Analysis of the molecular mechanisms linking the heat shock response and inflammatory gene expression in endothermic organisms

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CONTENTS

1. Abstract

Heat shock response is one of the ancient protective mechanisms in organisms against thermal and environmental stresses, which characterized by the induction of heat shock proteins (HSPs), and is regulated by heat shock factor (HSF) that binds to heat shock response element (HSE). Vertebrate HSF family consists of four members (HSF1-4). HSF1 is a master regulator of HSPs in mammals, whereas HSF3 in avians. Recently, we have demonstrated that heat shock suppresses lipopolysaccharide (LPS) – induced expression of pyrogenic cytokines such as interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α), at least partly through HSF1 directly or indirectly. The HSF1-mediated negative regulatory loop may be important to inhibit an excessive febrile response and injuries to tissues in mammals. However, the regulation of avian pyrogenic cytokines by heat shock has not been clarified. We here examined effects of heat shock on the expression of pyrogenic cytokines, IL-6 and IL-1β, in avian cells, whose body temperature is exceptionally high (40 to 44^{o}C) among vertebrate species. We here found that heat shock induces the expression of IL-6 in various kinds of chicken cells. HSF3, an avian orthologue of mammalian HSF1, directly bound to and activated IL-6 during heat shock in chicken cells. Other components of the febrile response mechanism, such as IL-1β and ATF3, were also differently regulated in mammalian and chicken cells. These results suggest that the febrile response is exacerbated by a feed-forward circuit composed of the HSF3-IL-6 pathway in birds, and the heat shock response is important for the regulation of body temperature in endothermic organisms.

2. Introduction

Endothermic organisms, mammals and birds, maintain high and constant body temperature over a wide range of environmental temperatures by expending great quantities of energy (Bennett, A.F., et al., 1979). In response to infection and diseases, these organisms further generate a fever, which is a physiological defensive response, that involves an increase in core body temperature mediated by pyrogenic cytokines such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and IL-6 (Mackowiak, P.A., et al., 1998; Leon, L.R. 2002). Fever or hyperthermia has been experimentally shown to play beneficial roles in disease prognosis in mammals (Hotchkiss, R., et al., 1993; Chu, E.K., et al., 1997), partly by suppressing the expression of cytokines including IL-1 and TNF-α (Kappel, et al., 1991; Fouqueray, et al., 1992; Ensor, J.E., et al., 1994; Kluger, M.J., et al., 1997).

A hallmark of stressed cells and organisms are the increased synthesis of heat shock proteins (HSPs) that function as molecular chaperones to prevent protein misfolding and aggregation to maintain protein homeostasis (Powers E. T. et al., 2009). The transcriptional activation of HSPs genes is mediated by heat shock factor (HSF). The vertebrate HSF family consists of four members: HSF1, HSF2, HSF3 and HSF4. Among the family members, HSF1 is master regulator in mammals, and by HSF3 in avian. HSF1 also plays important roles in developmental process such as oogenesis, spermatogenesis, and cilia formation (Akerfelt, M., et al., 2010; Fujimoto, M., et al., 2010). Previous studies demonstrated that HSF1 inhibits the expression of TNF-α and IL-1β by binding directly to the TNF-α promoter, or by physically interacting with NF-IL6, an activator of IL-1β (Xiao, X., et al., 1999; Singh, I.S., et al., 2002; Xie, Y., et al., 2002).

Furthermore, *IL-6* was shown to be a direct target of HSF1. HSF1 up-regulates basal IL-6 expression in HSF1-null primary MEF cells, and is required for its maximal induction in

response to LPS stimulation (Inouye, S., et al., 2004). HSF1 constitutively binds to HSE in the IL-6 promoter to open its chromatin structure partially to facilitate the binding of other transcription factor repressor ATF3 or activator NF-κB to this promoter (Inouye, S., et al., 2007)

Although HSF1 binding does not necessarily affect the transcription of *IL-6*, it also suppress IL-6 expression by inducing activating transcription factor 3 (ATF3) (Takii, R., et al., 2010; Janus, P., et al., 2011; Chen, S., et al., 2012; Ambade, A., et al., 2012), a negative regulator of inflammatory cytokines including IL-6 (Gilchrist, M., et al., 2006). Taken togenther, HSF1 plays an important role in a feedback regulation of the febrile response in mammals, which includes fever and the inflammatory response (Mackowiak, P.A. 1998).

Recently, avian IL-6 and IL-1β were also shown to be mediators of the febrile response (Marais, M., et al., 2011). Therefore, it is necessary to examine whether the same feedback regulation of the febrile response exists in birds, whose body temperature is maintained at markedly higher temperature than that of mammals (Rodbard, S. 1950).

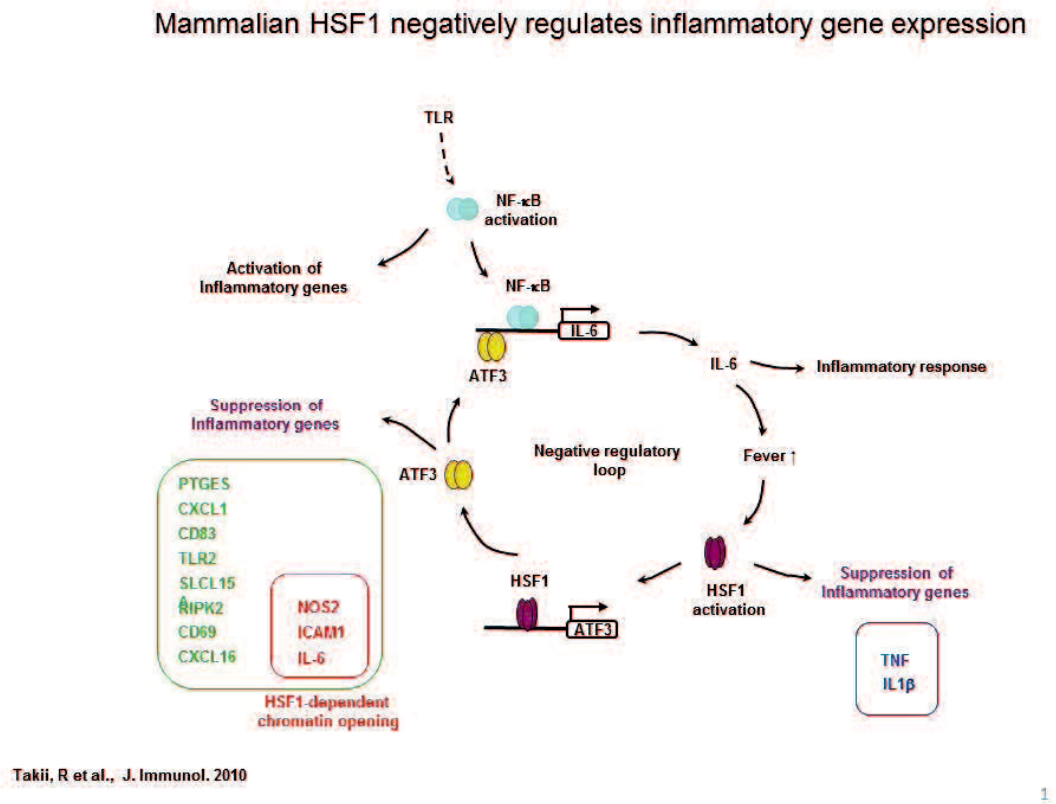

Fig.1. The feedback loop of IL-6 expression that negatively regulates the expression of inflammatory genes in mammals.

Toll-like receptor (TLR) induces NF-κB activation through multiple steps. Activated NF-κB induces the expression of inflammatory genes including IL-6, leading to febrile response. As a result, HSF1 is activated and directly suppresses the expression of inflammatory genes TNF-α, IL-1β. At the same time it induces the expression of ATF3, which inhibits the expression of many inflammatory genes, including IL-6 (Takii et al., 2010).

3. Materials and methods

3.1 Cell culture, screening, and treatments

Chicken B lymphocyte DT40 cells and HSF1- and HSF3-deficient cells were maintained at 37˚C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% chicken serum, and 2-mercaptoethanol (10 μM) (Nakai et al., 2001; Tanabe, M., et al., 1998). To generate HSF3-/- DT40 cells overexpressing human HSF1, pCMV/hHSF1 (Inouye, S., et al., 2003) and pZeoSV2 (Invitrogen) vectors were co-transfected into HSF3-/- (#21) cells (Tanabe, M., et al., 1998) and incubated in the presence of zeocin (300 μg/ml).

Primary chicken embryonic fibroblasts (CEF) and quail fibroblast QT6 cells were maintained at 37˚C as described previously (Nakai, A., et al., 1995). Chicken HD3 erythroblasts (a gift from Dr. T. Graf) were maintained in DMEM supplemented with 8% FBS and 2% chicken serum (Beug, H., et al., 1982), and chicken DF-1 fibroblast cell line (ATCC CRL-12203) in DMEM supplemented with 10% FBS (Himly, M., et al., 1992). Primary mouse embryonic fibroblasts (MEF) were maintained at 37˚C in 5% CO_2 in DMEM supplemented with 10% FBS. Spleen cells were prepared from dissected mouse spleens by removing the erythrocytes using Ack lysis buffer (Life Technologies), and were cultured in RPMI-1640 medium supplemented with 10% FBS, non-essential amino acid, and 2-mercaptoethanol (50 μM) for 24 h (Inouye, S., et al., 2004). Mouse macrophage Raw264.7 and B lymphoma A20 cells were cultured in RPMI-1640 medium supplemented with 10% FBS. Cells were treated with heat shock, lipopolysaccharide *E. coli* 0127:B8 (LPS, Sigma-Aldrich), sodium arsenite (Sigma-Aldrich), L-azetidine-2-carboxylic acid (Sigma-Aldrich), or tunicamycin (Merck Millipore) for the indicated periods.

3.2 Western blot analysis

Extracts of zeocin-resistant clones cell extracts were prepared in lysis buffer containing 1.0% Nonidet P-40, 150 mM Tris-HCl (pH 8.0), 1μg/ml leupeptin, 1μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 15,000 X g for 10 min, supernatant were removed. Equal amount of protein were loaded on 10% SDS-PAGE and transferred to nitrocellulose membranes by electrophoretic transfer with a buffer containing 5 mM sodium tetraborate. The membranes were blocked with 5% dry milk in phosphate-buffered saline (PBS) for 1 hour. Then the membranes were incubated with 1:1,000 dilution of α-mHSF1j antibody and α-β-actin (Sigma) in 2% dry milk at 4˚C overnight (Fujimoto, M., et al., 2008). After being washed with PBS, the membranes were incubated with a 1:1,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG and anti-mouse-IgG (Cappel) for 1hour at room temperature, and then signals were detected by the Amersham ECL detection kit.

3.3 Northern blot analysis

Total RNA was isolated from cultured cells using TRIzol (Invitrogen), and a Northern blot analysis was performed as described previously using ^{32}P-labelled cDNA probes for chicken HSP70 (Tanabe, M., et al., 1998), and for mouse IL-6, HSP70.1, and human-actin (Inouye, S., et al., 2004). A cDNA probe for chicken IL-6 (278 bp) was generated by RT-PCR using total RNA from DT40 cells and primers: 5'-ACC CGC ACC ATG AAC TTC ACC GAG GGC TGC G-3', and 5'-CGT CCT GCA GCT GGA CGG CGC GGT CGC GC-3'. Autoradiography was performed using images on X-ray film (RX-U, Fujifilm Co., Tokyo).

3.4 RT-qPCR

To estimate mRNA levels by quantitative PCR (qPCR), first-strand cDNA was

synthesized using 2 μg of total RNA isolated above, avian myeloblastosis virus reverse transcriptase (AMV-RT), and oligo $(dT)_{20}$ according to the manufacturer's instructions (Invitrogen). Real-time qPCR was performed using the StepOnePlus (Applied Biosystems) with Power SYBR Green PCR master mix (Applied Biosystems) according to the manufacturer's instructions. Relative quantities of mRNAs were normalized against chicken and mouse GAPDH mRNA levels, respectively. All reactions were performed in triplicate with samples derived from three experiments.

List of primer sequences used for RT-qPCR

Real-time PCR	**Forward primer**	**Reverse primer**
Chicken ATF3	5'-CGGCCAGGTGTCTGCATT-3'	5'-CCTGTGGGACTGACGTTTGTT-3'
Chicken IL-6	5'-CCTGTTCGCCTTTCAGACCTA-3'	5'-ACGTTCTGCTTTTCGCTATCGA-3'
Chicken IL-1β	5'-CAGCCTCAGCGAAGAGACCTT-3'	5'-ACTGTGGTGTGCTCAGAATCC-3'
Chicken HSP70	5'-GTCTGAGTAGGTGGTGAAGGTCTGT-3'	5'-TGACTGCTCTCATCAAGCGTAAC-3'
Chicken GAPDH	5'-CCTGCATCTGCCCATTT-3'	5'-GGCACGCCATCACTATC-3'
Mouse ATF3	5'-GCTGCTGCCAAGTGTCGAAA-3'	5'-CGGTGCAGGTTGAGCATGTA-3'
Mouse IL-6	5'-GCTTAATTACACATGTTCTCTGGGAAA-3'	5'-CAAGTGCATCATCGTTGTTCATAC-3'
Mouse IL-1β	5'-GAAGATGGAAAAGCGGTTTG-3'	5'-GTACCAGTTGGGGAACTCTGC-3'
Mouse HSP70-1	5'-GGCTGGTGAGCCACTTCGT-3'	5'-GTTCTGGCTGATGTCCTTCTTGT-3'
Mouse GAPDH	5'-CATGGCCTTCCGTGTTCCTA-3'	5'-GCGGCACGTCAGATCCA-3'

3.5 Transduction

To examine gene expression by HSF1 or HSF3, 50 x 10^4 immortalized HSF1-null MEF cells were plated on 60 mm dishes and then incubate in 1 ml serum free DMEM containing adenovirus (6 X 10^6 pfu/ml) expressing human HSF1 or chicken HSF3 for 2 hours.

After the cells were washed twice with phosphate buffered saline and 4 ml of DMEM containing 10% fetal bovine serum was added, cells were maintained for 48 hours, and treated with heat shock at 42°C 1 h (Fujimoto, M., et al., 2010).

3.6 Estimation of IL-6 activity

Mouse IL-6-dependent hybridoma 7TD1 cells (RIKEN RCB1190) were cultured in RPMI-1640 medium supplemented with 1% FBS, 2-mercaptoethanol (50 μM), and 0, 25, or 125 U/ml of mouse IL-6 (Van Snick, J., et al., 1986). At 3 days after the treatment, cell numbers were counted, and a standard curve was generated. DT40 and double-null (#54) cells were treated without or with heat shock at 45°C for 1 h, and allowed to recover at 37°C for 8 h. Cultured media were collected, and aliquots were added to 7TD1 cells cultured in RPMI-1640 medium supplemented with 1% FBS and 2-mercaptoethanol (50 mM) for 3 days. Cell numbers were then counted, and IL-6 activity was estimated.

3.7 Reporter analysis

DNA fragments of the chicken IL-6 promoter (-530 to +71) (Figure S2), mouse IL-6 promoter (-1265 to + 69), and human IL-6 promoter (-1154 to +100) relative to each transcription start site were isolated by PCR using the genomic DNA of DT40, MEF (ICR background), and HEK293 cells, respectively. The fragments were inserted upstream of the HSV-TK promoter of ptk-galp3-luc at the HindIII/SalI site to generate pcIL-6-luc, pmIL-6-luc, and phIL-6-luc reporter plasmids. We limited the promoter region of pcIL-6-luc by PCR to generate pcIL-6-luc-ΔN1 (-390 to +71), pcIL-6-luc-ΔN2 (-248 to +71), and pcIL-6-luc-ΔN3 (-170 to +71). Deletion mutants, pcIL-6-luc-ΔHSE3 (deletion of a region -345 to -327), pcIL-6-luc-ΔHSE4-5 (deletion of a region -323 to -284), and pcIL-6-luc-ΔHSE6-7 (deletion of a

region -283 to -254) were also generated by PCR-mediated site-directed mutagenesis. We similarly substituted conserved nucleic acids (G or C in a consensus HSE sequence nGAAn) to generate point mutants in HSE6 (-286 to -272) and HSE7 (-273 to -254) (Figure 2C). Sequences were verified using 3500 Genetic Analyzer (Applied Biosystems).

HEK293 or QT6 cells were transfected with 3 μg of the reporter plasmid and 2 μg of pact-LacZ as an internal control using the calcium phosphate method described previously (Takii, R., et a., 2010). Four hours after the transfection, cells were washed with phosphate-buffered saline and incubated for a further 44 h in normal medium. After heat shock at 42°C or 43°C for 1 h and recovery at 37°C for 12 h, cell extracts were prepared and luciferase and β-galactosidase activities were examined.

Primer sequences used to isolate the DNA fragments of vertebrate IL-6 promoters

	Forward primer	**Reverse primer**
Chicken IL-6 promoter	5'-CGC**GTCGAC**GTGTGCAGCGGTTGAACC-3'	5'-CGC**GGATCC**CGTCGCCTCGCAGCCCTC-3'
Mouse IL-6 promoter	5'-CGC**GTCGAC**TGAGAGTGTGTTTTGTAA-3'	5'-CGC**GGATCC**CTTGCAGAGAGGAACTTC-3'
Human IL-6 promoter	5'-CGC**GTCGAC**TCCTGCAAGAGACACCATCC-3'	5'-CGC**GGATCC**AAGGATTTCCTGCACTTACTTGTGG-3'

Bold face; Sal I and Bam HI

Primer sequences used to generate deletion mutants of the chicken IL-6 promoter

	Forward primer	**Reverse primer**
ΔHSE3	5'-CACAATAAAATGTGCGCAGGTACGGGACCC-3'	5'-CCTGCGCACATTTTATTGTGTTTTATACAT-3'
ΔHSE4-5	5'-ACACCTGTGCCCCCGCCCCGACCCCCGGCC-3'	5'-CGGGGCGGGGGCACAGGTGTGGCGCGTCTT-3'
ΔHSE6-7	5'-AGGCTCACCCGAGCGGCGGCGCGGCCCG CA-3'	5'-GCCGCCGCTCGGGTGAGCCTGGCAGCCTCC-3'

3.8 Electrophoretic mobility shift assay

DT40 whole cell extracts were prepared in buffer C (20 mM HEPES, pH7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF) from untreated and heat-shocked DT40 cells. Aliquots of extracts (25 ug) were subjected to EMSA using a ^{32}P-labeled annealed HSE7-oligonucleotide probe and each specific antibody as described previously (Inouye, S., et al., 2004; Nakai, A., et al., 2001). The probe sequences were: HSE7-top, 5'-gat tat aaC CCC CGG CCG AAG CCA GGT Gaa gtt gat-3' and HSE7-bottom, 5'-atc aac ttC ACC TGG CTT CGG CCG GGG Gtt ata atc-3' (unique sequences, which are flanked by random sequences, are indicated in capitals). Complex formation of the probe and HSF3 were competed with unlabelled oligonucleotides for HSE7 or its mutant (HSE7-m1 or HSE7-m2) (Inouye, S., et al., 2004).

3.9 Chromatin immunoprecipitation

DT40 cells were treated without or with heat shock at 45˚C for 1 h, and chromatin immunoprecipitation (ChIP) was performed using a ChIP assay kit (EMD millipore) according to the manufacturer's instructions. An antiserum for chicken HSF3 (α-cHSF3γ) was used to precipitate complexes of the HSF3 and DNA fragments. ChIP-enriched DNAs were amplified by PCR using primers corresponding to DNA fragments of the chicken IL-6 promoter (region a, +15 to -176; region b, -179 to -409; region c, -363 to -519), as well as that of chicken HSP70 (-38 to -240). Amplified DNA was stained with ethidium bromide and photographed.

Primer sequences used for ChIP analysis

ChIP	**Forward primer**	**Reverse primer**
Chicken IL-6	5'-GTTGAACCAAGGGCGTCCAGTTTCATGC-3'	5'-CATCTTATCCATTGATACGTTTTCG-3'

promoter (-519 to -363)		
Chicken IL-6 promoter (-409 to -176)	5'-GATGCATAACGAATAAAAGTCGCG-3'	5'-GAGGCGAGCCGGCCAGGCAGAGG-3'
Chicken IL-6 promoter (-176 to +15)	5'-CTCCCCGTCTCCGTTTCACAATCTCAATGC-3'	5'-GTGAAGTTCATGGTGCGGCTTCTCTGTTCG-3'
Chicken HSP70 promoter (-240 to -38)	5'-GCTAGAGAGTGGGCGCTACGC-3'	5'-CTGCTGTCTGCGGCGCGATCTGC-3'

3.10 Incubation and isolation of chicken embryos

Fertilized chicken eggs were incubated, and treated with heat shock as described previously (Kawazoe, Y., et al., 1999). Briefly, fertilized chicken eggs were incubated in a humidified incubator at 37°C and rotated two times a day. To examine IL-6, IL-1β, ATF3 expressions, the eggs were placed in plastic bag on day 6 and submerged in a water bath at 45°C for 1 hour. RNA was isolated as described above.

3.11 Statistical analysis

Data were analyzed with Student's t-test. Asterisks in figures indicate significant differences ($P < 0.05$ or 0.01). Error bars represent standard deviations (s.d.) for three independent experiments.

4. Results

4.1. IL-6 expression is induced during heat shock in chicken cells.

A comparison of mouse and human IL-6 promoter sequences revealed the conserved heat shock element (HSE)–like sequences, mammalian HSE2 (mHSE2) (overlapping with HSE1) and mHSE3, and HSF1 was previously shown to bind to the former in vivo (Inouye, S., et al., 2007). However, mHSE2 was not conserved in the chicken IL-6 promoter sequence, which contained 32 HSE-like sequences within -1309 bp from a transcription start site (Figure 1). Furthermore, the binding sites of activators including activator protein-1 (AP-1), nuclear factor-κB (NF-κB), nuclear factor-IL-6 (NF-IL6), and glucocorticoid response element (GRE) were conserved in chickens and mammals, whereas cAMP-responsive element (CRE or ATF/CRE), which was bound by ATF3, was not (Kaiser, P., et al., 2004). Therefore, we investigated whether chicken IL-6 expression is regulated in the same manner as mammalian IL-6.

Interestingly, we found that IL-6 mRNA was markedly induced during heat shock at 45^{o}C for 1 h, similar to HSP70 mRNA in chicken DT40 B lymphocytes, HD3 erythroblasts, DF-1 fibroblasts, and primary chicken embryonic fibroblasts (CEF) (Figure 2A). In contrast, it

was not induced in mouse A20 B lymphoma cells, Raw264.7 macrophage cells, mouse spleen cells, or primary mouse embryonic fibroblasts (MEF). Although IL-6 mRNA was induced less by LPS stimulation in chicken cells, it was not induced in mouse A20 cells, which suggested that the heat-mediated induction of IL-6 expression is independent of the responsiveness to LPS. We examined its expression in more detail in DT40 cells, and found that IL-6 mRNA, similar to HSP70 mRNA, was markedly induced by the treatment with heat shock, even at 43°C, sodium arsenite, and a proline analogue, but not by the tunicamycin treatment, which induces the endoplasmic reticulum-stress response (Figure 2B). Furthermore, the profile of IL-6 mRNA expression during heat shock at 45°C was similar to that of HSP70 mRNA expression (Figure 2C). IL-6 mRNA was also induced in wild-type and HSF1-null DT40 cells, but not in HSF3-null cells or double-null cells, indicating that HSF3 is required for IL-6 expression during heat shock (Figure 2D). The production of IL-6 actually increased during heat shock in the culture medium of wild-type and HSF1-null cells, but did not in that of HSF3-null or double-null cells (Figure 2E). These results clearly demonstrate that the *IL-6* gene is a *bona fide* heat-shock gene in chicken cells.

4.2. Chicken HSF3 directly binds to and activates the IL-6 gene during heat shock

To identify an HSE, which is responsible for the heat-mediated induction of chicken IL-6 expression, we first cloned a DNA fragment of the chicken IL-6 promoter (-530 to +71) and determined its nucleotide sequence (Figure 3). Within this region, we found seventeen HSE-like sequences, which we referred to as HSE1 to HSE17. To identify a functional HSE, we performed luciferase reporter analyses in quail fibroblasts (QT6) by first generating reporter constructs having different IL-6 promoter lengths (Figure 4A). We found that the heat-shock treatment markedly increases luciferase activity in pcIL-6-luc (-530 to +71) and pcIL-6-luc-ΔN1

(-390 to +71), but does not in pcIL-6-luc-ΔN2 (-248 to +71) or pcIL-6-luc-ΔN3 (-170 to +71) (Figure 4A). These results indicate that the region containing HSE3 to HSE7 is required for the induction of chicken IL-6.

We next performed analyses using reporter constructs lacking regions containing HSEs, and found that luciferase activity in pcIL-6-luc-ΔHSE3 and pcIL-6-luc-ΔHSE4-5 increases following heat shock, whereas that in pcIL-6-luc-ΔHSE6-7 does not (Figure 4B). Furthermore, analyses using reporter constructs having mutations in HSE6 and HSE7 revealed that luciferase activity in pcIL-6-luc-HSE6m, but not that in pcIL-6-luc-HSE7m, increases after heat shock (Figure 4B, C). Thus, HSE7 is responsible for the heat-mediated induction of chicken IL-6.

We examined whether HSF3 is able to bind to HSE7, which is composed of four inverted repeats of an nGnnn sequence, by EMSA *in vitro* using a ^{32}P-labelled HSE7 probe. In unstressed DT40 cells, two non-specific HSE7-binding activities were detected, which were composed of factors that did not react with the antibodies for HSFs (Figure 5A). In contrast, the above non-specific HSE7-binding activity disappeared in heat-shocked cells, and an HSE7-HSF3 complex, which was super-shifted with the anti-HSF3 antibody, was detected (Figure 5A) (Nakai, A., et al., 2001). This complex was competed with unlabelled HSE7, but not with mutated oligonucleotides, in which conserved “G” was substituted with “A” (Figure 5A, B). Furthermore, we examined HSF3-binding to the IL-6 promoter *in vivo* a ChIP assay with three primer sets, which amplified regions containing HSE1-2 (region a), HSE3-11 (region b), or HSE12-17 (region c) (Figure 5C). The results revealed that HSF3 binds directly to the region b containing the HSE7 in the IL-6 promoter, as well as the HSP70 promoter, in heat-shocked DT40 cells. Binding of HSF3 to these promoters was hardly detected in unstressed cells *in vivo*. Taken together, chicken HSF3 directly binds to HSE7 and activates *IL-6* during heat shock.

4.3. Regulation of the febrile response components evolutionally diverged in mammals and birds

As *HSF1* and *HSF3* genes evolved differently in mammals and birds (Fujimoto, M., et al., 2010), we examined whether the different responsiveness of IL-6 expression against heat shock is caused by functional differences in these HSFs. We evaluated the transcriptional activity of chicken, mouse, and human IL-6 promoters by performing luciferase reporter analyses in quail QT6 cells and human HEK293 cells. We found that luciferase activity under the control of the chicken IL-6 promoter was elevated by heat shock not only in QT6 cells but also in HEK293 cells, whereas that of the mouse and human IL-6 promoters was not in any cell type (Figure 6A). Therefore, the different heat responsiveness of IL-6 expression is not caused by functional differences in mammalian HSF1 and chicken HSF3.

Since IL-6 is a major pyrogenic cytokine, regulation of the components of mammalian feedback mechanism of the febrile response may have evolved differently in birds. We first examined the expression of IL-6, IL-1β, and ATF3 in HSF1-null MEF cells and HSF1-null cells overexpressing human HSF1 or chicken HSF3 (Figure 6B). We found that the overexpression of human HSF1 or chicken HSF3 increases the expression of ATF3 mRNA during heat shock, but does not that of IL-6 or IL-1β mRNAs in MEF cells. The expression of these genes was then examined in wild-type DT40 cells, HSF3-null cells, and HSF3-null cells overexpressing human HSF1 (Figure 6C). The results revealed that the expression of IL-1β mRNA as well as IL-6 mRNA is induced by the presence of chicken HSF3 or overexpression of human HSF1 during heat shock in chicken cells. The expression of IL-6 and IL-1β mRNAs was also markedly induced in chicken embryos on day 6 by *in vivo* hyperthermia (Figure 6D). These results demonstrate that elevations in temperature induces the expression of at least two pyrogenic

cytokines, IL-6 and IL-1β, in chicken cells, but does not induce the expression of ATF3, a negative regulator of IL-6 (Gilchrist, M., et al., 2006). Furthermore, human HSF1 and chicken HSF3 were confirmed to be able to play same roles in regulating the components of the febrile response mechanism.

5. Discussion

The HSF family consists of four members in vertebrates, including HSF1, HSF2, HSF3, and HSF4, which bind to HSE (Fujimoto, M., et al., 2010). HSF1 was shown to be necessary for the induced expression of major HSPs during heat shock in mammalian cells, whereas it is dispensable for that in avian cells (Nakai, A., et al., 2001). In birds, HSF1 only slightly induces HSP70 expression during heat shock (Inouye, S., et al., 2003). In contrast, chicken HSF3 is necessary for the induction of HSP expression during heat shock, and its deficiency results in reduced thermotolerance (Tanabe, M., et al., 1998). We here showed that chicken HSF3, but not chicken HSF1, also induces the expression of the major avian pyrogenic cytokine IL-6 during heat shock (Figure 1) (Marais, M., et al., 2011). Human HSF1 was also able to induce IL-6 expression during heat shock in HSF3-null DT40 cells (Figure 6C), and chicken IL-6 promoter activity was induced during heat shock not only in avian cells, but also in human cells (Figure 4A). Although functional HSE (HSE7) in the IL-6 promoter does not contain a perfect nGAAn unit, unlike that in the promoters of major HSPs (Figures 4 and 5), these results indicate that the differential regulation of IL-6 expression in mammals and birds is

caused by changes in its promoter sequences during evolution.

IL-6 is one of the main pyrogenic and inflammatory cytokines (Leon, L.R. 2002; Medzhitov, R. 2008), and its expression in mammals is regulated by various transcription factors including the activators, NF-κB and NF-IL6 (Matsusaka, T., et al., 1993), and repressor, ATF3 (Gilchrist, M., et al., 2006). Uniquely, HSF1 plays at least two important roles in the regulation of mammalian IL-6 expression. First, HSF1 constitutively binds to many of its target genes (Trinklein, N.D., et al., 2004), including *IL-6*, which facilitates binding of the other transcription factors to these promoters (Inouye, S., et al., 2007). Therefore, knockout or knockdown of HSF1 may result in increased or decreased expression of IL-6, depending on the cell type (Inouye, S., et al., 2007; Rokavec, M., et al., 2012; Fujimoto, M., et al., 2012). Second, when cells are exposed to elevated temperatures, activated HSF1 directly induces the expression of ATF3, a repressor of inflammatory cytokine genes including *IL-6* (Takii, R., et al., 2010). Because IL-6 itself generates fever or high temperature, this HSF1-ATF3 pathway constitutes a feedback mechanism of the febrile response in mammals (Takii, R., et al., 2010). Conversely, we first demonstrate in birds that an avian master regulator HSF3 directly binds to and robustly activates *IL-6*, an avian pyrogenic cytokine gene (Marais, M., et al., 2011) when cells are exposed to a high temperature (Figures 2, Hotchkiss, R., et al., 1993; Chu, E.K., et al., 1997). Our results further emphasize the roles of HSFs in regulating vertebrate febrile and inflammatory responses.

6. Conclusions

1. Il-6 expression is induced during heat shock in chicken cells.
2. Chicken HSF3 directly binds to and activates the *IL-6* gene during heat shock.
3. Regulation of IL-6 and other febrile response components evolutionally diverged in mammals and birds.

7. Perspective

Analysis of the components of the febrile response mechanism revealed that the expression of not only IL-6, but also the other pyrogenic cytokine IL-1β, is heat-inducible in chicken cells (Figure 6). In contrast, expression of the negative regulator ATF3 was not induced during heat shock (Figure 6), and the ATF/CRE element, which exists in human and mouse IL-6 promoters, was not conserved in the chicken IL-6 promoter (Figure 1). Taken together, these results strongly suggest that the febrile response is exacerbated at least by the feed-forward circuit composed of the HSF3-IL-6 pathway in birds, which prefer relatively high body temperatures. Body temperatures in birds vary between 35 and 41 ° C during a resting phase and between 40 and 47 ° C during a highly active phase (see below; figure 7) (Prinzinger, R., et al., 1991). If activated chicken HSF3, similar to mammalian HSF1, can suppress pyrogenic cytokines, body temperature may not be so high during active phases. Furthermore, the febrile response in birds was shown to be significantly affected by ambient temperature, and high ambient temperatures during fever could lead to harmful body temperatures (Gray, D.A., et al., 2013). This exacerbated febrile response in birds may also be partly due to the unique

feed-forward circuit, as similar elevations in body temperature markedly induce the heat shock response in chicken (Mahmoud, K.Z., et al., 2003). The acquisition of these high body temperatures during avian evolution, which supports sustained activity to fly or run promptly (Bennett, A.F., et al., 1979), may have to be accompanied by marked regulatory changes in the components of the febrile response mechanism, as well as the functional diversification of HSF1 and HSF3 (Fujimoto et al., 2010; Inouye, S., et al., 2003).

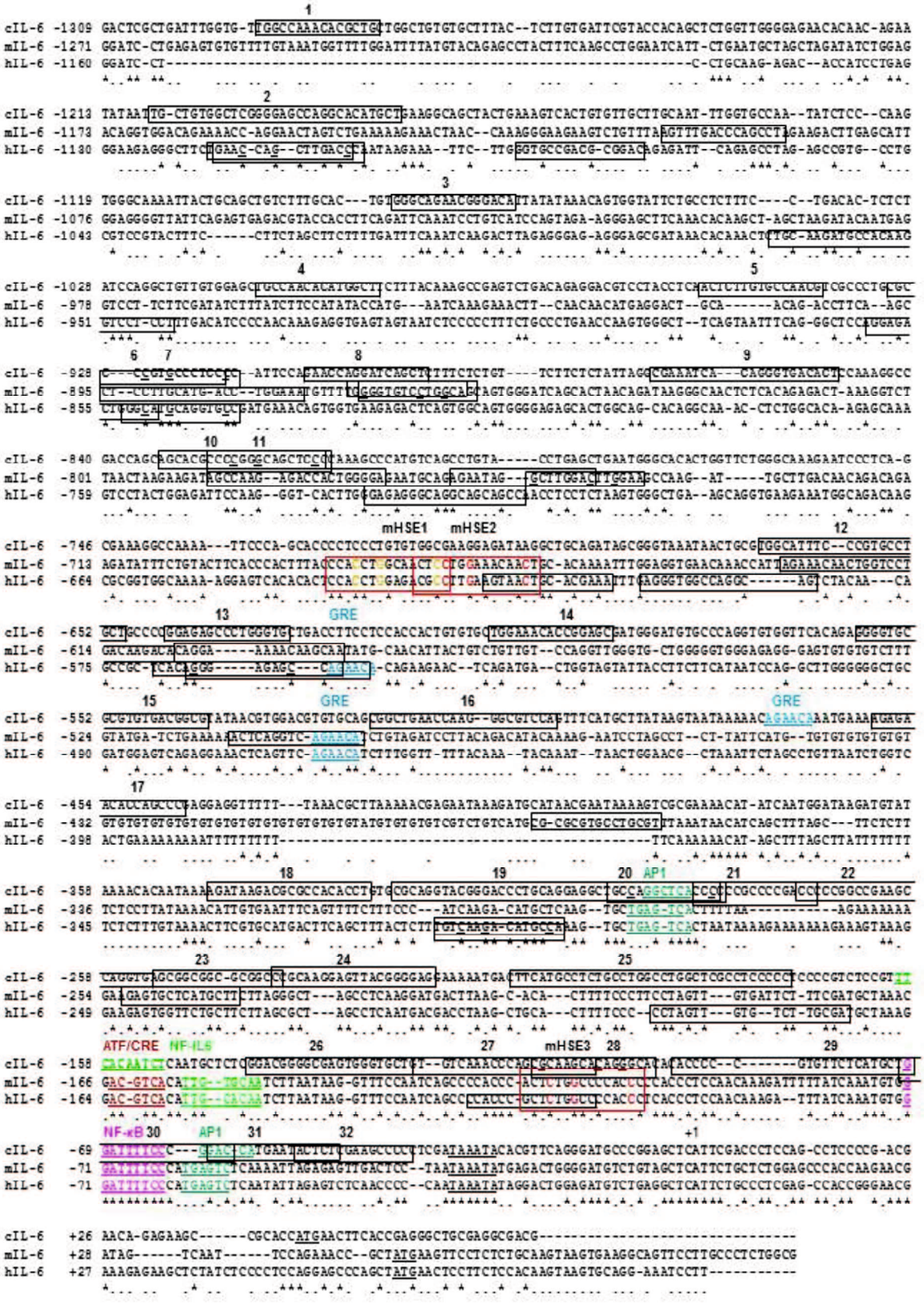
Vertebrate IL-6 promoters
cIL-6 -1309
mIL-6 -1271
hIL-6 -1160
cIL-6 -1213
mIL-6 -1173
hIL-6 -1130
cIL-6 -1119
mIL-6 -1076
hIL-6 -1043
cIL-6 -1028
mIL-6 -978
hIL-6 -951
cIL-6 -928
mIL-6 -895
hIL-6 -855
cIL-6 -840
mIL-6 -801
hIL-6 -759
mHSE1
mHSE2
cIL-6 -746
mIL-6 -713
hIL-6 -664
GRE
cIL-6 -652
mIL-6 -614
hIL-6 -575
GRE
GRE
cIL-6 -552
mIL-6 -524
hIL-6 -490
AP1
cIL-6 -458
mIL-6 -432
hIL-6 -398
cIL-6 -358
mIL-6 -336
hIL-6 -345
ATF/CRE
NF-IL6
mHSE3
cIL-6 -158
mIL-6 -166
hIL-6 -164
NF-κB
AP1
+1
cIL-6 -69
mIL-6 -71
hIL-6 -71
cIL-6 +26
mIL-6 +28
hIL-6 +27

Fig. 1. Nucleotide sequence alignment of vertebrate IL-6 promoters.

Nucleotide sequences of chicken (-1307 to +74), mouse (-1271 to +101), and human (-1160 to +101) IL-6 promoters from Ensembl genome databases (*http://asia.ensembl.org/index.html*) were aligned with GENETYX software (GENETYX Co., Tokyo). Identical (*) and semi-conserved (.) sequences are shown among all three species. The black boxes indicate HSE-like sequences, which consists of at least three inverted repeats of nGAAn (the G nucleotide must be conserved). There are 32 HSE-like sequences in the chicken IL-6 promoter within -1307 bp. The red boxes indicate three HSE-like sequences (mHSE1 to mHSE3), which are conserved in the mouse and human promoters [17], but not in the chicken promoter. Transcription factor binding sites, AP1 (Fos/Jun), NF-κB, NF-IL6, ATF/CRE, and GRE, are shown by underlines.

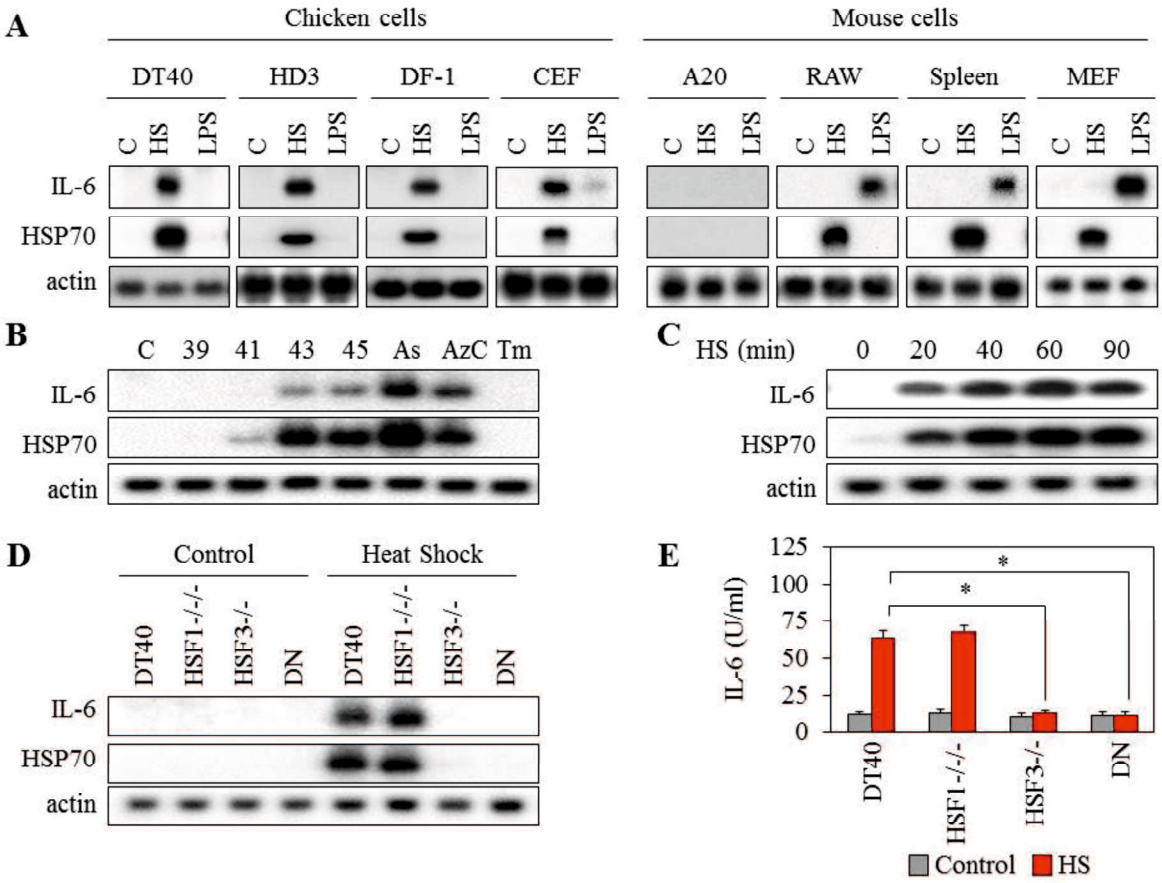

Fig. 2. IL-6 expression is induced during heat shock in chicken cells.

A, Chicken and mouse cells were untreated (C), or treated with heat shock at 45°C (chicken DT40, HD3, DF-1, and CEF cells) or 42°C (mouse A20, Raw, MEF, and Spleen cells) for 1 h (HS) or LPS (1 μg/ml) for 6 h. Total RNA was isolated and Northern blot analysis was performed using ^{32}P-labelled cDNA probes for chicken IL-6 and HSP70 (chicken cells), and those for mouse IL-6 and Hsp70-1 (mouse cells). A cDNA probe for mouse β-actin was used to detect β-actin mRNA in both mouse and chicken cells.

B, DT40 cells maintained at 37°C were treated with heat shock at 39°C, 41°C, 43°C, or 45°C for 1 h, sodium arsenite (50 μM) for 8 h (As), L-azetidine-2-carboxylic acid (5 mM) for 8 h (AzC), and tunicamycin (2 μg/ml) for 8 h (Tm). Northern blot analysis was performed as described above.

C, DT40 cells were treated with heat shock at 45°C for indicated periods. Northern blot analysis was performed as described above.

D, Northern blot analysis was performed using untreated (Control) or heat-shocked wild-type DT40, HSF1-/-/- (#59), HSF3-/- (#21), and double-null (HSF1-/-/-; HSF3-/-, #54) cells [26].

E, Cells described in D were untreated (C) or heat-shocked at 45°C for 1 h (HS), and allowed to recover at 37°C for 8 h. The production of IL-6 in the cultured medium was assessed by

determining IL-6 activity. Error bars show the mean ± s.d. (n = 3). Asterisks indicate *$p < 0.01$ determined using an unpaired *t*-test.

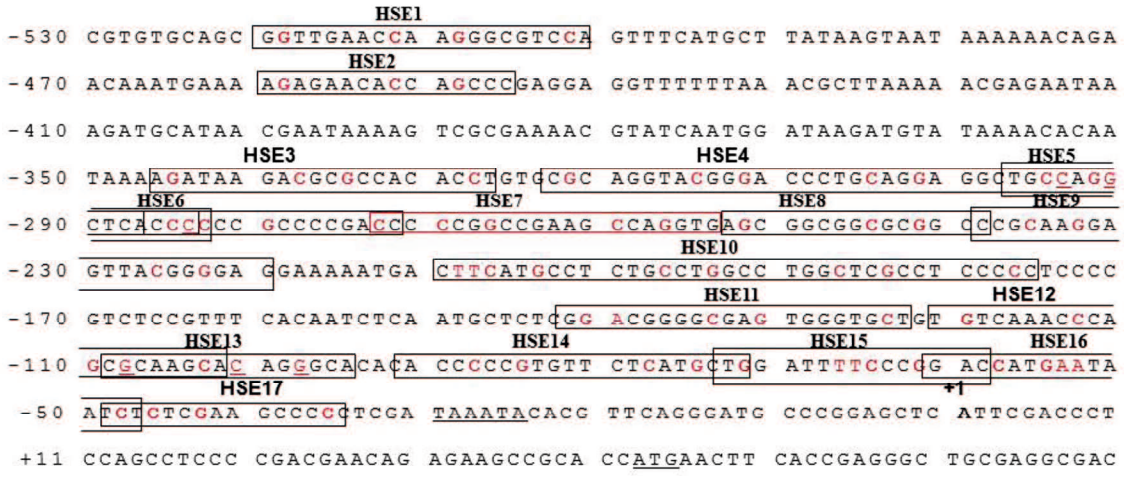

Fig. 3. Nucleotide sequence of the chicken IL-6 promoter, which was used for the reporter assay.

The chicken IL-6 promoter (-530 to +71) was molecularly cloned, and its nucleotide sequence was determined. Boxes indicate 17 HSE-like sequences within this region. Among them, HSE7 (red box) was responsible for the heat-mediated activation of chicken IL-6 promoter activity. The TATA box and initiation codon are marked by underlines. The transcription start sites are shown by +1.

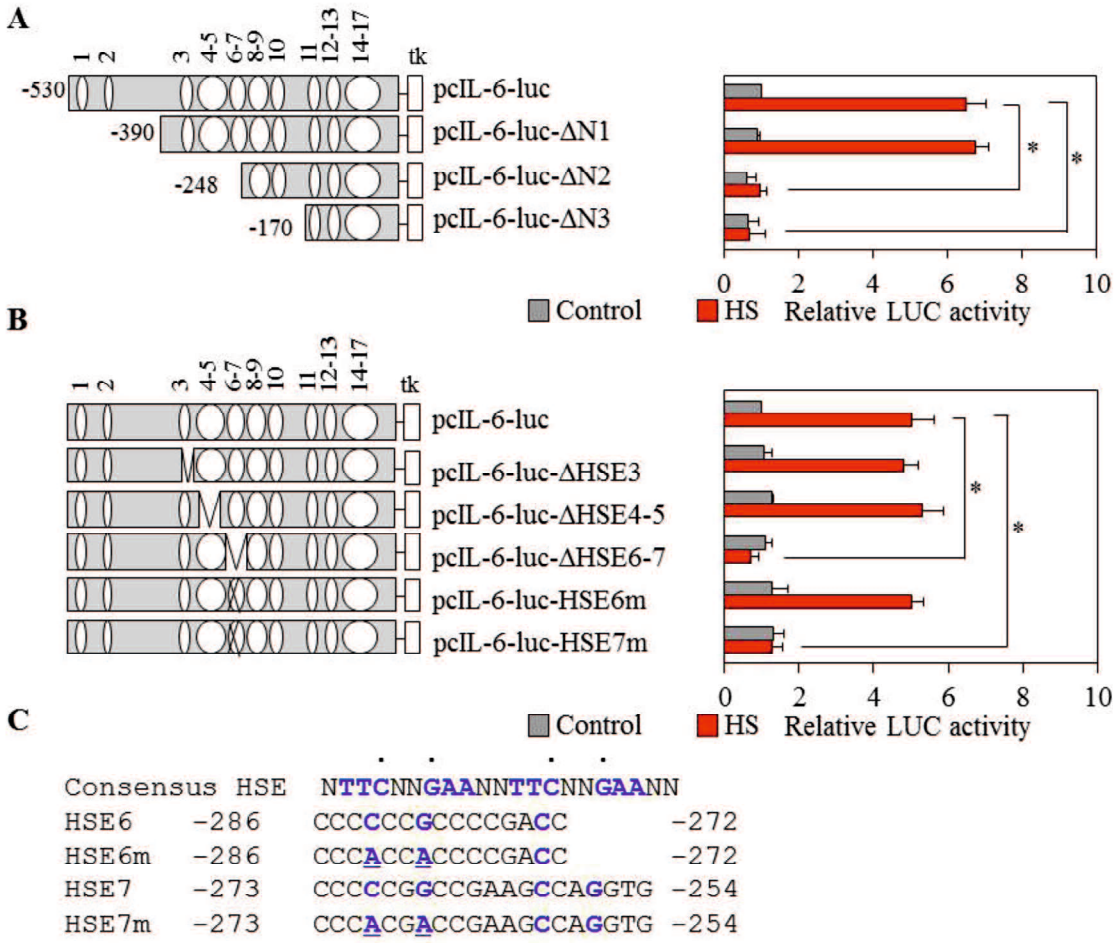

Fig. 4. Identification of HSE in the chicken IL-6 promoter.

A, Reporter plasmids were transfected into QT6 cells for 48 h. Cells were untreated (Control, open bars) or treated with heat shock at 43°C for 1 h and allowed to recover for 12 h (HS, black bars). LUC activity relative to that in untreated cells is shown (right). Error bars show the mean ± s.d. (n = 3). Asterisks indicate *$p < 0.01$ determined using an unpaired *t*-test. Putative HSEs (HSE1 to HSE17) in the cIL-6 promoter within -530 from a transcription start site is shown (left).

B, Reporter analysis was performed as described in A, using cIL-6 reporter plasmids having deletion (pcIL-6-luc-ΔHSE3, ΔHSE4-5, ΔHSE6-7) and point mutations (pcIL-6-luc-6m and pcIL-6-luc-7m).

C, Nucleotide sequences of the HSE6, HSE7, and their mutants.

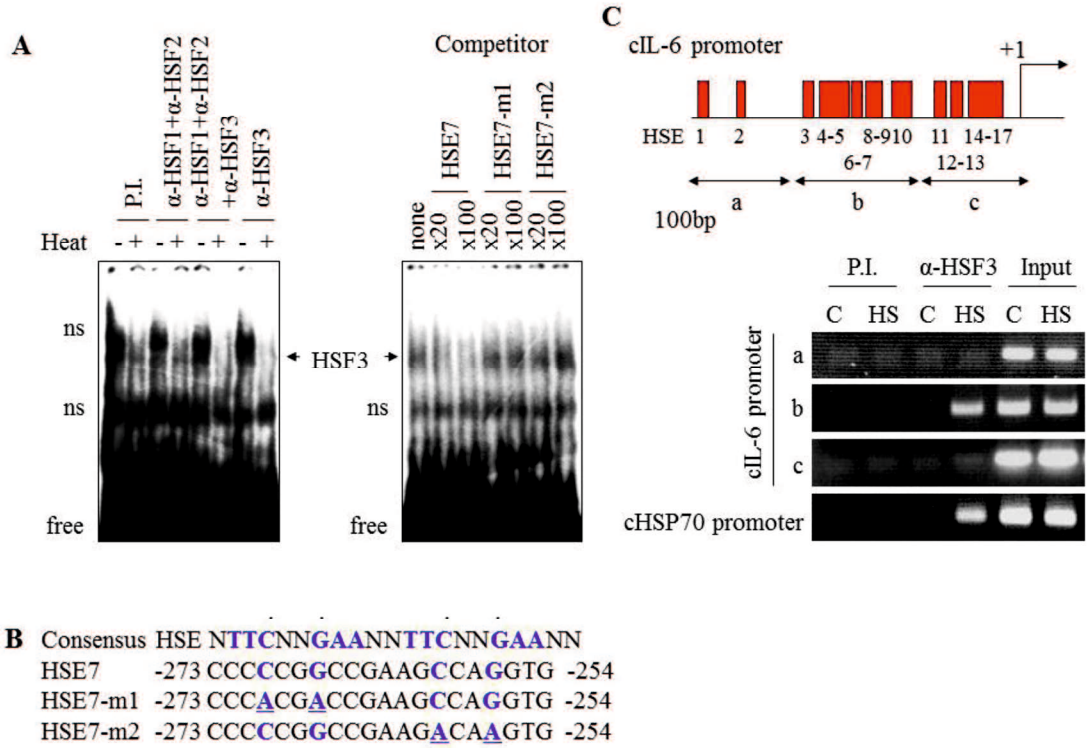

Fig. 5. HSF3 binds directly to the chicken IL-6 promoter.

A, Whole cell extracts were prepared from DT40 cells treated without (-) or with (+) heat shock at 45°C for 1 h. EMSA was performed using a ^{32}P-labeled HSE7 probe in the presence of pre-immune serum (P.I.), α-cHSF1γ, α-cHSF2δ, or α-cHSF3γ [26] (left). The HSF3-HSE7 complex was detected when an extract from heat-shocked cells was mixed with the HSE7 probe, and was competed with unlabelled oligonucleotides for HSE7 or its mutant (HSE7-m1 or HSE7-m2) [16] (right). Arrows indicate HSE7-HSF3 complexes. Nonspecific binding activities are indicated as "ns", and free probes as "free".

B, Unique nucleotide sequences of the HSE7 and its mutants are shown.

C, DT40 cells were untreated (C) or treated with heat shock at 45°C for 1 h (HS). ChIP assay was performed using the antibody for chicken HSF3 (α-cHSF3γ), and DNA fragments of cIL-6 promoter (region a, +15 to -176; region b, -179 to -409; region c, -363 to -519) as well as that of chicken HSP70 (-38 to -240) were amplified by PCR.

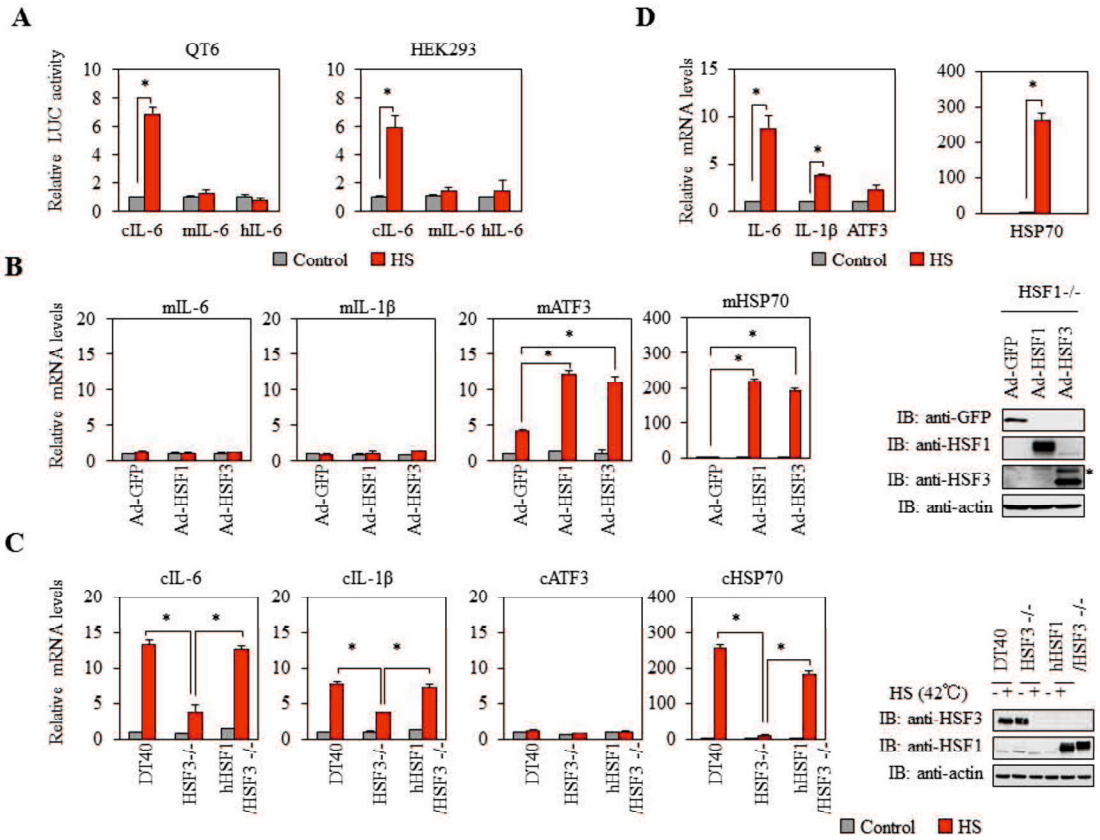

Fig. 6. Chicken HSF3 induces IL-6 expression during heat shock.

A, Reporter analysis of the chicken, mouse, and human IL-6 promoters. QT6 cells (left) and HEK293 cells (right) were transfected for 48 h with a reporter plasmid pcIL-6-luc (-530 to +71), pmIL-6-luc (-1265 to + 69), or phIL-6-luc (-1154 to +100). Cells were untreated (Control, open bars) or treated with heat shock (HS, black bars) at 43°C (QT6 cells) or 42°C (HEK293 cells) for 1 h, and allowed to recover at 37°C for 12 h. Luciferase (LUC) activities of each reporter plasmid relative to that in untreated cells are shown. Error bars show the mean ± s.d. (n = 3). Asterisks indicate *$p < 0.01$ determined using an unpaired *t*-test.

B, HSF1-/- immortalized MEF cells were infected with Ad-cHSF3, Ad-hHSF1, or Ad-GFP for 48 h, and untreated or treated with heat shock at 42°C for 1 h. The mRNA levels of mIL-6, mIL-1β, mATF3, and mHSP70 were quantified by RT-qPCR, and levels relative to those in Ad-GFP-infected untreated cells are shown. Error bars show the mean ± s.d. (n = 3). Asterisks indicate *$p < 0.01$ determined using an unpaired *t*-test. Extracts of these cells were prepared and subjected to Western blotting using an antibody for HSF1 (α-mHSF1j), HSF3 (α-cHSF3γ), GFP, or α-actin.

C, Wild-type DT40, HSF3-/- (#21), and hHSF1/HSF3-/- (#–Ch6) cells were untreated (Control, open bars) or treated with heat shock at 45°C for 1 h (HS, black bars). The mRNA levels were quantified and levels relative to those in untreated wild-type DT40 cells are shown as described

in B. Western blotting was also performed as described in B.

D, Expression of IL-6, IL-1β, and ATF3 in chicken embryos in vivo during heat shock. Chicken embryos at day 6 were untreated or treated with heat shock at 45^{o}C for 30 min. Total RNA from the bodies of embryos was isolated and each mRNA level was quantified by RT-qPCR. The mRNA levels relative to those in untreated embryos are shown. Error bars show the mean ± s.d. (n = 3). Asterisks indicate *$p < 0.001$ determined using an unpaired *t*-test.

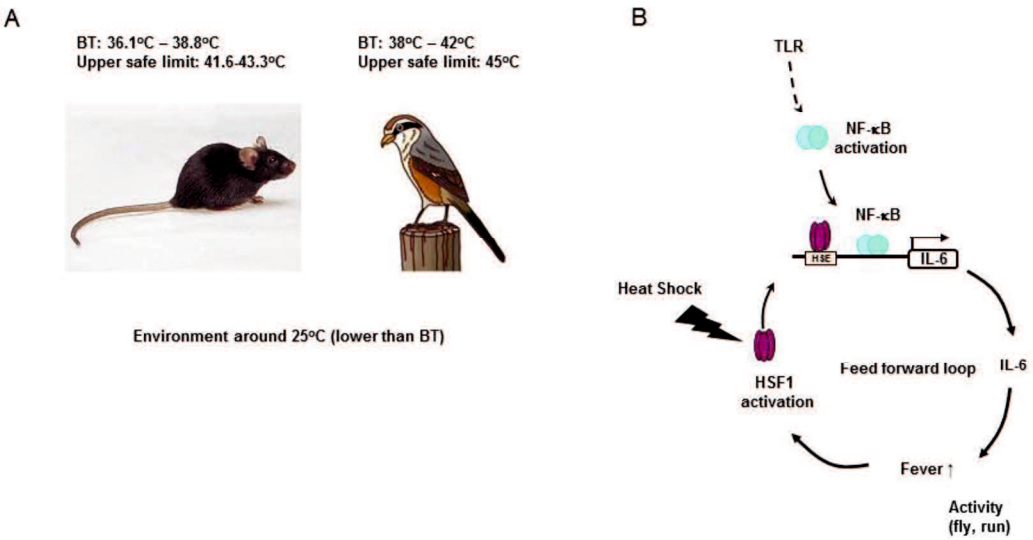

Fig.7. Proposed model

A, Endothermic organisms body temperature (BT). Endothermic organisms maintain BT higher than surrounding environment. Birds keep body temperature always higher than mammals during resting and active phases. When, mammalian body temperature cross upper safe level, HSF1 protects febrile response by suppressing inflammatory cytokines. In birds, HSF3 exacerbated febrile response.

B, Avian master regulator chicken HSF3 accelerates expression of pyrogenic cytokines. In birds, chicken HSF3 directly binds to and activates IL-6 expression during heat shock. The enhanced IL-6 expression leads febrile response by feed forward circuit loop. TLR pathway also expected to activate IL-6 through NF-kB activation.

References

1) Bennett, A.F., and Ruben, J.A. (1979) Endothermy and activity in vertebrates. Science 206, 649-54.

2) Mackowiak, P.A. (1998) Concepts of fever. Arch. Intern. Med. 158, 1870-1881.

3) Leon, L.R. (2002) Invited review: cytokine regulation of fever: studies using gene knockout mice. J. Appl. Physiol. 92, 2648-2655.

4) Hotchkiss, R., Nunnally, I., Lindquist, S., Taulien, J., Perdrizet, G., and Karl, I. (1993) Hyperthermia protects mice against the lethal effects of endotoxin. Am. J. Physiol. 265, R1447-1457.

5) Chu, E.K., Ribeiro, S.P., and Slutsky, A.S. (1997) Heat stress increases survival rates in lipopolysaccharide-stimulated rats. Crit. Care Med. 25, 1727-1732.

6) Kappel, M., Diamant, M., Hansen, M.B., Klokker, M., and Pedersen, B.K. (1991) Effects of in vitro hyperthermia on the proliferative response of blood mononuclear cell subsets, and detection of interleukins 1 and 6, tumour necrosis factor-α and interferon-γ. Immunology 73, 304-308.

7) Fouqueray, B., Philippe, C., Amrani, A., Perez, J., and Baud, L. (1992) Heat shock prevents lipopolysaccharide-induced tumor necrosis factor-α synthesis by rat mononuclear

phagocytes. Eur. J. Immunol. 22, 2983-2987.

8) Ensor, J.E., Wiener, S.M., McCrea, K.A., Viscardi, R.M., Crawford, E.K., and Hasday, J.D. (1994) Differential effects of hyperthermia on macrophage interleukin-6 and tumor necrosis factor-α expression. Am. J. Physiol. 266, C967-974.

9) Kluger, M.J., Rudolph, K., Soszynski, D., Conn, C.A., Leon, L.R., Kozak, W., Wallen, E.S., and Moseley, P.L. (1997) Effect of heat stress on LPS-induced fever and tumor necrosis factor. Am. J. Physiol. 273, R858-863.

10) Powers, E. T., Morimoto, R. I., Dillin, A., Kelly, J. W. & Balch, W. E. Biological and chemical approaches to diseases of proteostasis deficiency. Annu. Rev. Biochem. 78, 959–991 (2009).

11) Akerfelt, M., Morimoto, R.I., and Sistonen, L. (2010) Heat shock factors: integrators of cell stress, development and lifespan. Nat. Rev. Mol. Cell Biol. 11, 545-55.

12) Fujimoto, M., and Nakai, A. (2010) The heat shock factor family and adaptation to proteotoxic stress. FEBS J. 277, 4112-25.

13) Xiao, X., Zuo, X., Davis, A.A., McMillan, D.R., Curry, B.B., Richardson, J.A., and Benjamin, I.J. (1999) HSF1 is required for extra-embryonic development, postnatal growth and protection during inflammatory responses in mice. EMBO J. 18, 5943-5952.

14) Singh, I.S., He, J.R., Calderwood, S., and Hasday, J.D. (2002) A high affinity HSF-1 binding site in the 5'-untranslated region of the murine tumor necrosis factor-α gene is a transcriptional repressor. J. Biol. Chem. 277, 4981-4988.

15) Xie, Y., Chen, C., Stevenson, M.A., Auron, P.E., and Calderwood, S.K. (2002) Heat shock factor 1 represses transcription of the IL-1β gene through physical interaction with the nuclear factor of interleukin 6. J. Biol. Chem. 277, 11802-11810.

16) Inouye, S., Izu, H., Takaki, E., Suzuki, H., Shirai, M., Yokota, Y., Ichikawa, H., Fujimoto, M., and Nakai, A. (2004) Impaired IgG production in mice deficient for heat shock transcription factor 1. J. Biol. Chem. 279, 38701-38709.

17) Inouye, S., Fujimoto, M., Nakamura, T., Takaki, E., Hayashida, N., Hai, T., and Nakai, A. (2007) Heat shock transcription factor 1 opens chromatin structure of interleukin-6 promoter to facilitate binding of an activator or a repressor. J. Biol. Chem. 282, 33210-33217.

18) Takii, R., Inouye, S., Fujimoto, M., Nakamura, T., Shinkawa, T., Prakasam, R., Tan, K., Hayashida, N., Ichikawa, H., Hai, T., and Nakai, A. (2010) Heat shock transcription factor 1 inhibits expression of IL-6 through activating transcription factor 3. J. Immunol. 184, 1041-1048.

19) Janus, P., Pakuła-Cis, M., Kalinowska-Herok, M., Kashchak, N., Szołtysek, K., Pigłowski, W., Widlak, W., Kimmel, M., and Widlak, P. (2011) NF-κB signaling pathway is inhibited by heat shock independently of active transcription factor HSF1 and increased levels of

inducible heat shock proteins. Genes Cells 16, 1168-11675.

20) Chen, S., Zuo, X., Yang, M., Lu, H., Wang, N., Wang, K., Tu, Z., Chen, G., Liu, M., Liu, K., and Xiao, X. (2012) Severe multiple organ injury in HSF1 knockout mice induced by lipopolysaccharide is associated with an increase in neutrophil infiltration and surface expression of adhesion molecules. J. Leukoc. Biol. 92, 851-857.

21) Ambade, A., Catalano, D., Lim, A., and Mandrekar, P. (2012) Inhibition of heat shock protein (molecular weight 90 kDa) attenuates proinflammatory cytokines and prevents lipopolysaccharide-induced liver injury in mice. Hepatology 55, 1585-1595.

22) Gilchrist, M., Thorsson, V., Li, B., Rust. A.G., Korb, M., Roach, J.C., Kennedy, K., Hai, T., Bolouri, H., and Aderem, A. (2006) Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4. Nature 441, 173-178.

23) Mackowiak P.A. (1998) Concepts of fever. Arch Intern Med. 158(17):1870-81.

24) Marais, M., Maloney, S.K., and Gray, D.A. (2011) Brain IL-6- and PG-dependent actions of IL-1β and lipopolysaccharide in avian fever. Am. J. Physiol. Regul. Integr. Comp. Physiol. 301, R791-800.

25) Rodbard, S. (1950) Weight and body temperature. Science 111, 465-466.

26) Nakai, A., and Ishikawa, T. (2001) Cell cycle transition under stress conditions controlled by vertebrate heat shock factors. EMBO J. 20, 2885-95.

27) Tanabe, M., Kawazoe, Y., Takeda, S., Morimoto, R. I., Nagata, K., and Nakai, A. (1998) Disruption of the HSF3 gene results in the severe reduction of heat shock gene expression and loss of thermotolerance. EMBO J. 17, 1750-1758.

28) Nakai, A., Kawazoe, Y., Tanabe, M., Nagata, K., and Morimoto, R. I. (1995) The DNA-binding properties of two heat shock factors, HSF1 and HSF3 are induced in the avian erythroblast cell line HD6. Mol. Cell. Biol. 15, 5168-5178.

29) Beug, H., Palmieri, S., Freudenstein, C., Zentgraf, H., and Graf, T. (1982) Hormone -dependent terminal differentiation in vitro of chicken erythroleukemia cells transformed by *ts* mutants of avian erythroblastosis virus. Cell 28, 907-919.

30) Himly, M., Foster, D.N., Bottoli, I., Iacovoni, J.S., and Vogt, P.K. (1998) The DF-1 chicken fibroblast cell line: transformation induced by diverse oncogenes and cell death resulting from infection by avian leukosis viruses. Virology 248, 295-304.

31) Fujimoto, M., Oshima, K., Shinkawa, T., Wang, B., Inouye, S., Hayashida, N., Takii, R., and Nakai, A. (2008) Analysis of HSF4 binding regions reveals its necessity for gene regulation during development and heat shock response in mouse lenses. J. Biol. Chem. 283, 29961-29970.

32) Fujimoto, M., Hayashida, N., Katoh, T., Oshima, K., Shinkawa, T., Prakasama, R., Tan, K., Inouye, S., Takii, R., and Nakai, A. (2010) A novel mouse HSF3 has the potential to activate nonclassical heat-shock genes during heat shock. Mol. Biol. Cell 21, 106-116.

33) Van Snick, J., Cayphas, S., Vink, A., Uyttenhove, C., Coulie, P.G., Rubira, M.R., and Simpson, R.J. (1986) Purification and NH2-terminal amino acid sequence of a T-cell-derived lymphokine with growth factor activity for B-cell hybridomas. Proc. Natl. Acad. Sci. USA 83, 9679-9683.

34) Kawazoe, Y., Tanabe, M., Sasai, N., Nagata, K., and Nakai, A. (1999) HSF3 is a major heat shock responsive factor during chicken embryonic development. Eur. J. Biochem. 265, 688-697.

35) Kaiser, P., Rothwell, L., Goodchild, M., and Bumstead, N. (2004) The chicken proinflammatory cytokines interleukin-1beta and interleukin-6: differences in gene structure and genetic location compared with their mammalian orthologues. Anim. Genet. 35, 169-175.

36) Inouye, S., Katsuki, K., Izu, H., Fujimoto, M., Sugahara, K., Yamada, S., Shinkai, Y., Oka, Y., Katoh, K., and Nakai, A. (2003) Activation of heat shock genes is not necessary for protection by heat shock transcription factor 1 against cell death due to a single exposure to high temperatures. Mol. Cell. Biol. 23, 5882-5895.

37) Medzhitov, R. (2008) Origin and physiological roles of inflammation. Nature 454, 428-435.

38) Matsusaka, T., Fujikawa, K., Nishio, Y., Mukaida, N., Matsushima, K., Kishimoto, T., and Akira, S. (1993) Transcription factors NF-IL6 and NF-kappa B synergistically activate

transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. Proc. Natl. Acad. Sci. USA 90, 10193-10197.

39) Trinklein, N.D., Murray, J.I., Hartman, S.J., Botstein, D., and Myers, R.M. (2004) The role of heat shock transcription factor 1 in the genome-wide regulation of the mammalian heat shock response. Mol. Biol. Cell 15, 1254-1261.

40) Rokavec, M., Wu, W., and Luo, J.L. (2012) IL6-mediated suppression of miR-200c directs constitutive activation of inflammatory signaling circuit driving transformation and tumorigenesis. Mol. Cell 45, 777-789.

41) Fujimoto, M., Takaki, E., Takii, R., Tan, K., Prakasam, R., Hayashida, N., Iemura, S., Natsume, T., and Nakai, A. (2012) RPA assists HSF1 access to nucleosomal DNA by recruiting histone chaperone FACT. Mol. Cell 48, 182-194.

42) Prinzinger, R., Preßmar, A., and Schleucher, E. (1991) Body temperature in birds. Comp. Biochem. Physiol. 99A, 499-506.

43) Gray, D.A., Marais, M., and Maloney, S.K. (2013) A review of the physiology of fever in birds. J. Comp. Physiol. B 183, 297-312.

44) Mahmoud, K.Z., Edens, F.W., Eisen, E.J., Havenstein, G.B. (2003) Effect of ascorbic acid and acute heat exposure on heat shock protein 70 expression by young white Leghorn chickens. Comp. Biochem. Physiol. C 136, 329-335.

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