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

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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
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 **1. Introduction**

2

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

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

24 Pérez-Ramírez et al. (2014) indicated that the avian species could be categorized into three
25 classes; highly competent birds (susceptible) such as *Passeriformes* (*Corvidae* and *Passeridae*) and
26 *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.

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1 **2. Materials and methods**

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3 *2.1. Blood samples*

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5 Blood samples were collected from adult mallard ducks and white Chinese geese (reared in
6 Sapporo Maruyama Zoo, Sapporo, Hokkaido, Japan) in heparinized blood collection tubes to prevent
7 blood coagulation. Blood samples were centrifuged at 400 Xg for 15 min at room temperature (RT),
8 pellets were suspended in 10 ml of phosphate-buffered saline (PBS), and then, carefully layered on
9 3.0 ml of HISTOPAQUE-1077 (Sigma-Aldrich, St. Louis, MO, USA) followed by centrifugation at
10 400 Xg for 30 min at RT. The opaque interface layer containing leukocytes was collected and
11 transferred into a new tube, washed twice with 10 ml of PBS, and then centrifuged at 250 Xg for 10
12 min at RT. Thereafter, the leukocytes were suspended and cultured in 10 ml of RPMI 1640 medium
13 (Gibco/Invitrogen, Carlsbad, CA, USA) containing 1% penicillin/streptomycin/glutamine
14 (Gibco/Invitrogen), 10% fetal bovine serum (FBS) (Atlas Biologicals, Inc. Fort Collins, CO, USA),
15 1µg/ml phytohaemagglutinin P (Sigma-Aldrich), and 10 µg/ml pokeweed mitogen (Sigma-Aldrich)
16 in a 5% CO₂ incubator at 37 °C for 48 h.

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18 *2.2. Cell culture*

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20 BHK-21, 293FT, and primary culture cells of ostrich renal epithelial cells (kindly gifted from
21 Prof. Mayumi Ishizuka, Laboratory of Toxicology, Faculty of Veterinary Medicine, Hokkaido
22 University, Japan) were cultured in DMEM (Gibco/Invitrogen) supplemented with 10% FBS (Atlas
23 Biologicals) and 1% penicillin/streptomycin/glutamine (Gibco/Invitrogen). Cells were cultured in a
24 5% CO₂ incubator at 37 °C and passaged every two days.

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26 *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 *osOASI*

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 *osOASI*.

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

1 95 °C for 5 min, followed by 35 cycles of denature at 95 °C for 30 sec, annealing at 60 °C for 30 sec,
2 and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 2 min. The amplified
3 fragments were extracted, ligated into pGEM-T-Easy vector, and then transfected into DH5α *E. coli*
4 competent cells. Plasmids were then extracted and sequenced.

5 The open reading frames (ORF) of *duOASL*, *goOASL*, *osOASL*, and *osOASI* were amplified
6 using specific primers (Table 1) to conjugate the FLAG-TAG sequence at the 3'-end as well as the
7 NotI restriction site at both 5'- and 3'-ends using the KOD FX NEO polymerase (Toyobo Co., Ltd.)
8 according to the manufacturer's protocol. To amplify *duOASL* and *goOASL*, pGEM-T-Easy plasmids
9 described above were used as template. To amplify *osOASL* and *osOASI*, cDNA prepared from total
10 RNA of ostrich renal cells was used as template. A *chOASL-A* clone conjugated with FLAG-TAG and
11 NotI restriction site was previously prepared (Tag-EL-Din-Hassan et al., 2012 and 2017). The
12 amplified fragments were extracted and digested with NotI restriction enzyme (Toyobo Co., Ltd.),
13 and then purified and cloned into the NotI restriction site of the pIRES-EGFP (the mammalian
14 expression vector, Clontech Bio. Inc., Shiga, Japan). Similarly, the ORFs of *duRNASEL* and
15 *osRNASEL* were amplified using specific primers (Table 1), extracted and cloned to pGEM-T-Easy
16 vector, digested with NotI restriction enzyme, and then cloned into pIRES-EGFP. In this study, all
17 DNA fragment extraction from agarose gel was performed using FastGene Gel/PCR Extraction Kit
18 (Nippon Genetics Co., Ltd, Tokyo, Japan). All cloning reactions were performed using 2X Ligation-
19 Convenience Kit (Wako Nippon Gene Co., Ltd., Toyama, Japan) according to the manufacturer's
20 protocol. All transfections were performed using DH5α *E. coli* competent cells. All plasmid DNA
21 extractions were performed using FastGene Plasmid Mini Kit (Wako Nippon Gene Co., Ltd.). The
22 large-scaled plasmid purification was performed using CsCL₂-ethidium bromide gradient purification
23 method as described previously (Sambrook and Russell, 2001).

24

25 2.6. Alignment analysis and neighboring tree

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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 Oas11, NP_660210 and mouse Oas12, NP_035984), *Rattus*
22 *norvegicus* (rat Oas11, NP_001009681 and rat Oas12, 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),

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

3

4 2.7. Preparation of cell lysate

5

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

18

19 2.8. Western blotting

20

21 Western blotting was performed as described previously (Tag-EL-Din-Hassan et al., 2012 and
22 2017). Briefly, 12 μ g proteins of each sample were separated in 10% SDS-polyacrylamide gel
23 electrophoresis on 100 V for 1 h and blotted onto PVDF membrane. Blotted membrane was blocked
24 with 3% non-fatty milk in PBS containing 0.1% Tween-20 (PBS-Tween-20) for 1 h at RT. Then, the
25 membrane was incubated with diluted (1/2,000) mouse anti-FLAG M2 antibody (Sigma-Aldrich) in
26 PBS-Tween-20 at RT for 2 h, washed 3 times with PBS-Tween-20 for 10 min, incubated with diluted

1 (1/5,000) horse radish peroxidase-conjugated goat anti-mouse antibody in PBS-Tween-20 at RT for
2 1 h, and washed 3 times with PBS-Tween-20 for 10 min each. The immune-reactive bands were
3 detected by ECL™ Western Blotting Detection Reagents (GE Healthcare UK Limited, Little Chalfont,
4 UK) according to manufacturer's protocol.

5

6 *2.9. Assay of enzymatic activity*

7 The enzymatic activity was measured as described previously (Sarkar et al., 2005) with a slight
8 modification. An aliquot of 2.5 µl of whole cell lysates of 293FT cells expressing duOASL, goOASL,
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 µg/ml poly(I):(C), and 5
11 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
12 stopped by boiling at 95 °C for 5 min, followed by adding loading buffer containing 25% formamide,
13 0.5% bromophenol blue, and 0.5% xylene cyanol. The synthesized 2'-5'-adenylates (2'-5'A) products
14 were run in a 20% polyacrylamide-urea (7 M) gel. The electrophoresis was performed on 200 V for
15 4 h, and then products were visualized by autoradiography with a BAS2500 Bio-Imaging analyzer
16 (FUJIFILM, Tokyo, Japan).

17

18 *2.10. Inhibitory activity on WNV replicon replication*

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20 WNV genomic RNA harboring the secreted alkaline phosphatase (SEAP) reporter gene instead
21 of viral structural genes, WNV/SEAP-replicon RNA was propagated as described previously
22 (Moritoh, et al., 2011; Tag-EL-Din-Hassan, et al., 2017). BHK-21 cells were cultured in a 100-mm
23 plate at the density of 1.5×10^6 cells/plate for 24 h and then, 10 µg of pIRES-EGFP (empty vector as
24 the control), pduOASL-FL-EGFP, pgoOASL-FL-EGFP, posOASL-FL-EGFP, posOAS1-FL-EGFP,
25 and pchOASL-A-FL-EGFP were transfected using Lipofectamine 2000® (Invitrogen) according to
26 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.

10

11 *2.11. RNase L activation assay*

12

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.).

24

25 *2.12. Localization of the OAS proteins*

26

1 BHK-21 cells were transfected with 0.1 μ g 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 μ l 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).

16

17 2.13. Statistical analysis

18

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

1 3. Results

2

3 3.1. Bioinformatics information

4

5 The sequence results demonstrated that the full length of *duOASL* (KU569292), *goOASL*
6 (KU569293) and *osOASL* (XM_009673088) mRNA were 1,689, 1,649, and 1,640 bp with open
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
9 *duOASL*, *goOASL*, and *osOASL* mRNA exhibited 76%, 76%, and 73% homology to *chOASL-A*
10 mRNA, respectively. Moreover, *duOASL*, *goOASL*, and *osOASL* proteins exhibited 64%, 64%, and
11 63% homology to *chOASL-A* protein, respectively. The sequence alignment between *duOASL* and
12 *goOASL* mRNA showed 88% homology as well as both mRNA showed 75% homology to *osOASL*
13 mRNA. In addition, the protein sequence alignment between *duOASL* and *goOASL* showed 80%
14 homology as well as both proteins showed 64% homology to *osOASL*. Domain structure analysis of
15 *duOASL*, *goOASL*, and *osOASL* showed that these proteins were OAS-like proteins conserving three
16 main domains, nucleotidyltransferase, OAS1_C, and two ubiquitin-like domains, UBL1 and UBL2,
17 whereas the *osOAS1* possessed only the nucleotidyltransferase and OAS1_C domains similar to
18 OAS1 protein family.

19 The phylogenetic tree was constructed by using neighbor-joining analyses based on various
20 species OAS proteins (Fig. 1). Both avian and mammalian OASL families descend from the same
21 ancestor B. All avian OASL families descend from the same ancestor F and show high homology
22 each other than the mammalian OASL families which descend from the ancestor E. The *goOASL* and
23 *duOASL* show high homology each other than the other avian OASL proteins. On the other hand,
24 *chOASL* protein is more closely related to the quail and turkey OASL proteins than *duOASL* and
25 *goOASL*. Moreover, *osOASL* protein shows higher homology and evolutionarily more closely related
26 to pigeon, parrot, crows, and zebra fish 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 [Perehygin et al. \(2006\)](#).

4 5 *3.2. The enzymatic activity*

6
7 OAS enzymatic activity was assessed using 293FT cells transfected with pIRES-EGFP empty
8 vector as a control, pduOASL-EGFP, pgoOASL-EGFP, posOASL-EGFP, posOAS1-EGFP, and
9 pchOASL-A-EGFP. The expression of all genes was confirmed by RT-PCR and Western blotting (Fig.
10 2A). Fig. 2B shows that duOASL, goOASL, osOAS1, and chOASL-A proteins possess the enzymatic
11 activity to convert the ATP to 2'-5' A up to tetramer, whereas only the osOASL protein expresses very
12 weak enzymatic activity.

13 14 *3.3. RNase L activation assay*

15
16 The results of the RNase L activation assay showed that lysates from BHK-21 cells transfected
17 with duOASL, goOASL, osOASL, osOAS1, and chOASL-A emitted fluorescence as little as did the
18 negative control (non-transfected and pIRES-EGFP-transfected BHK-21 cells) in all time points,
19 indicating that ectopically expressed proteins were all unable to activate RNase L (Fig. 3). On the
20 other hand, when BHK-21 cells were stimulated with the IFN, the RNase L activity was significantly
21 up-regulated.

22 However, when exogenous RNase L proteins (duRNase L and/or osRNase L) were added to the
23 reaction mixture, duOASL, goOASL, osOAS1, and chOASL-A proteins significantly emitted
24 fluorescence with almost similar extent as did the positive control (293FT and BHK-21 cells treated
25 with IFN). However, the osOASL emitted very low extent of fluorescence, approximately 30% of the
26 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.

2

3 *3.4. Inhibitory activity on WNV replicon replication*

4

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).

14

15

1 4. Discussion

2

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

15 We demonstrated for the first time the full-length sequences of *duOASL* (KU569293) and
16 *goOASL* mRNA (KU569292), with open reading frame of 1,512 and 1,527 bp, encoding 503 and 508
17 amino acids with predicted molecular weight of 57.44 and 58.09 kDa, respectively. Domain structure
18 analysis of duck and goose *OAS* proteins showed that both proteins have three conserved domains;
19 nucleotidyltransferase and *OAS1_C* domains as well as ubiquitin-like domains (two repetitive
20 ubiquitin-like domains, UBL1 and UBL2), indicating that both proteins are *OAS*-like proteins (Player
21 and Torrence, 1998). Moreover, although *OAS* gene families in mammals include four types of *OAS*
22 genes (*OAS1*, *OAS2*, *OAS3*, and *OASL*), the avian taxa possess decreasing or limiting *OAS* genes
23 during the evolution process. In all known avian genome database there is no evidence for the
24 presence of *OAS1*, *OAS2*, and *OAS3* genes (Pereygin et al., 2006). Magor et al. (2013) discussed this
25 theory for other missing genes in avian taxa such as *TLR8* and *ISG15* genes. Furthermore, based on
26 database for *OAS* proteins of almost all avian species except for the ostrich, we can find only one

1 OAS protein, OASL that possesses both enzymatic and antiviral activities. On the other hand in
2 ostrich, we can find two different OAS proteins, osOASL (XP_009671383) and osOAS1
3 (XP_009667960), indicating the duplication of the small OAS proteins into osOAS1 and osOASL,
4 resulting in a unique evolutionary process from the other avian species. Moreover, the osOAS proteins
5 show development of a semi-functional specification not seen in the other avian species; osOAS1
6 possesses enzymatic activity but not antiviral activity, whereas osOASL possesses antiviral activity
7 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
9 or indirect pathways. The classical antiviral OAS/RNase L pathway or the enzymatic activity results
10 in viral and cellular RNA degradation including ribosomal RNA within infected cells, subsequently
11 effectively inhibits the viral replication (Silverman, 2007). In the current study, we investigated the
12 classical OAS/RNase L activity. We demonstrated that duOASL, goOASL and osOAS1 proteins
13 showed enzymatic activity resembling chOASL-A to convert the ATP into mono, di, tri, and tetra 2'-
14 5'(A)s, except for the osOASL protein which expressed very low enzymatic activity (Fig. 2B and 4A-
15 E). Similarly, [Sokawa et al. \(1984\)](#) conducted one of the most earlier studies with avian species, where
16 they investigated the 2'-5'-oligoadenylate synthetase activity in erythrocytes. They found that all
17 studied avian species (chicken, goose, and pigeon) expressed 2-5A synthetase in erythroid cells. In
18 the current study, the conserved domain analysis in the OAS unit of duOASL, goOASL, osOASL as
19 well as osOAS1 showed high homology to those of chOASL-A (Fig. 7 A and B). However, the LxxxP
20 domain that is important for the enzymatic activity of OAS proteins showed one amino acid
21 substitution in osOASL protein in position 8 from proline to threonine (P8T). This amino acid
22 substitution 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
24 domain of mouse 9-2 OAS protein resulted in almost 60% deactivation of the enzymatic activity
25 compared with the unmutated protein. However, this mutation seems to have more stronger effects
26 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

1 birds became resistant to WNV infection. Similar to WNV infection in ducks, Ramadori and Armbrust
2 (2001) and Song et al. (2014) clearly illustrated the age-related susceptibility to duck hepatitis virus-
3 1 (DHV-1). Moreover, the behavioral and managerial problems such as feather picking and
4 cannibalism (Banet-Noach et al., 2003) as well as pathogen co-infection (Glávits et al., 2005) have a
5 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
7 antiviral response to IBDV infection and might play a role in viral sensing in chicken regardless of
8 the IFN pathway. As well, Cong et al. (2013) reported that the chOASL/RNase L pathway might be
9 involved in anti-avian-IBV infection. Sokawa et al. (1984) indicated that chOASL might be involved
10 in other regulatory functions other than the immune activity. Moreover, Song et al. (2014) found that
11 the duOASL might play a role in the resistance to DHV-1 infection in duck. As well, Yang et al.
12 (2016) recently indicated that the goOASL significantly increased antiviral activity against gosling
13 plaque virus, duck-TMUV and H9N2 avian influenza virus. Furthermore, the expression of goOASL
14 in the goose embryonic fibroblast showed significant inhibitory effect on the Newcastle diseases virus
15 replication in comparison with the control group. These facts indicate that the avian OAS proteins are
16 very important and playing crucial roles in the avian immune response.

17 In summary, the function of the avian OAS proteins is rarely studied and incompletely
18 understood. Recently, we have reported that the chOASL protein possesses antiviral activity against
19 WNV independent of its enzymatic activity (Tag-EL-Din-Hassan et al., 2012 and 2017). In the current
20 study we have demonstrated that duOASL and goOASL are dual-function proteins similar to the
21 chOASL, where they express antiviral activity through both the OAS/RNase L-dependent and
22 independent pathways. However, we have demonstrated that ostrich OAS proteins undergo a
23 duplication to produce two OAS proteins (osOAS1 and osOASL) during the evolution process. It is
24 of interest that, unlike chicken, duck, and goose OASs, the function of ostrich OAS family are split,
25 where the osOAS1 expresses the OAS-enzymatic activity to stimulate RNase L, whereas the osOASL
26 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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3

1 **Figure legends**

2

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, osOASL, 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
24 using FRET assay. A), duOASL; B), GoOASL; C), chOASL; D), osOAS1; and E), osOASL using
25 FRET probe for periods of 0, 60, and 120 min at 21 °C. The background fluorescence activity was
26 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 reign (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.

23

Fig. 1

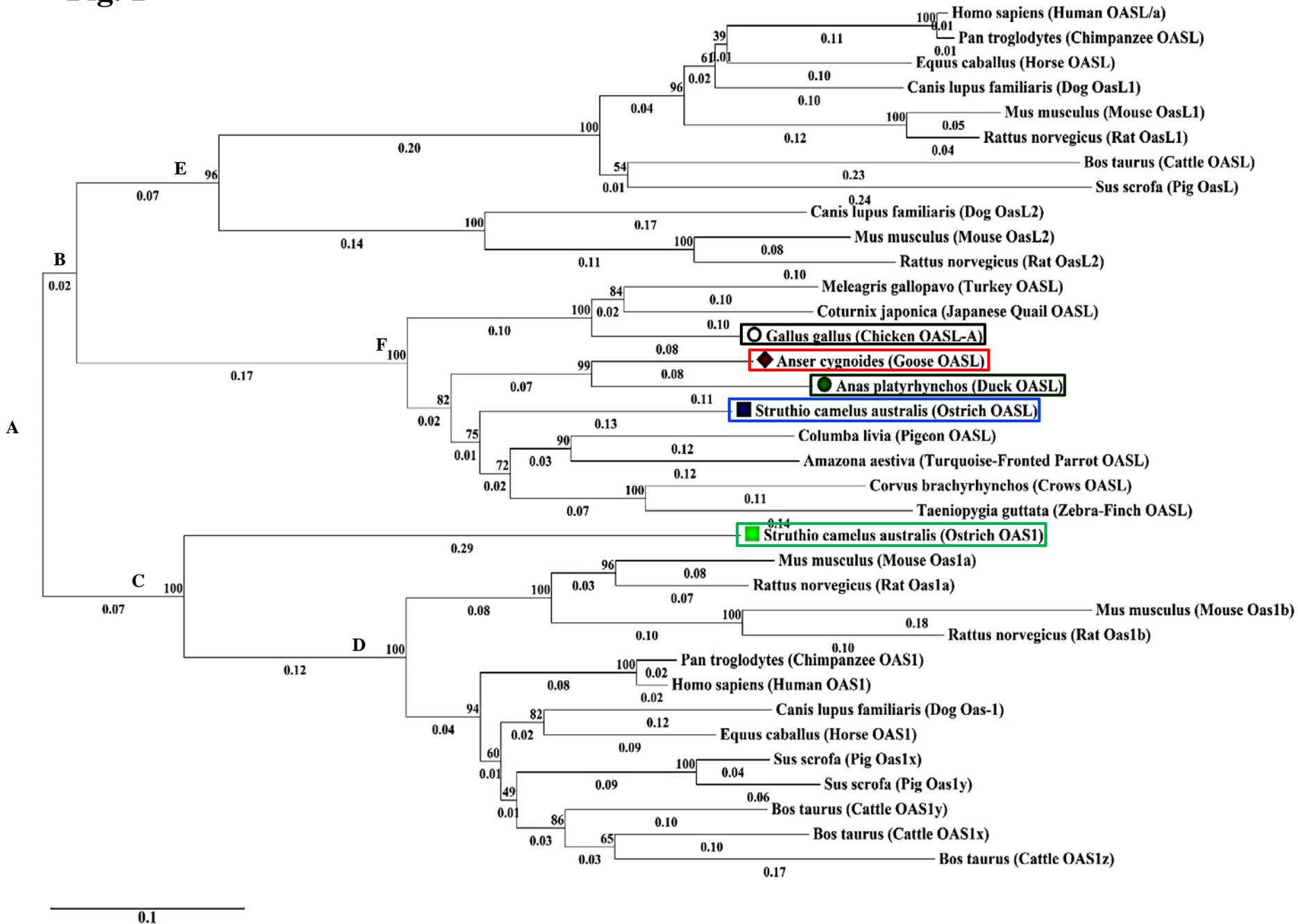


Fig. 2

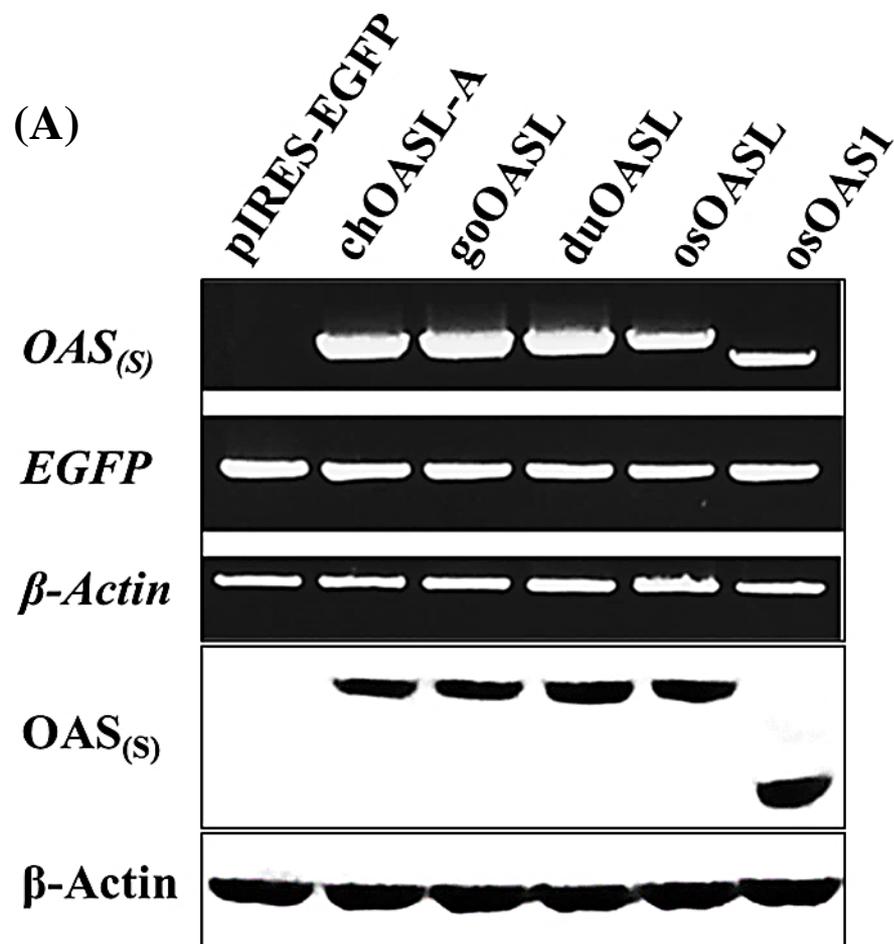


Fig. 2

(B)

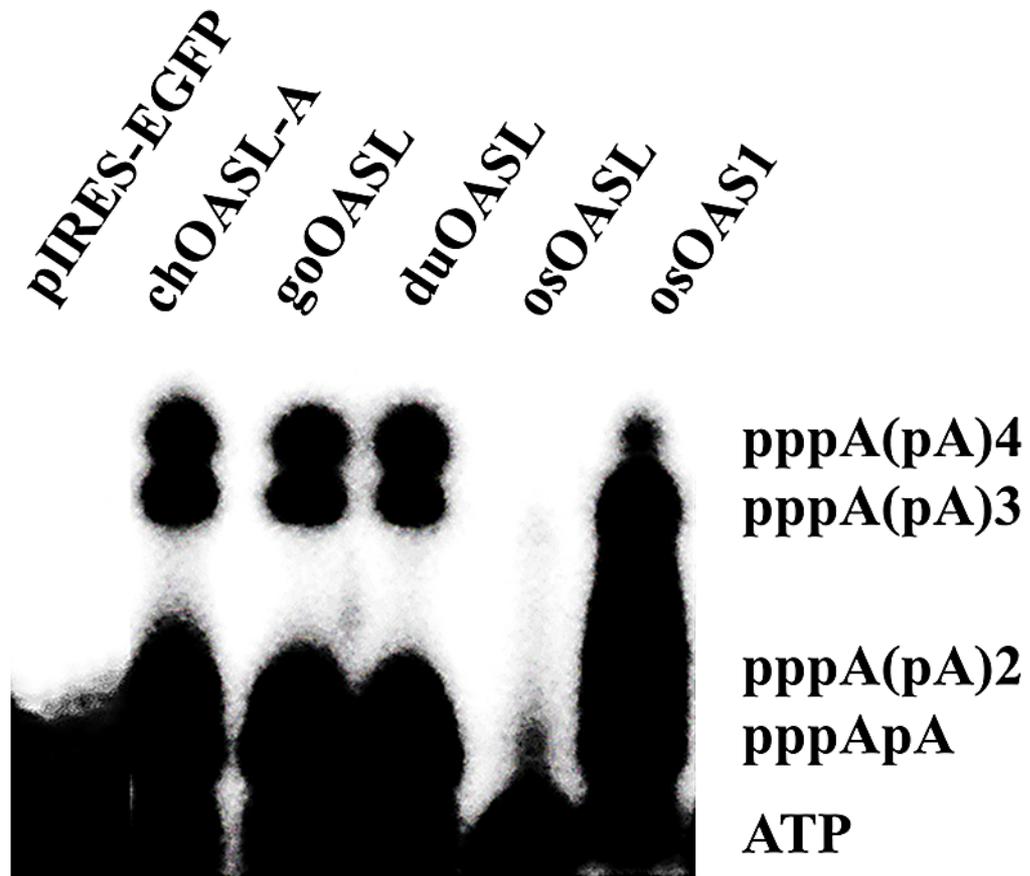


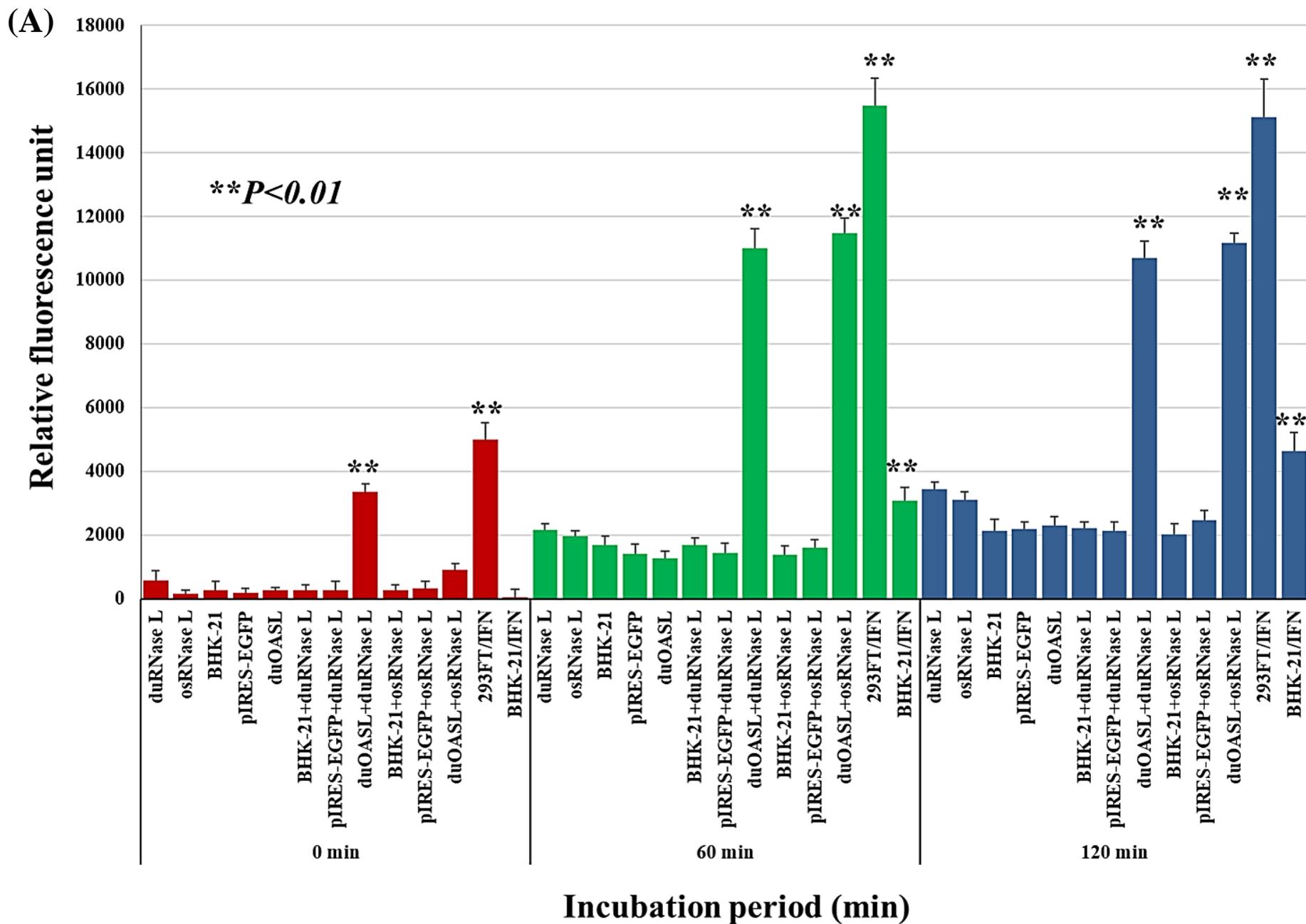
Fig. 4**duOASL**

Fig. 4**(B)**

Relative fluorescence unit

goOASL

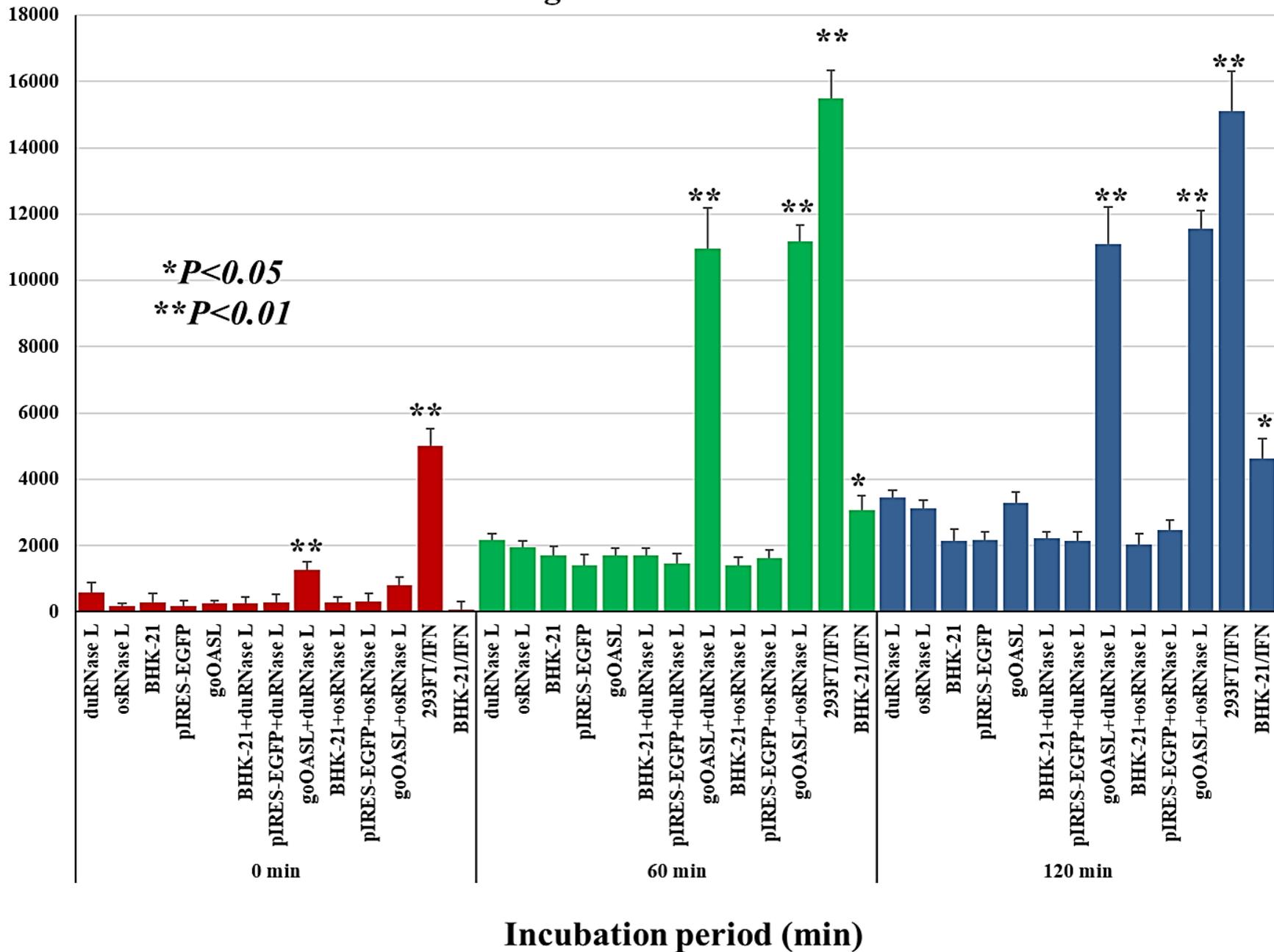


Fig. 4

(C)

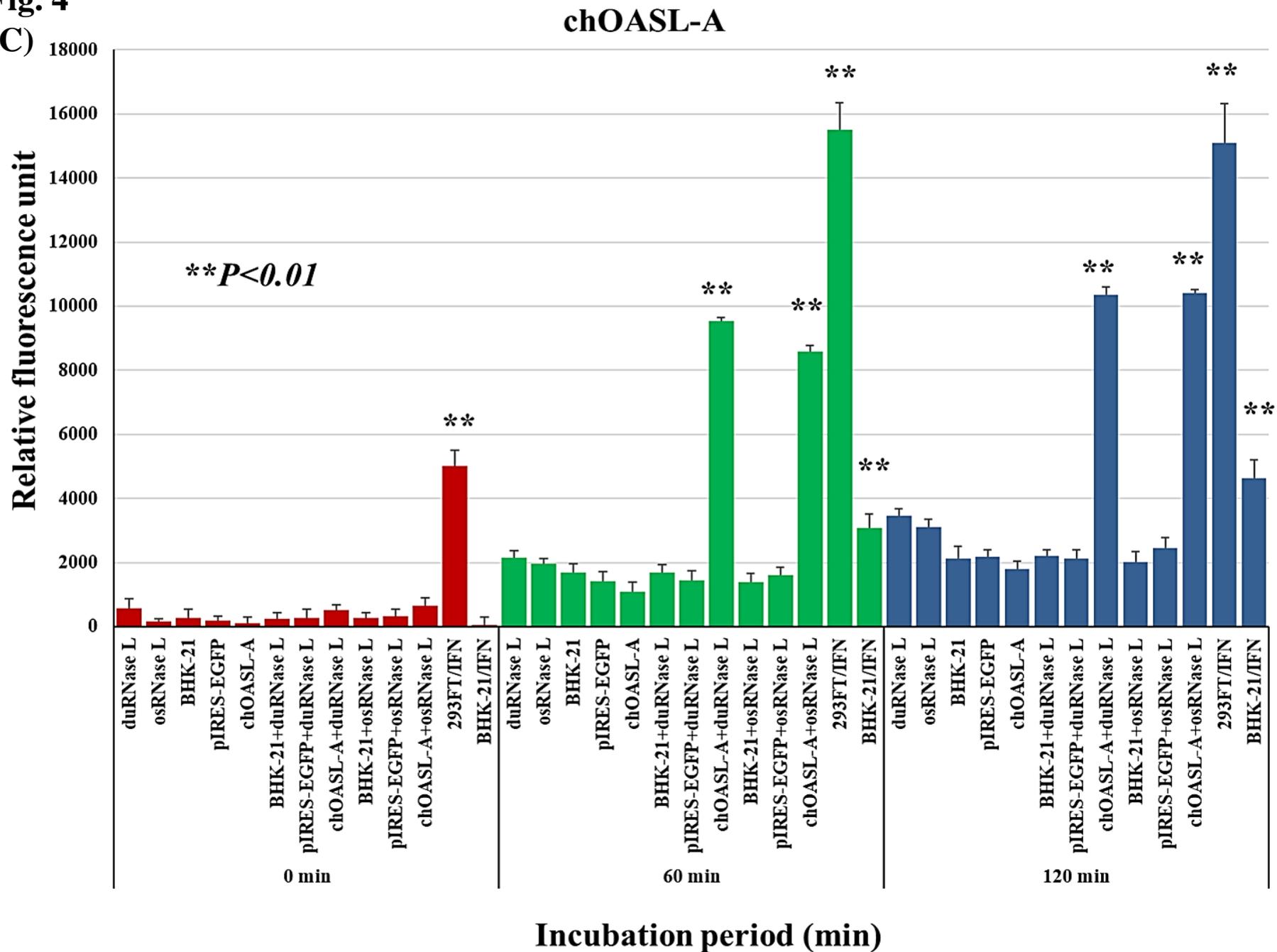


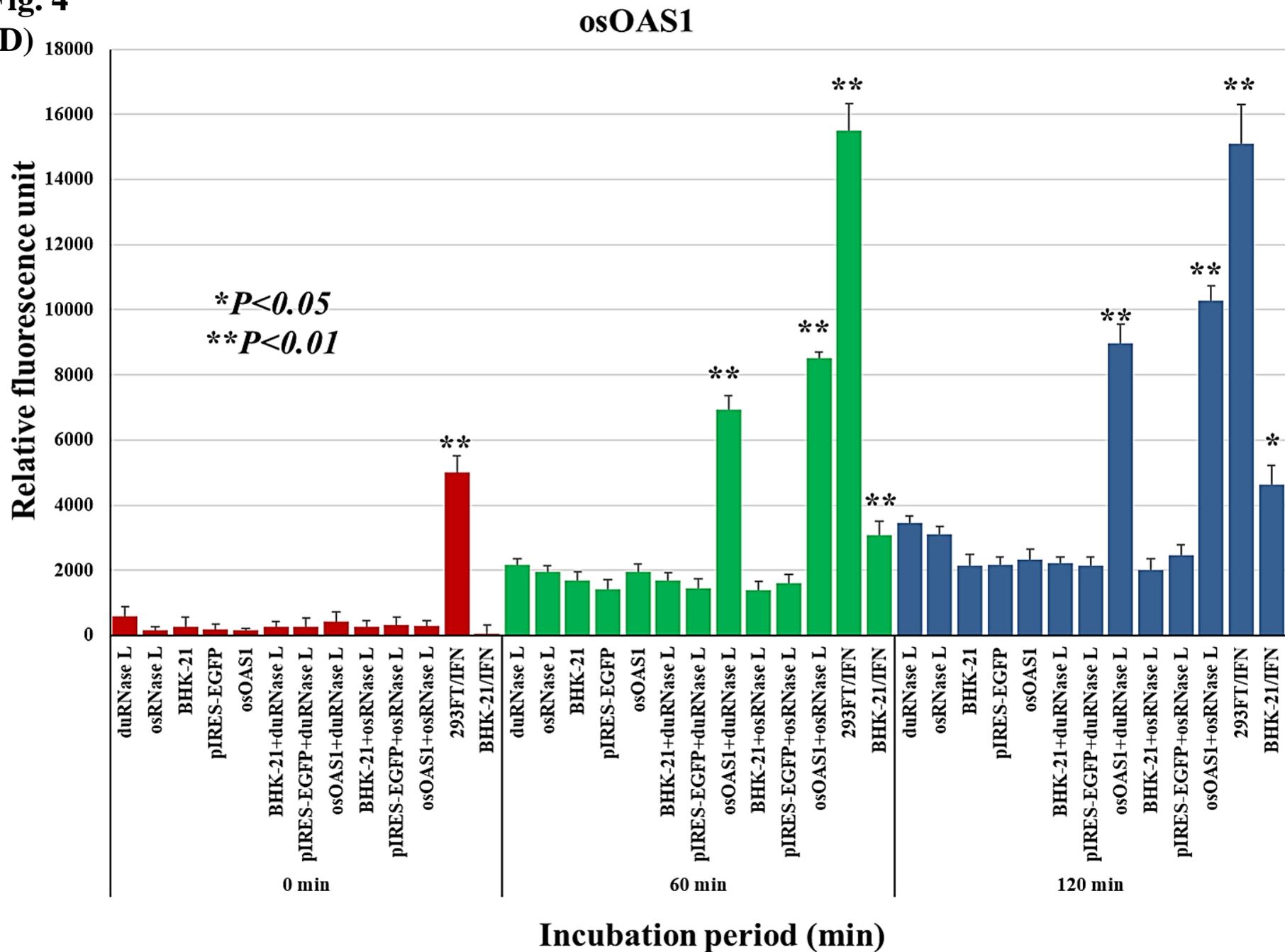
Fig. 4**(D)**

Fig. 4

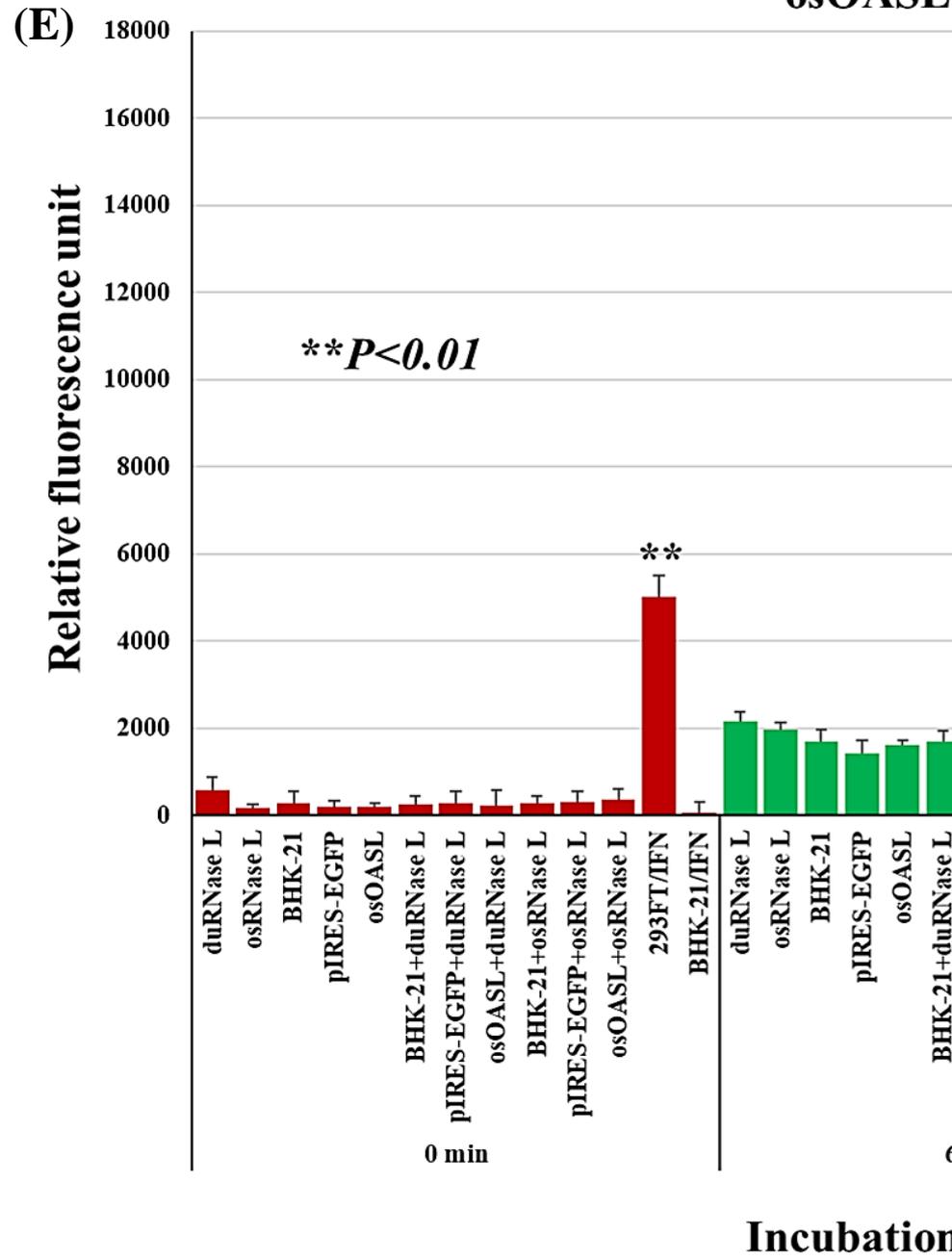


Fig. 5

Amplification of rep WNV/SEAP replicon

(A)

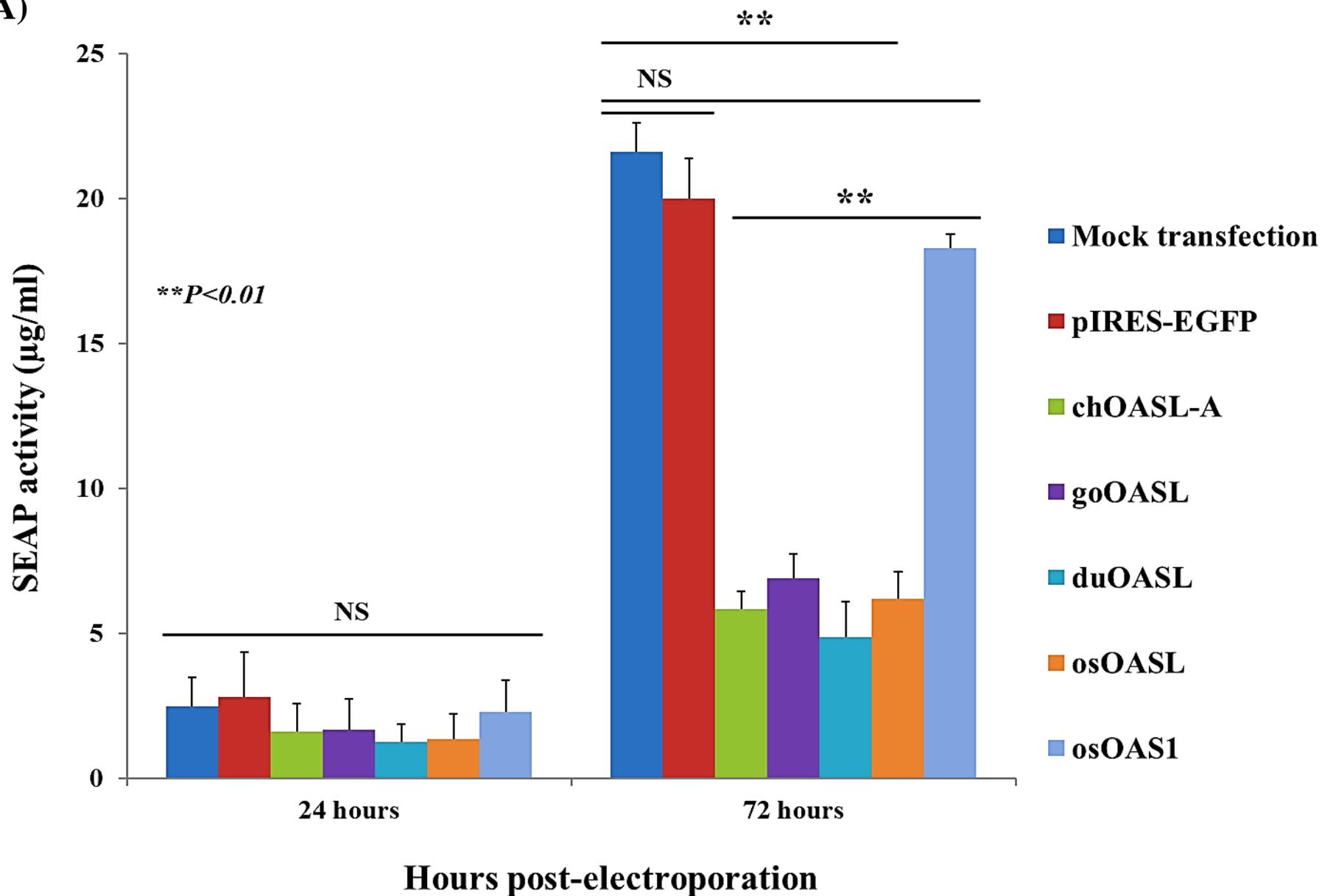


Fig. 5

(B)

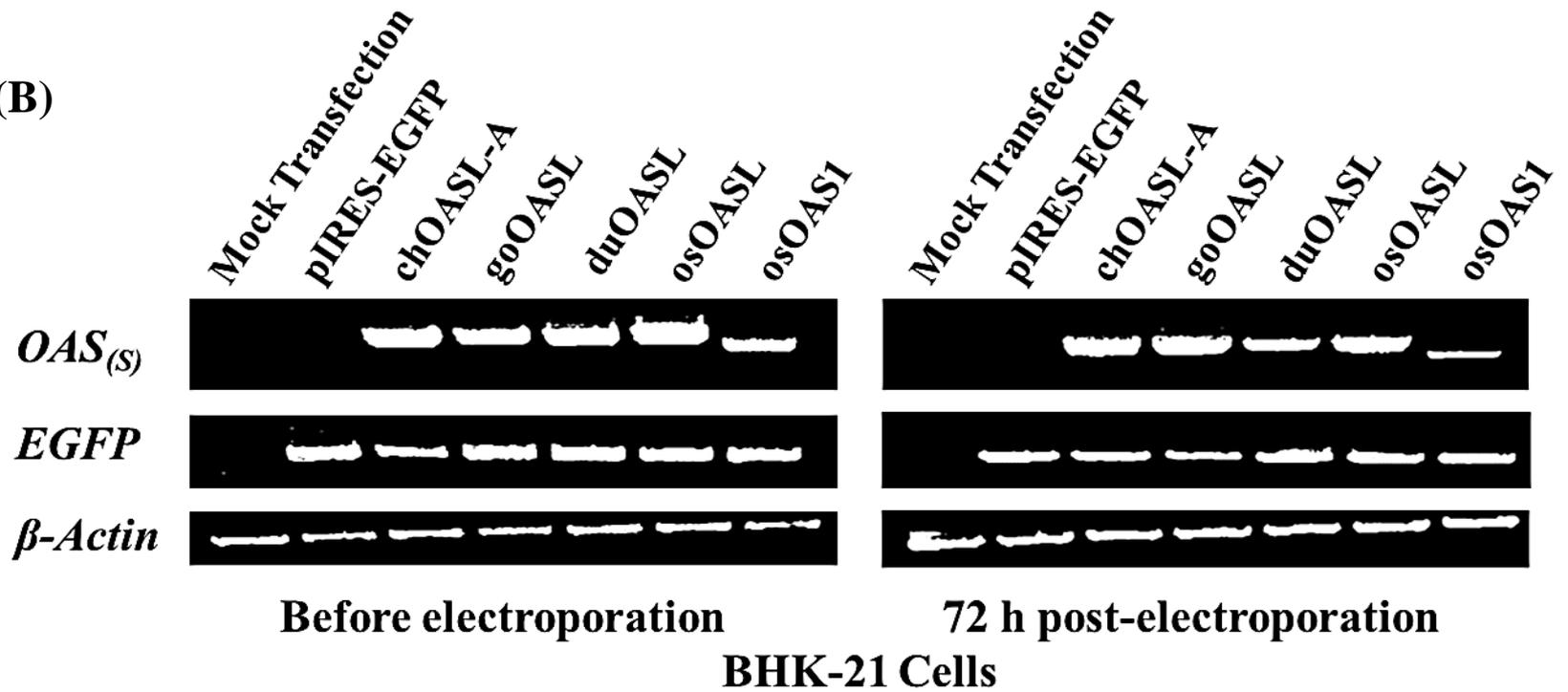


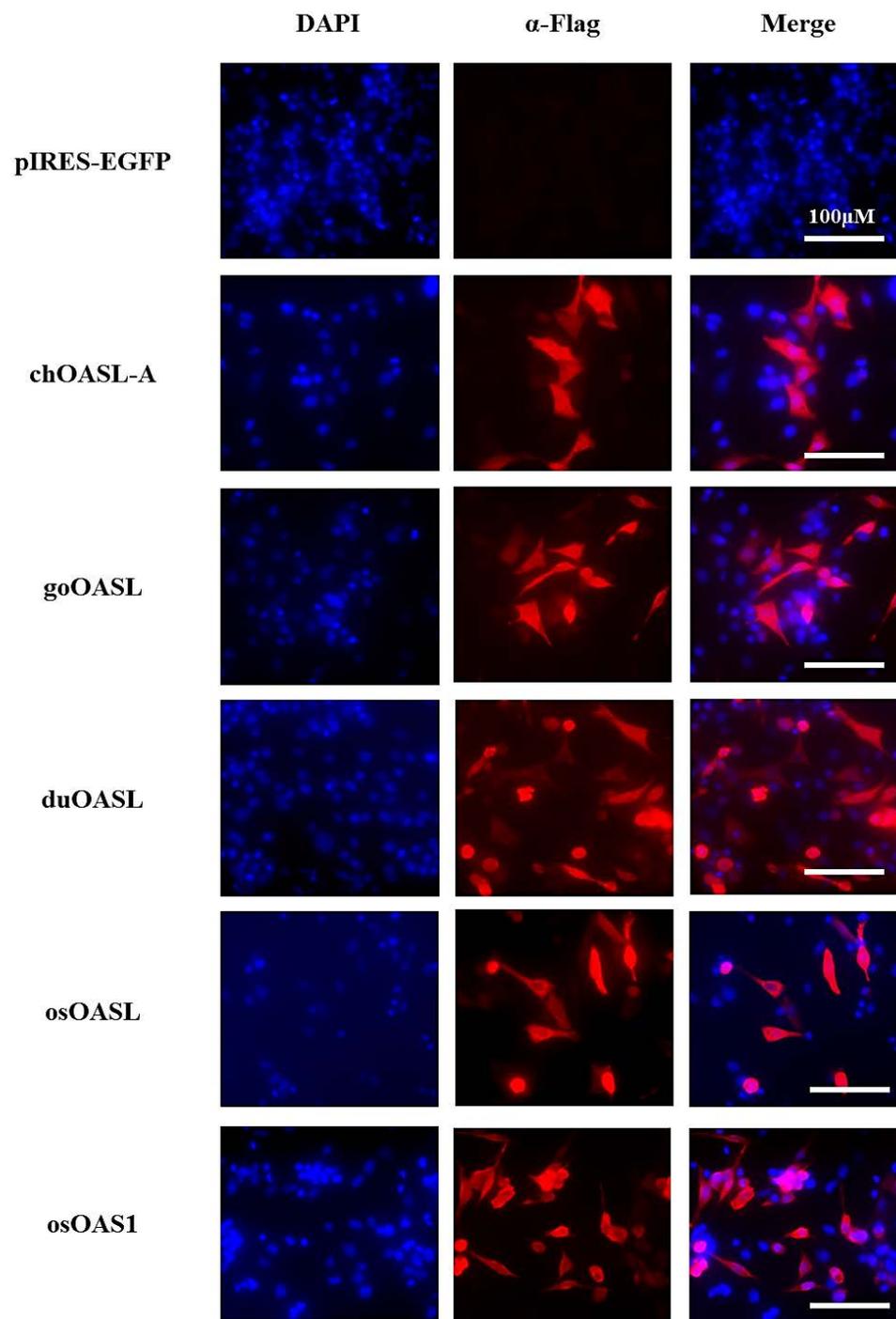
Fig. 6

Fig. 7

(A)

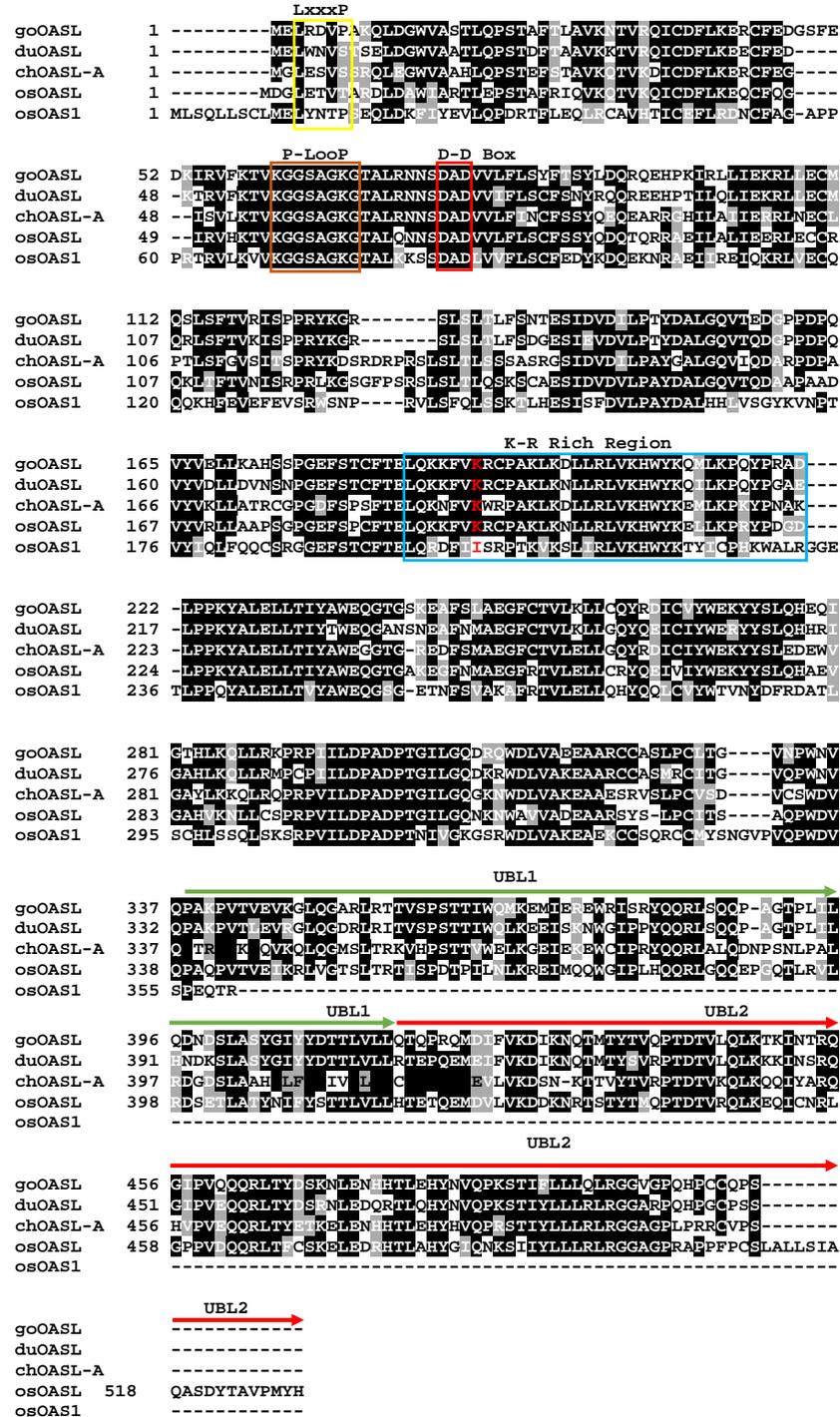
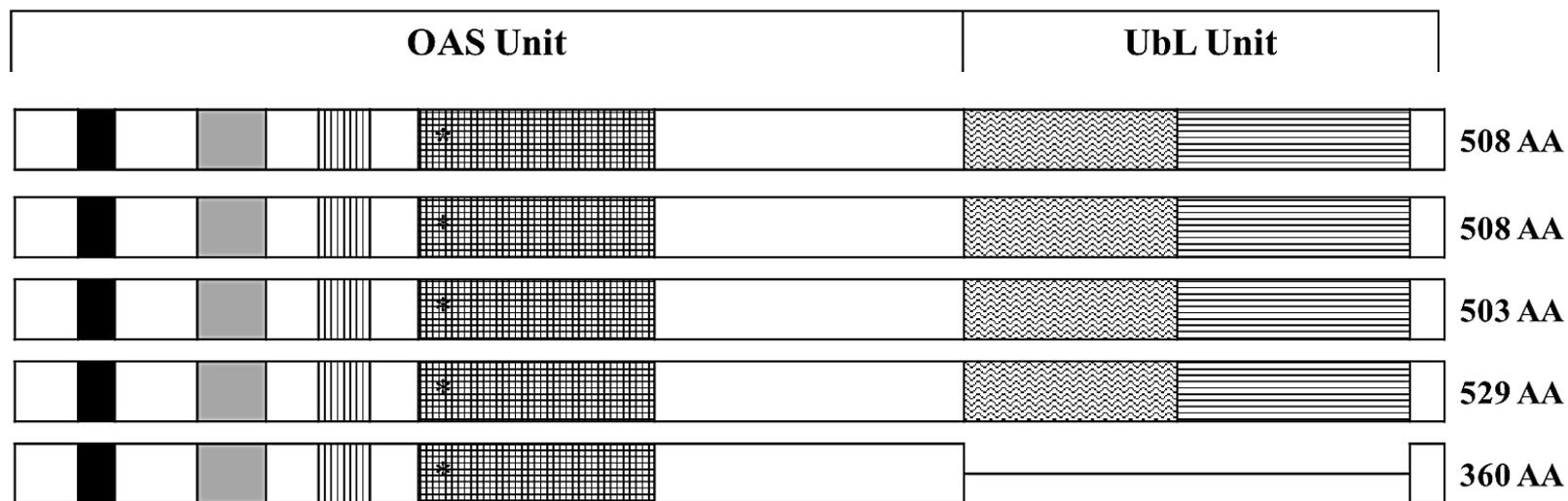


Fig. 7

(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		