenesis of DHV-1 in Pekin ducks and on the innate immune responses of ducks to infection. Arch. Virol. 159, 905-914.

Swayne, D.E., Beck, J.R., Smith, C.S., Shieh, W.J., Zaki, S.R., 2001. Fatal encephalitis and myocarditis in young domestic geese (*Anser anser domesticus*) caused by West Nile virus. Emerg. Infect. Dis. 7, 751-753.

Tag-EL-Din-Hassan, H.T., Sasaki, N., Moritoh, K., Torigoe, D., Maeda, A., Agui, T., 2012. The chicken 2'-5' oligoadenylate synthetase A inhibits the replication of West Nile virus. Jpn. J. Vet. Res. 60, 95-103.

Tag-EL-Din-Hassan, H.T., Sasaki, N., Torigoe, D., Morimatsu, M., Agui, T., 2017. Analysis of the Relationship Between Enzymatic and Antiviral Activities of the Chicken Oligoadenylate Synthetase-Like. J. Interferon Cytokine Res. 37, 71-80.

Tatsumi, R., Hamada, K., Sekiya, S., Wakamatsu, M., Namikawa, T., Mizutani, M., Sokawa, Y., 2002. 2'-5'-oligoadenylate synthetase gene in chicken: gene structure, distribution of alleles and their expression. Biochim. Biophys. Acta 1494, 263-268.

1 Tatsumi, R., Sekiya, S., Nakanishi, R., Mizutani, M., Kojima, S., Sokawa, Y., 2003. Function of
2 ubiquitin-like domain of chicken 2'-5' oligoadenylate synthetase in conformational stability. *J.*
3 *Interferon Cytokine Res.* 23, 667-676.

4 Thakur, C.S., Xu, Z., Wang, Z., Novince, Z. and Silverman, R.H., 2005. A convenient and sensitive
5 fluorescence resonance energy transfer assay for RNase L and 2'-5'-oligoadenylates. *Methods*
6 *Mol. Med.* 116, 103-113.

7 Thomas, L.A., Eklund, C.M., 1960. Overwintering of Western equine encephalitis virus in
8 experimentally infected garter snakes and transmission to mosquitoes. *Proc. Soc. Exp. Biol.*
9 *Med.* 105, 52-55.

10 Tkachuk, Z.Yu., 2013. 2'-5'-Oligoadenylates as a «tool» of innate immunity. *Biopolym. Cell* 29, 266–
11 276.

12 Van der Meulen, K.M., Pensaert, M.B., Nauwynck, H.J., 2005. West Nile virus in the vertebrate world.
13 *Arch. Virol.* 150, 637-57.

14 Wang, A., Liu, F., Chen, S., Wang, M., Jia, R., Zhu, D., Liu, M., Sun, K., Wu, Y., Chen, X., Cheng A.,
15 2015. Transcriptome Analysis and Identification of Differentially Expressed Transcripts of
16 Immune-Related Genes in Spleen of Gosling and Adult Goose. *Int. J. Mol. Sci.* 16, 22904-
17 22926.

18 Yamamoto, A., Iwata, A., Koh, Y., Kawai, S., Murayama, S., Hamada, K., Maekawa, S., Ueda, S.,
19 Sokawa, Y., 1998. Two types of chicken 2'-5' oligoadenylate synthetase mRNA derived from
20 alleles at a single locus. *Biochim. Biophys. Acta* 1395, 181-191.

21 Yang, C., Liu, F., Chen, S., Wang, M., Jia, R., Zhu, D., Liu, M., Sun, K., Yang, Q., Wu, Y., Chen, X.,
22 Cheng, A., 2016. Identification of 2'-5'-Oligoadenylate Synthetase-Like Gene in Goose: Gene
23 Structure, Expression Patterns, and Antiviral Activity Against Newcastle Disease Virus. *J.*
24 *Interferon Cytokine Res.* 36, 563-572.

25 Zhu, J., Zhang, Y., Ghosh, A., Cuevas, R.A., Forero, A., Dhar, J., Ibsen, M.S., Schmid-Burgk, J.L.,
26 Schmidt, T., Ganapathiraju, M.K., Fujita, T., Hartmann, R., Barik, S., Hornung, V., Coyne, C.B.,

1 Sarkar, S.N., 2014. Antiviral activity of human OASL protein is mediated by enhancing
2 signaling of the RIG-I RNA sensor. *Immunity* 40, 936-948.

3

Figure legends

Fig. 1. Thirty-five OAS proteins were subjected to the neighborhood tree analysis using the MEGA 7 software. Blue rectangles, chOASL; green rectangles, duOASL; purple rectangles, goOASL; orange rectangles, osOASL; red rectangles, osOAS1. A, D, C, D, E, and F indicate the common ancestor for specific descendants. Numbers shown on branches represent the percentage of replicate trees, in which the associated taxa clustered together in the bootstrap test. Numbers below branches represent the evolutionary distances. The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Poisson correction method.

Fig. 2. Enzymatic activity of duOASL, goOASL, oSOASL, osOAS1, and chOASL-A. A) The RT-PCR and western blot of RNA and protein samples extracted at 48 h post transfection from 293FT cells transfected with pIRES-EGFP, pduOASL-FL-EGFP, pgoOASL-FL-EGFP, posOASL-FL-EGFP, posOAS1-FL-EGFP, and pchOASL-A-FL-EGFP. B) The measurement of the enzymatic activity of duOASL, goOASL, osOASL, osOAS1, and chOASL-A proteins.

Fig. 3. RNase L activation assay of duOASL, goOASL, osOASL, osOAS1, and chOASL-A proteins using FRET assay. The samples were incubated in cleavage buffer with 180 ng of FRET probe for periods of 0, 5, 10, 15, 30, 45, 60, 90, and 120 min at 21 °C. The background fluorescence activity was subtracted from all reads before statistical analysis. ** and * indicate statistical difference from other samples in the same incubation period with $P < 0.01$ and $P < 0.05$, respectively.

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.

2
3 **Fig. 5.** Antiviral activity assay of duOASL, goOASL, osOASL, osOAS1, and chOASL-A. A) RT-
4 PCR of RNA extracted from the mock-transfected BHK-21 cells, pIRES-EGFP as well as all
5 transfectants before and 72 h post WNV-replicon RNA electroporation. B) Amplification of WNV-
6 replicon replication as determined by the SEAP expression level at 24 and 72 h post WNV-replicon
7 RNA electroporation in all transfectants (mean $\pm$ SD).

8
9 **Fig. 6.** Cellular localization of duOASL, goOASL, osOASL, osOAS1, and chOASL-A proteins. The
10 immunofluorescence analysis was performed at 48 h after BHK-21 cells transfected with pIRES-
11 EGFP as well as all transfectants using mouse α -FLAG M2 antibody and Alexa Fluor 568-conjugated
12 goat anti-mouse IgG to detect the protein expression. DAPI was used for the nucleus counterstaining.
13 The white bars indicate the length of 100 μ m.

14
15 **Fig. 7.** Alignment for protein sequences and conserved domains. A) The important conserved
16 domains are as follows; the LxxxP (yellow box), P-loop (brown box), D-D box (red box) and K-R
17 rich region (blue box) as well as high homology in the UBL1 (green arrow) and UBL2 (red arrow)
18 units in OASL proteins. B) Schematic diagram of the OAS protein. The amino acids important for
19 the enzymatic activity in the LxxxP domain are shown as the yellow, green, and light blue highlights.
20 Amino acids with the purple highlight show the amino acid substitution, P8T in the LxxxP domain.
21 The grey highlight shows the conserved sequence in P-Loop. The red highlight shows the conserved
22 amino acids in D-D box. The pink highlight shows the conserved amino acid in K-R rich region.

Fig. 1

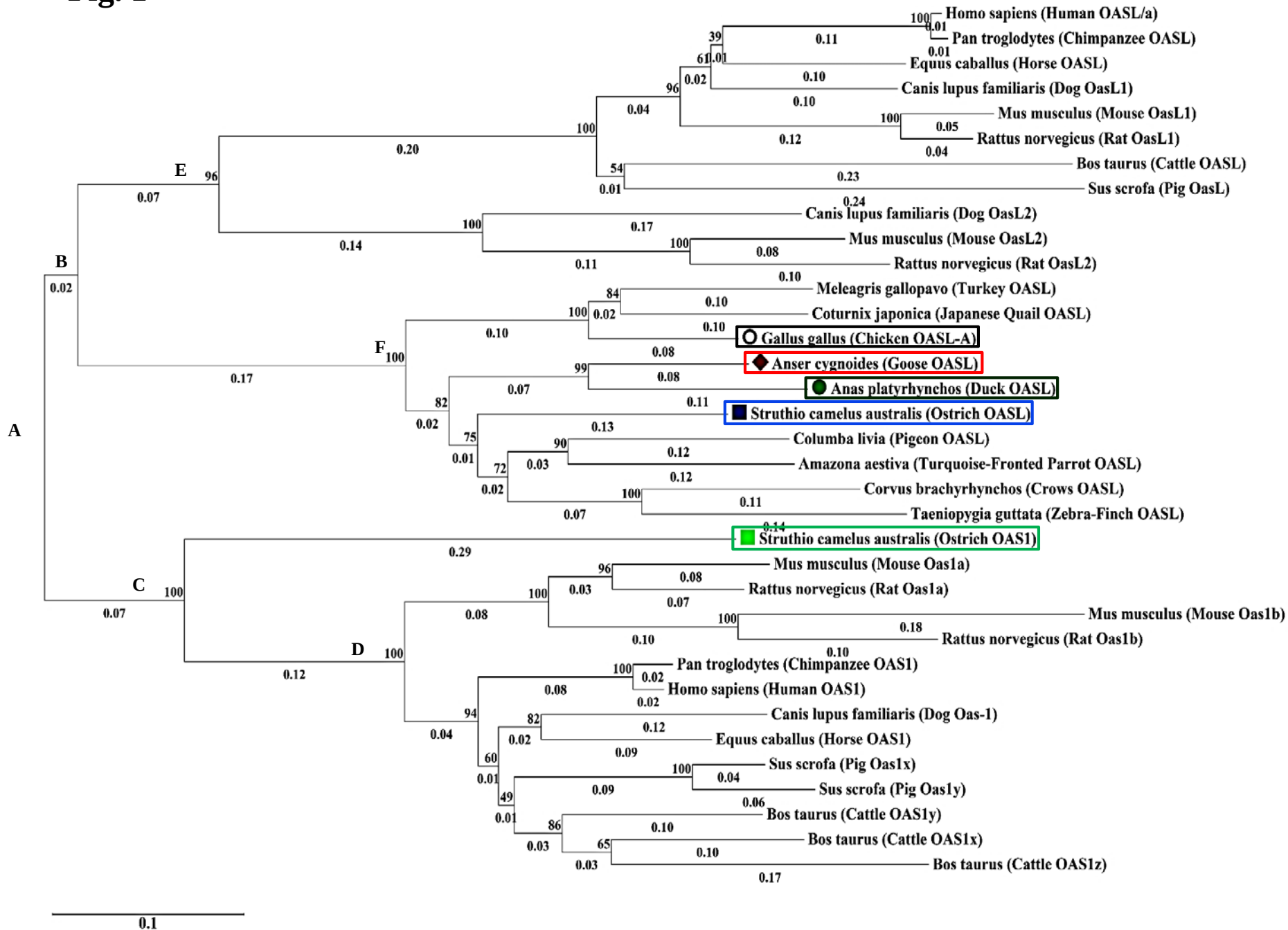


Fig. 2

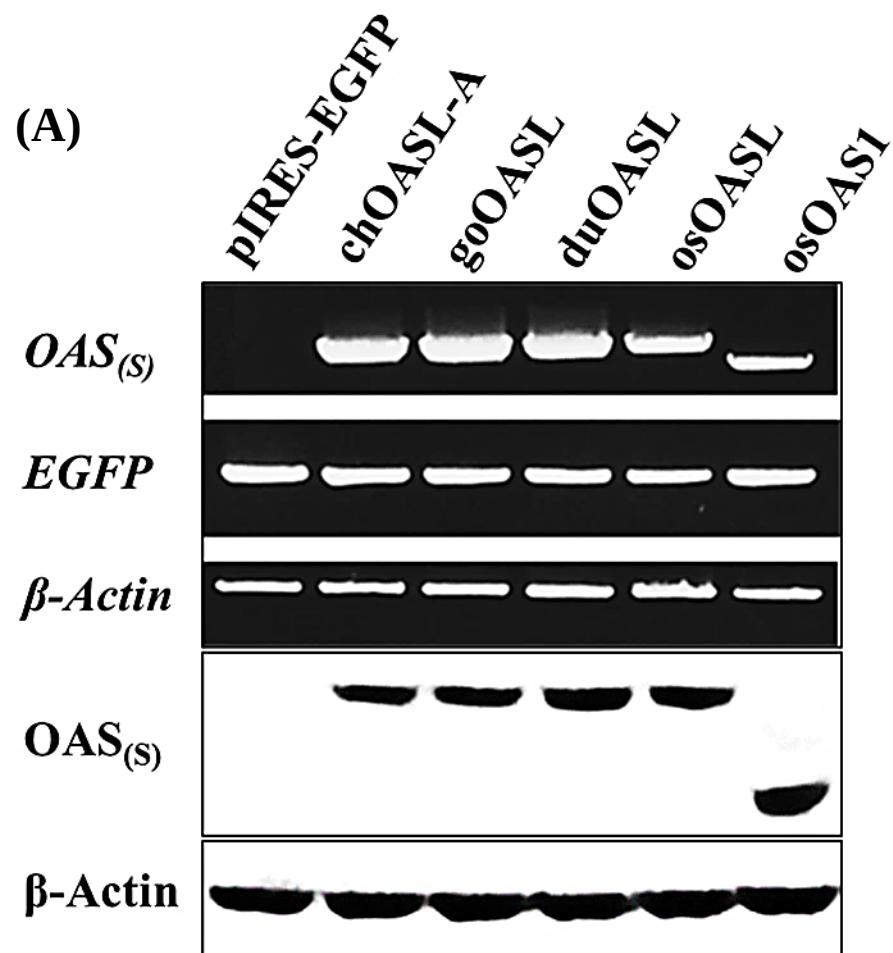


Fig. 2

(B)

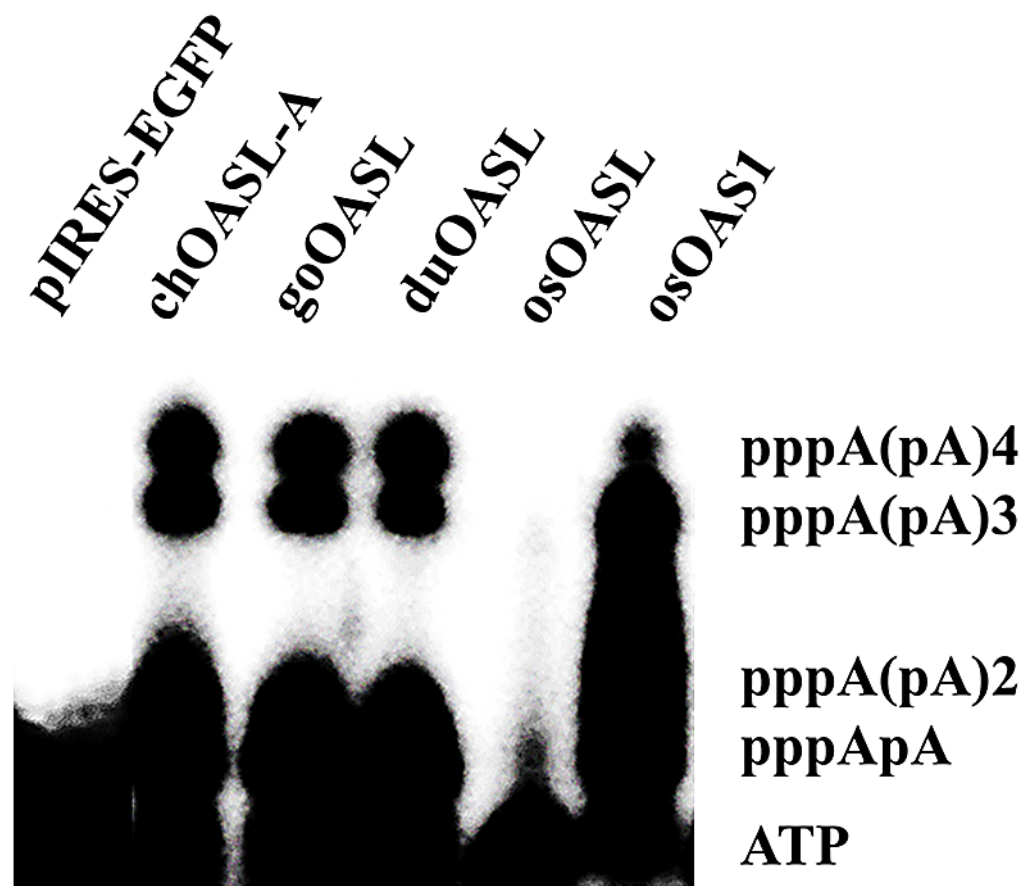


Fig. 3

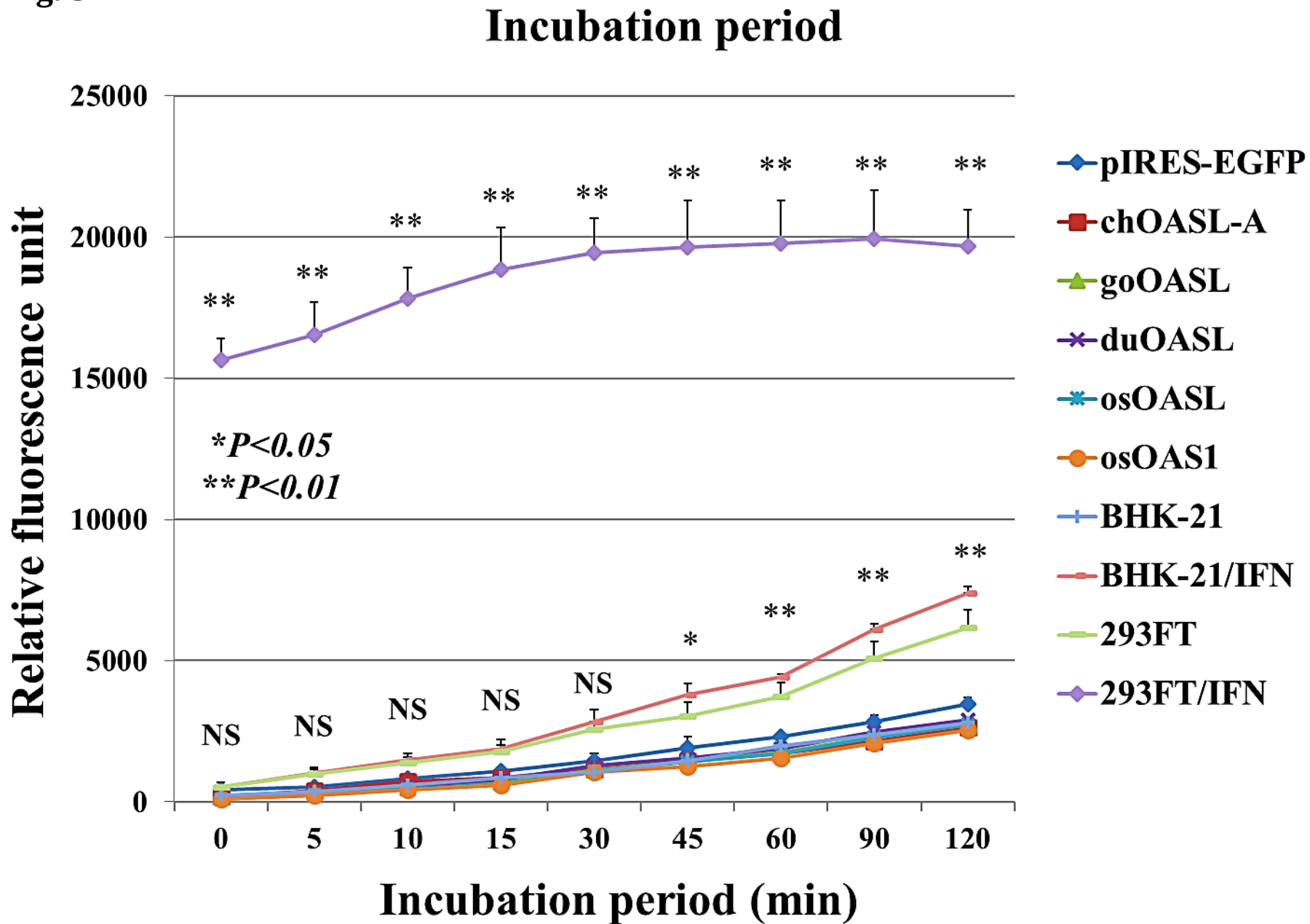


Fig. 4

(A)

Relative fluorescence unit

duOASL

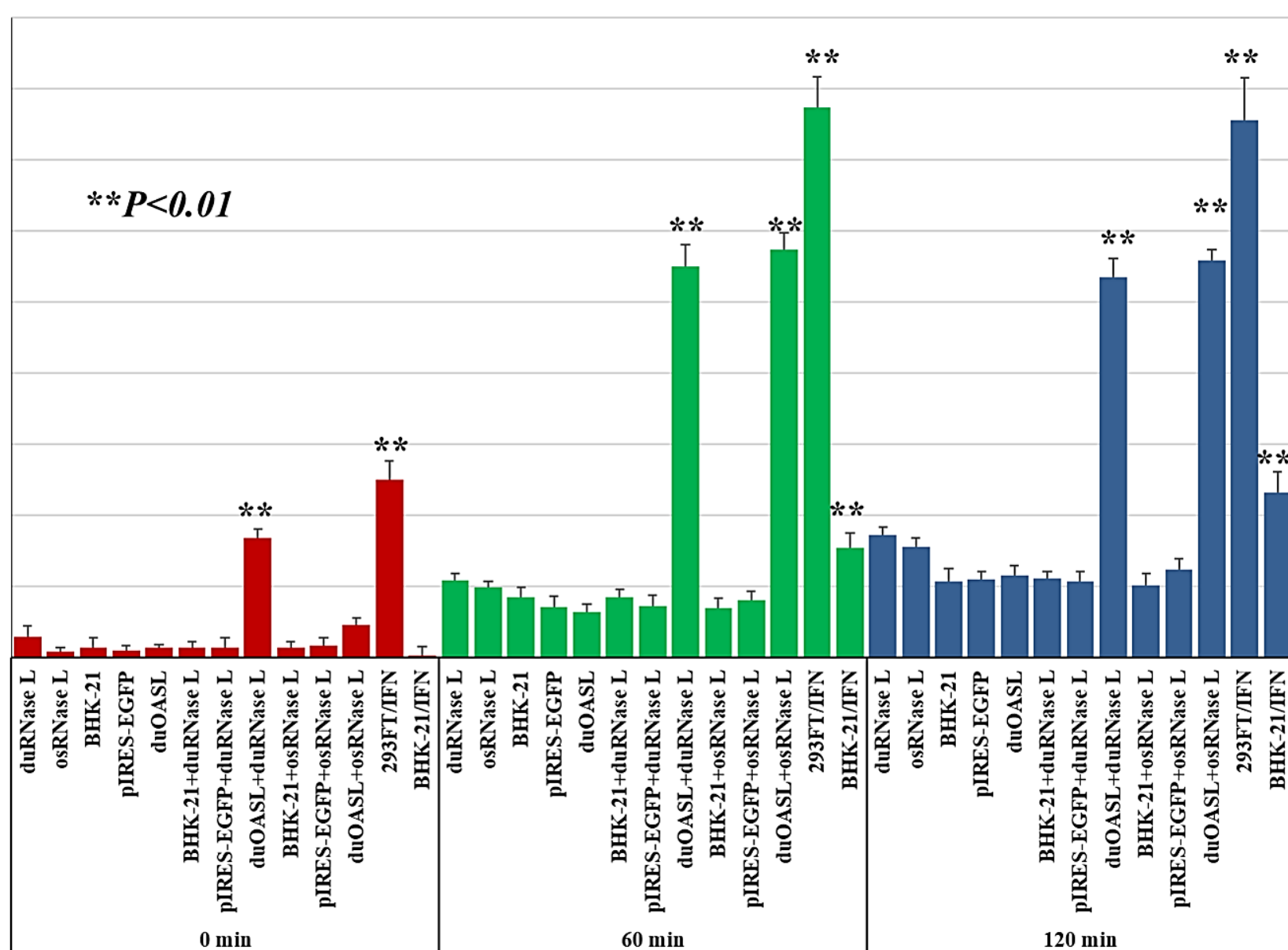


Fig. 4
(B)

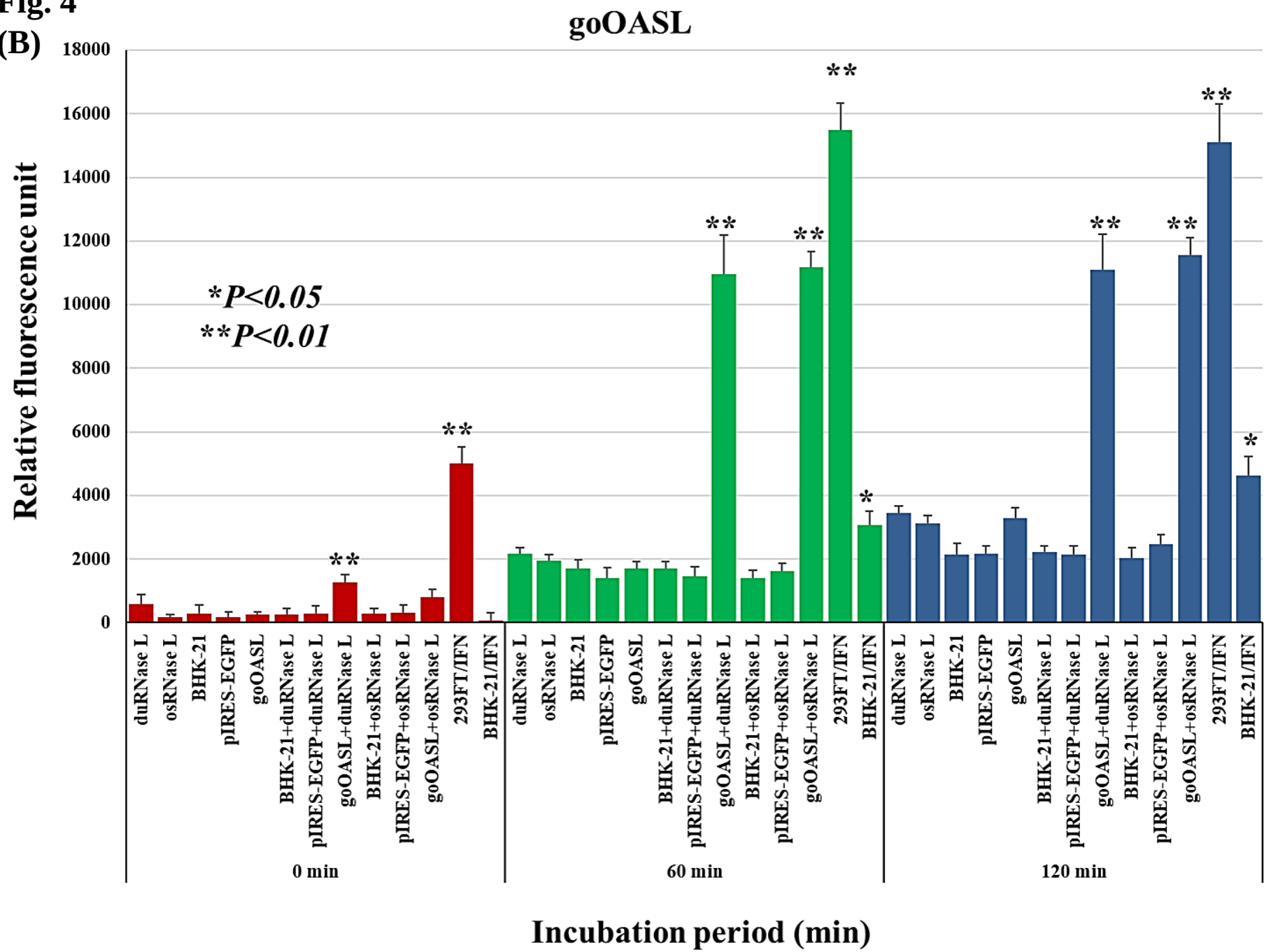
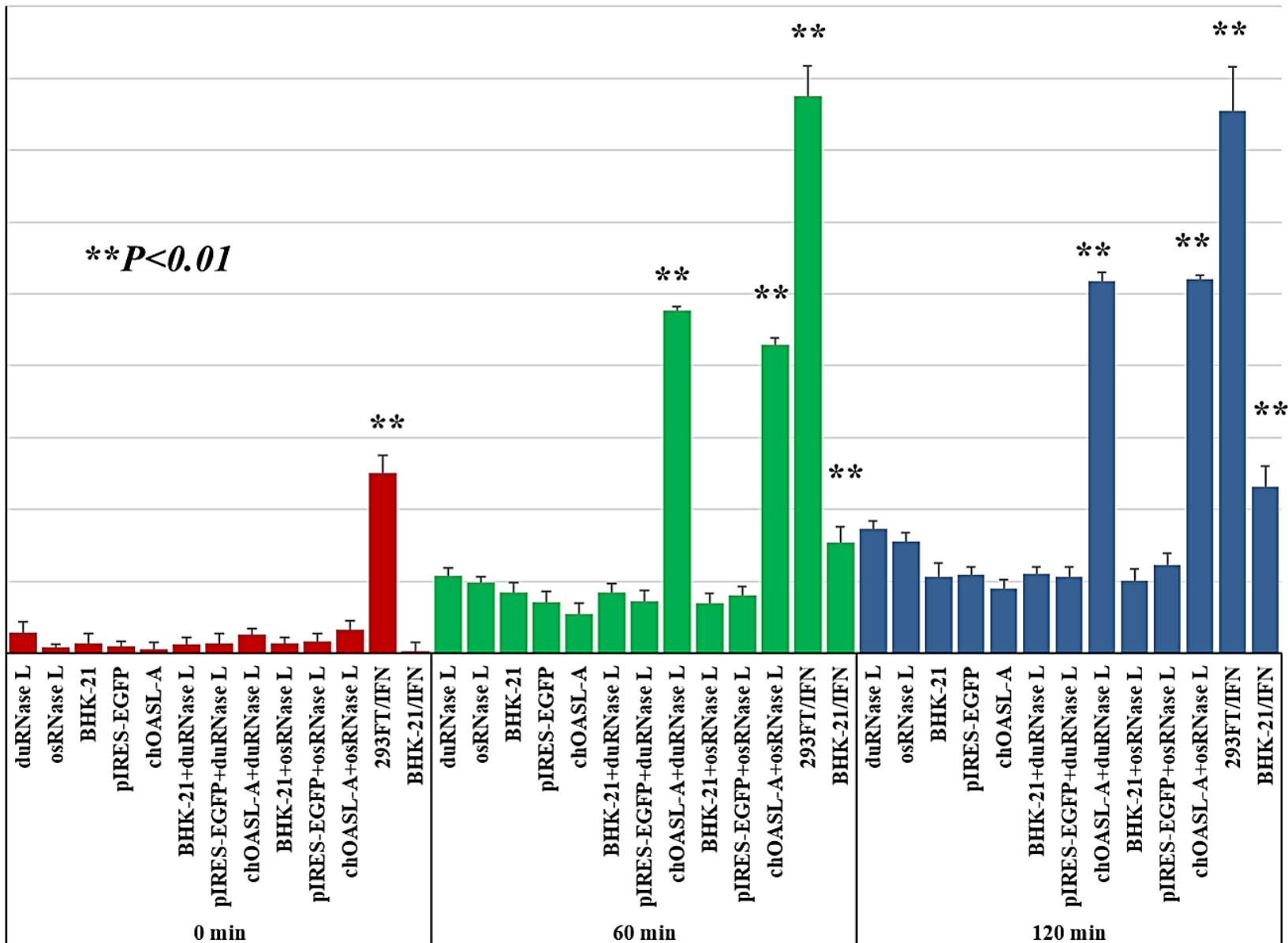


Fig. 4
(C)

chOASL-A

Relative fluorescence unit



Incubation period (min)

Fig. 4

(D)

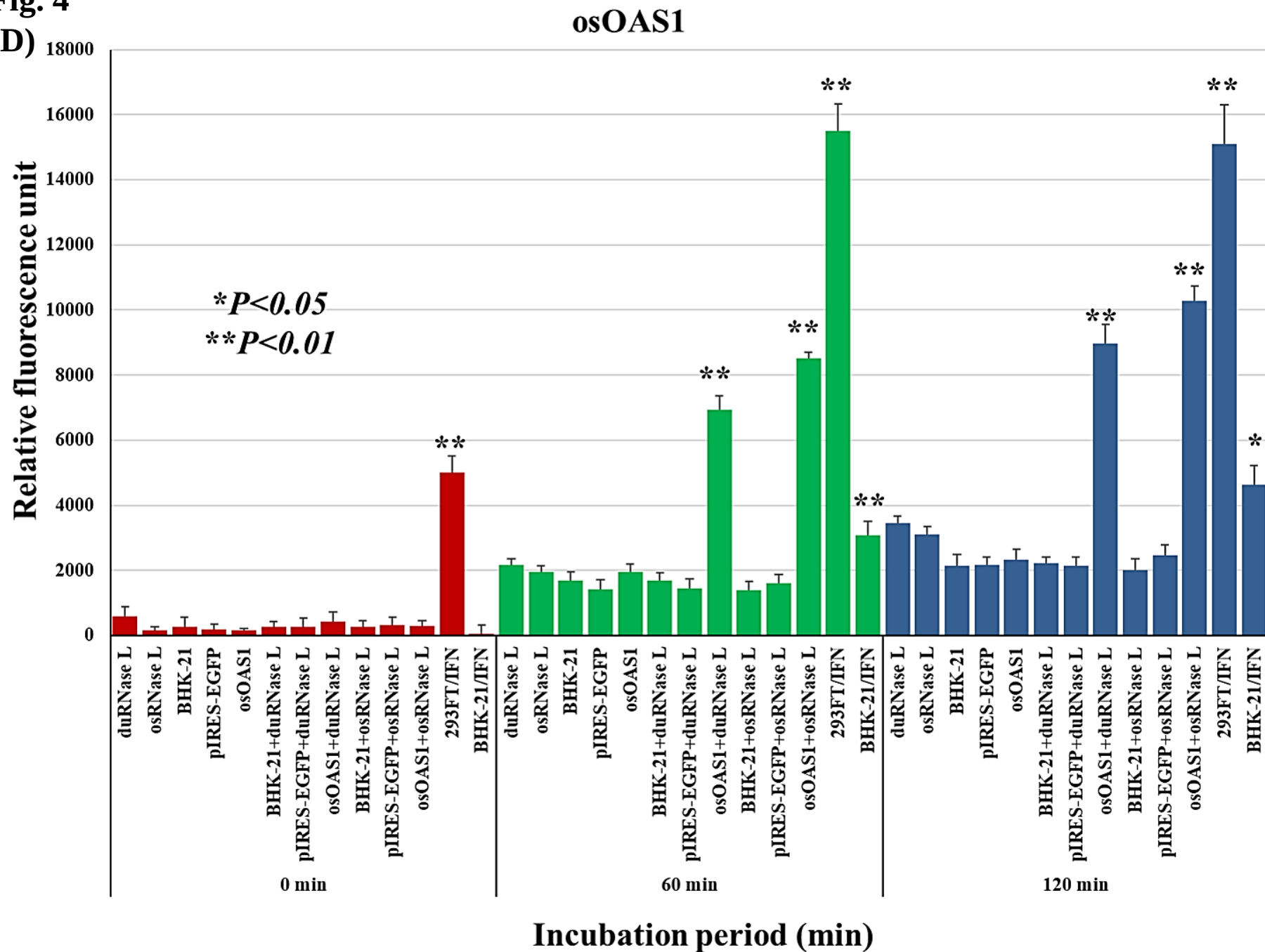
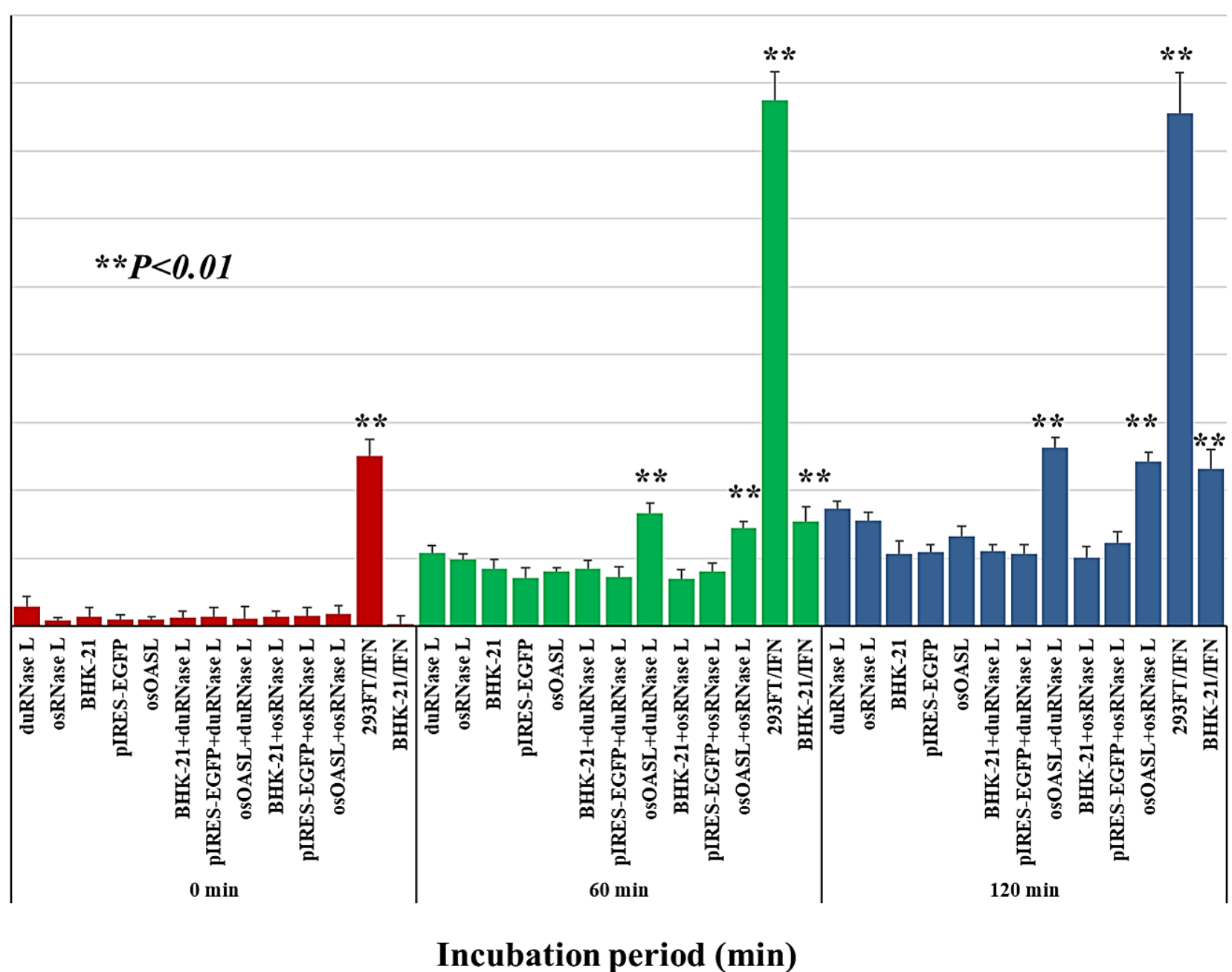


Fig. 4

(E) **osOASL**

Relative fluorescence unit



Amplification of rep WNV/SEAP replicon

Fig. 5

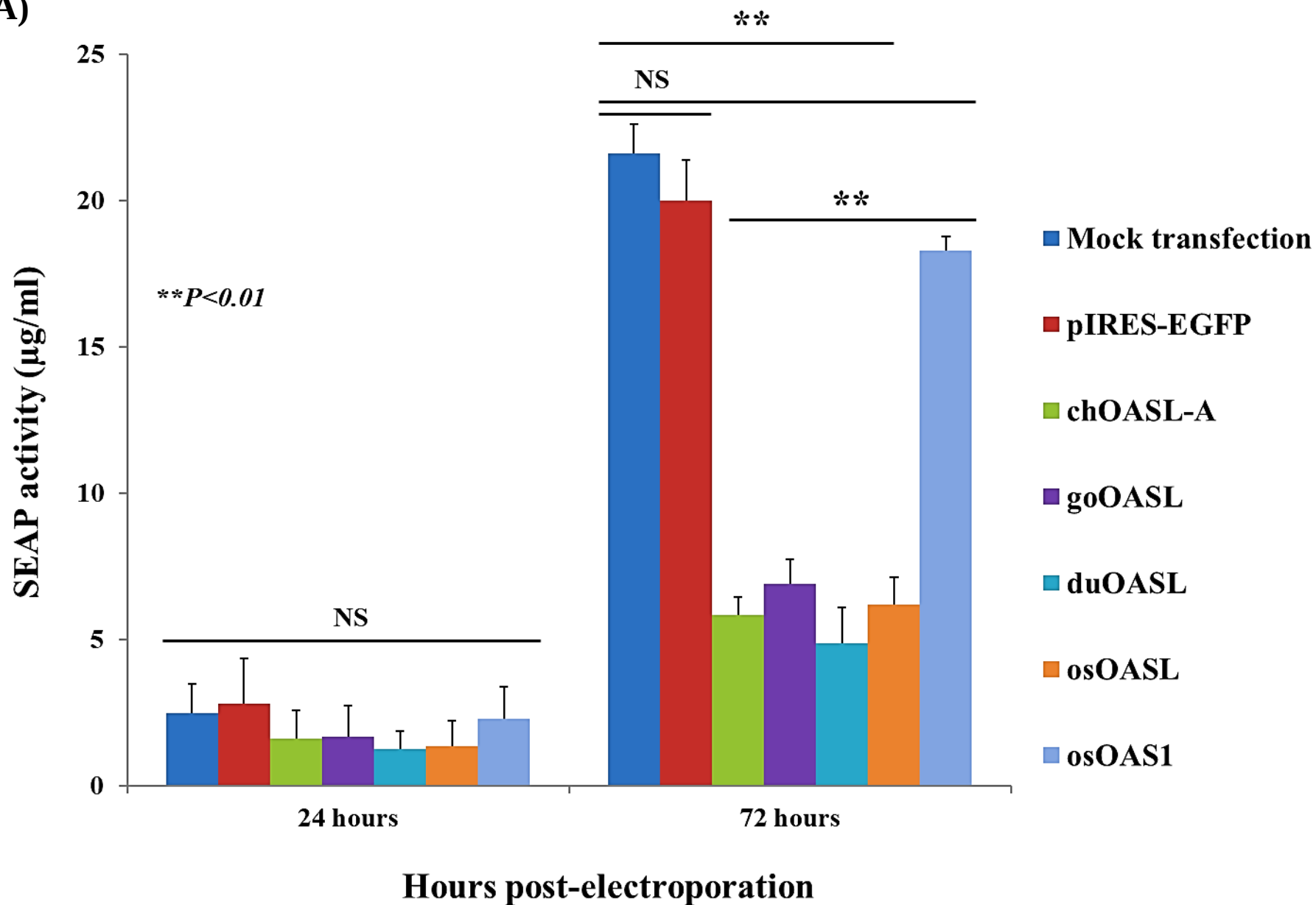


Fig. 5

(B)

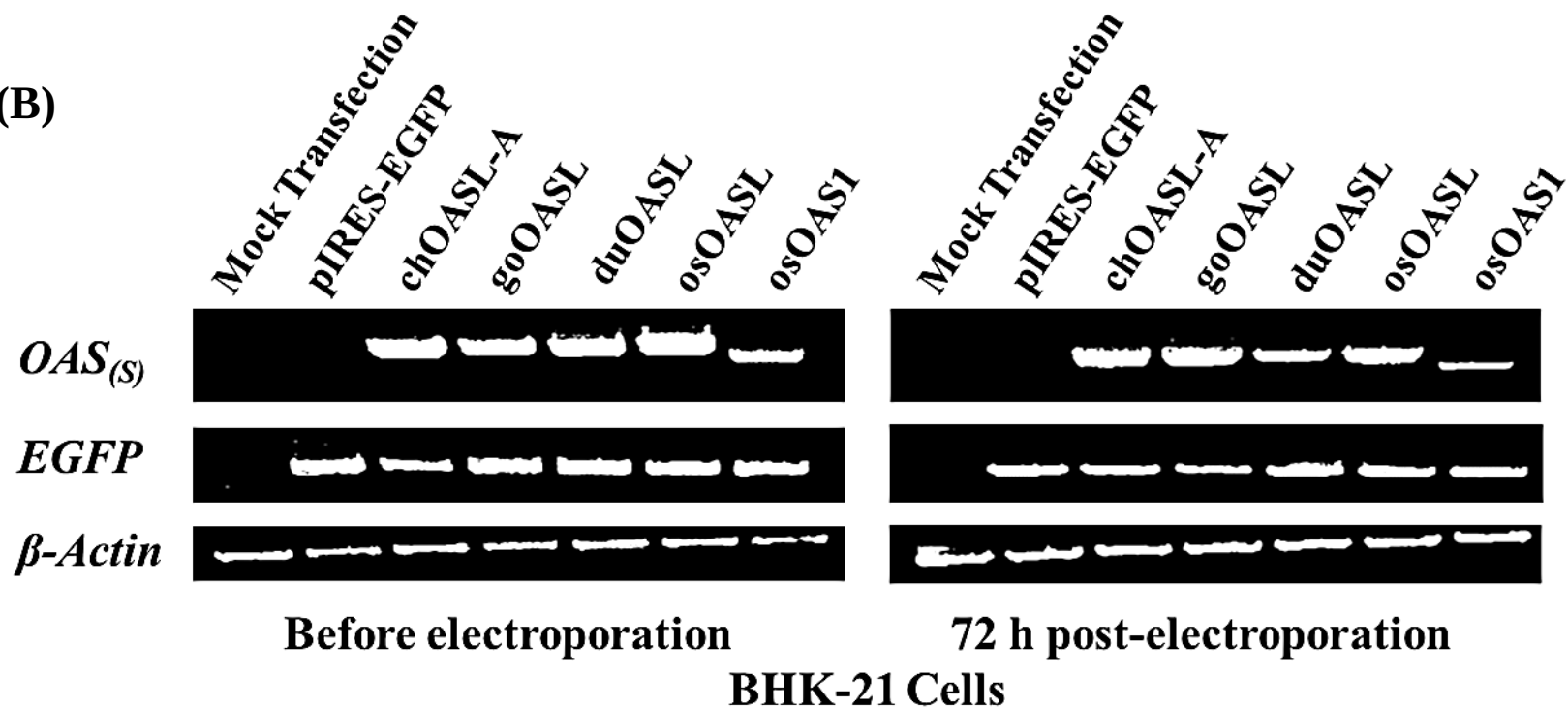
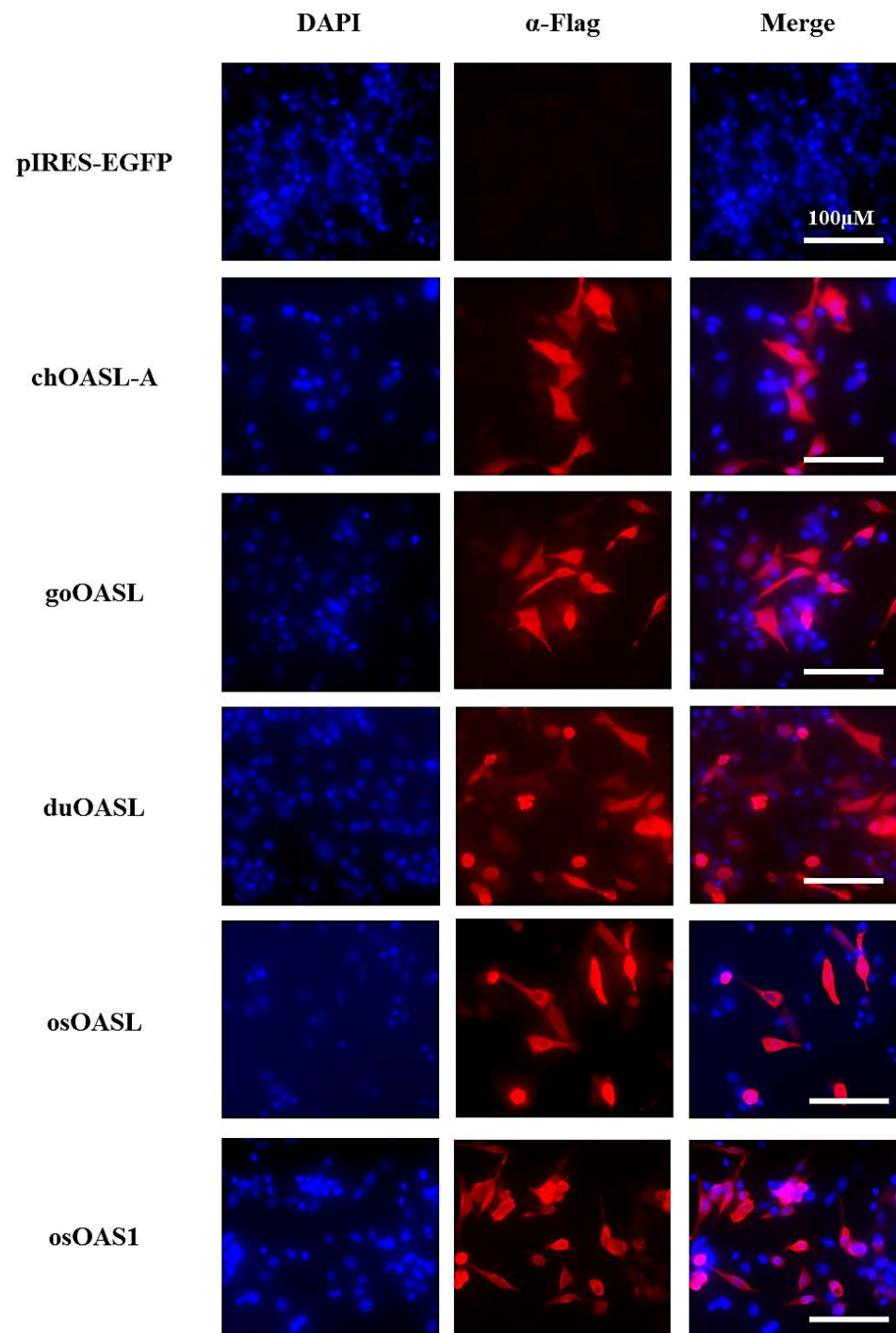


Fig. 6



(A)

LxxxP

go0ASL	1	-----	MELRDVP	AKQLDGVVAATLPQSTAF	LVAVK	ITV	QICDGLFKERCFEDGSFE
du0ASL	1	-----	MELWNVS	ISELDGVVAATLPQSTDF	AAVKKTV	QICDGLFKESCFED	----
ch0ASL - A	1	-----	MGLESVS	SRQLGFWAAHLPQSTF	TAVKQTV	QICDGLFKERCFEG	----
os0ASL	1	-----	MDGLETV	ARDLDWARTLEPSTAF	RIQVQKTV	QICDGLFKERCFEG	----
os0AS1	1	-----	MLSQLLSCL	MELYNTPEQLDKFIYEVLQPDRTF	LEQLCAVHTICEFLRNC	CFAG	-APP

P-Loop **D-D Box**

go0ASL	52	D	IRVFKTV	KGGSAGKGTALRNNS	DADV	VFLFSY	SYLDQRQEHPKTRL	LEKRL	EC
du0ASL	48	-	KTRVFKTV	KGGSAGKGTALRNNS	DADV	VFLFS	CSNYRQOREEHTPIQL	TEKRL	EC
ch0ASL - A	48	-	ISVLKTV	KGGSAGKGTALRNNS	DADV	VFLF	NCSSSYQ	QEARHILATIER	RLNEC
os0ASL	49	-	IRVHKTV	KGGSAGKGTALQNNNS	DADV	VFLFS	CSFYQDQTRR	EILALTEERLE	CCR
os0AS1	60	P	TRVRLKVV	KGGSAGKGTALKSSD	DADV	VFLFS	CFEDYKQDEKNRA	EITREIQKRL	VEQ

K-Rich Region

go0ASL	112	Q	SLSFTV	RISPPRYKGR	-----	SLS	L	LFS	N	T	E	S	I	D	V	D	L	P	T	D	A	L	G	O	V	T	E	D	P	P	D	Q		
du0ASL	107	Q	RLSFTV	KISPPRYKGR	-----	SLS	L	L	F	S	D	G	E	S	I	D	V	D	L	P	T	D	A	L	G	O	V	T	E	D	P	P	D	Q
ch0ASL - A	106	P	TLSFGV	SIRPVYKDSRDRPRS	SLS	L	L	S	S	A	R	G	S	I	D	V	D	L	P	A	T	A	L	G	O	V	T	E	D	P	P	D	A	
os0ASL	107	Q	KLFTFV	NISRPRLKSGGSPRS	SLS	L	L	S	S	A	R	G	S	I	D	V	D	L	P	A	T	A	L	G	O	V	T	E	D	P	P	D	A	
os0AS1	120	Q	QKHFEV	FEFVSRLSNP	----	R	V	S	F	S	L	K	S	K	E	S	I	S	F	D	V	P	A	L	H	H	V	S	G	Y	K	N	T	

K-Rich Region

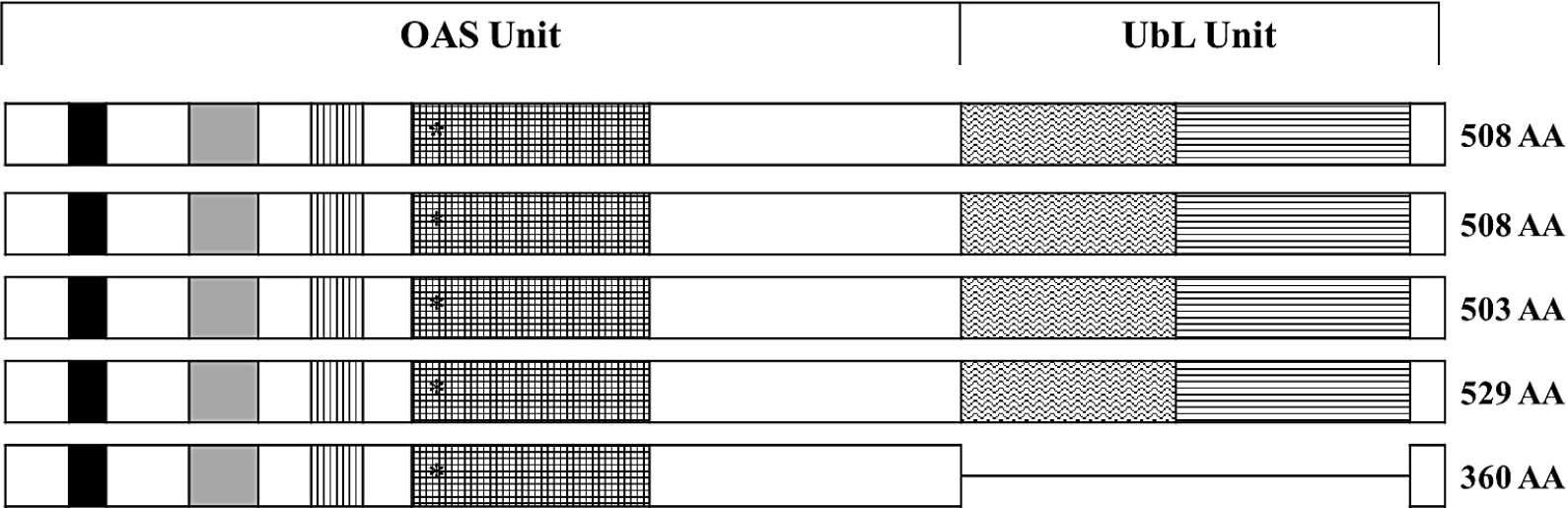
go0ASL	165	V	YV	E	L	L	K	A	H	S	S	P	G	E	F	S	T	C	F	T	E	L	Q	K	K	F	V	K	R	C	P	A	K	L	K	N	L	R	L	V	K	H	W	Y	K	Q	L	K	P	O	Y	P	A	E	----		
du0ASL	160	V	YV	D	L	L	D	V	N	S	N	P	G	E	F	S	T	C	F	T	E	L	Q	K	K	F	V	K	R	C	P	A	K	L	K	N	L	R	L	V	K	H	W	Y	K	Q	L	K	P	O	Y	P	A	E	----		
ch0ASL - A	166	V	YV	K	L	L	A	T	R	C	G	P	F	S	P	F	T	E	L	Q	K	N	F	V	K	R	C	P	A	K	L	K	N	L	R	L	V	K	H	W	Y	K	E	L	K	P	Y	P	N	A	K	----					
os0ASL	167	V	YV	R	L	L	A	A	P	S	G	P	G	E	F	S	T	C	F	T	E	L	Q	K	K	F	V	K	R	C	P	A	K	L	K	N	L	R	L	V	K	H	W	Y	K	E	L	K	P	Y	P	D	Q	----			
os0AS1	176	V	Y	I	Q	L	F	Q	Q	C	S	R	G	E	F	S	T	C	F	T	E	L	Q	R	D	F	I	S	R	P	T	K	V	K	S	L	R	L	V	K	H	W	Y	K	T	Y	C	P	I	K	W	A	L	R	G	E	----

K-Rich Region

go0ASL	222	L	P	P	K	A	E	L	L	T	I	Y	A	W	E	Q	G	T	S	E	F	S	A	E	G	F	C	T	V	L	K	L	C	O	Y	R	I	C	I	Y	E	K	Y	S	L	O	H	E	Q	----
du0ASL	217	L	P	P	K	A	E	L	L	T	I	Y	A	W	E	Q	G	T	S	E	F	S	A	E	G	F	C	T	V	L	K	L	C	O	Y	R	I	C	I	Y	E	K	Y	S	L	O	H	R	Q	----
ch0ASL - A	223	L	P	P	K	A	E	L	L	T	I	Y	A	W	E	Q	G	T	S	E	F	S	A	E	G																									

Fig. 7

(B)



	LxxxP	P-LooP	D-D Box	K-R Rich Region	UBL1	UBL2
chOASL-A	LESVS	KGGSAGKG	DAD	K	79 AA	92 AA
goOASL	LRDVP	KGGSAGKG	DAD	K	78 AA	93 AA
duOASL	LWNVS	KGGSAGKG	DAD	K	78 AA	93 AA
osOASL	LETVT	KGGSAGKG	DAD	K	79 AA	112 AA
osOAS1	LYNTP	KGGSAGKG	DAD	I		