

Drosophila DAXud1: A New Element in Transcriptional Pausing Complex Stabilization

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Abstract

Background

A rapid transcriptional response under an acute stimulus is common in all cellular systems and is an adaptation that allows tolerance to environmental changes. A gene group that has been studied because of its fast response and cytoprotective effects are the *hsp* genes (encoding Heat Shock Proteins (HSPs), conserved chaperones). Under normal conditions, the mRNA and protein levels of the main *hsp* genes are low but they increase rapidly upon heat shock (HS). This is achieved due to the presence of an RNA Polymerase II pausing complex located +30-50 bp from TSS. This complex maintains a partially synthesized RNA strand of said length, poised to resume synthesis, and undergoes subsequent transcriptional inactivation to restore transcript levels after environmental stabilization.

Methods

The Gal4/UAS system was used to modify *dAxud1* expression in a tissue specific manner. A DAxud1-GFP fusion was expressed in salivary glands to perform polytene chromosome immunofluorescence and chromatin immunoprecipitation. DAxud1 genome occupancy data was achieved expressing Dam-DAxud1 in imaginal wing discs using Gal4/UAS (TaDa-seq).

Results

Using TaDa-seq, we demonstrate that DAxud1 protein is present mainly near the TSS of significant occupied genes, most frequently in the first intron. This results also revealed DAxud1 is present in *hsp* genes, mainly in promoter zone. Following these results, we found that, under *dAxud* knockdown, larvae and adult flies have a diminished thermotolerance, despite showing an increase in *hsp* transcripts in larval tissues. We performed polytene chromosome immunofluorescence for DAxud1-GFP, revealing extensive, but dynamic localization on chromatin in *hsp70* loci. This was confirmed with chromatin immunoprecipitation. We also found that DAxud1 overexpression leads to an enrichment of RNA Polymerase II at the 5' end of the *hsp70* gene, with a decrease in its transcripts. Importantly, we show interaction of DAxud1 with NELF-B, a component of the transcriptional pausing complex, and knockdown of both genes individually has similar effects on *hsp70* transcription.

Conclusion

DAxud1 protein is a component of chromatin, that relocates under stress conditions such as heat shock, playing a role in maintaining RNA Polymerase II stalled at the 5' of *hsp70*, possibly through a pausing mechanism based on its interaction with NELF-B.

Introduction

In natural environments, organisms are exposed to different stresses that affect their survival. During evolution, different mechanisms have emerged to respond to stress, some of them widely conserved

among various kingdoms, but with differences in their temporal regulation and cell types involved. One of the conserved mechanisms for responding to stress is rapid biosynthesis of Heat Shock Proteins (HSPs), in which their mRNA levels can rise several fold in a few minutes, mainly due to transcriptional promoting elements preloaded at the core promoter and within the 5' end of the gene body [1, 2]. HSPs were initially associated with heat stress responses. Further research revealed that these proteins also behave as important cytoprotectors under other stress conditions, including oxidative stress, accumulation of protein aggregates, an acidic environment, and osmolarity changes [3]. In animals, *hsp* transcriptional induction is very fast; the *hsp70* promoter recruits specific factors in response to stress conditions [4], resulting in significant rise of *hsp70* mRNA in less than 5 minutes [5]. This recruitment during heat shock is commanded mainly by HSF (Heat Shock Factor), a transcriptional activator, one of five known chromatin proteins involved in the heat stress transcriptional response, that induces Hsp70 and other small HSPs (Hsp27, Hsp26) after acute thermal stress. However, the repertoire of chromatin associated proteins varies for other stress conditions and the differences are still poorly understood [6–8]. We will focus mainly on the *hsp70* paralogs because there is significant knowledge implicating the chromatin state of their promoters and the pausing phenomenon [9–14], as well as additional factors regulating their efficient transcriptional induction.

In *Drosophila melanogaster*, the control of *hsp70* transcription depends on at least 12 proteins that relocate to the *hsp70* loci after two minutes of exposure to 37°C in 3rd instar larvae [4, 13–16]. These proteins participate regulating chromatin condensation, recruiting transcription factors like HSF, engaging in transcriptional pausing, and controlling the initiation/elongation of *hsp70* transcription. In *hsp70* genes, transcriptional pausing is crucial for a quick transcriptional response upon stress or extracellular signaling. When pausing, RNA Polymerase II elongates only 30-50 bp from the transcription start site (TSS) and remains in that position until induced, where it resumes elongation and provides a transcriptional burst of the stress response genes [14, 17]. Transcriptional pausing is mediated by the NELF (NELF A, B, C/D, E) and DSIF (Spt5, Spt6) complexes, which retain RNA Polymerase II. After induction by heat shock, HSF recruits the p-TEFb kinase that phosphorylates NELF, DSIF and RNA Polymerase II, causing complete dissociation of NELF and the and the escape of RNA Polymerase, with DSIF acting as an elongation factor [4, 18]. In this work, we present evidence that introduces DAXud1 as an additional regulatory factor that participates in *hsp* transcription regulation, through its positioning at the transcription start site (TSS) and by stabilizing the pausing complex via its interaction with NELF and DSIF complexes.

The *dAxud1* gene is the only *Drosophila* ortholog of the human and mice CSRNP protein family (Cysteine Serine Rich Nuclear Proteins). Members of this protein family, which are conserved only in metazoans, have been proposed to be putative transcription factors with specific roles in apoptosis and neural development [19–21]. Initial studies proposed a physiological function for one of these proteins as a tumor suppressor in human tissues [19]. Further characterization of its ortholog in *Drosophila* suggested that its proapoptotic function relies on the activation of the JNK pathway [21]. Other studies indicate a strong association of the CSRNP family with stress responses as their mRNAs rise in cells

exposed to different kinds of stressful stimuli such as cold [22], oxidative stress [23, 24], bacterial infection [25], pressure [26], and acute stress by sprint running in skeletal muscle [27]. These observations prompted us to analyze the molecular bases of the adaptive and non-adaptive roles of DAxud1 during the stress response. Here, in a screen using whole-genome occupancy analysis (TaDa-seq), we reveal how DAxud1 has a connection to the general stress response by analyzing its occupancy of the *hsp70 locus*. In this gene, DAxud1 associates with the Pol II pausing complex through its interaction with the NELF complex and thereby directly influences *hsp* transcription during the stress response.

Methods

Fly stocks

Loss of function was achieved by expressing *dAxud1* RNAi through the GAL4/UAS system using the Vienna stock V26479. For the experiment for polytene chromosomes, we used flies with the genotype *nub>Gal4/Y; UAS-dAxud1::GFP/+*. For DamID-seq experiments (TaDa-seq), the genotypes were *nub>Gal4/Y; UAS-mCherry.Dam* and *nub>Gal4; UAS-mCherry.Dam::dAxud1*, both generated from the plasmid pUAST-attb-LT3-Dam donated by the Andrea Brand Laboratory [41]. The Dam fusion plasmids were injected with pBS130 that express the *phi*-131 integrase. The genotype of injected flies was *P{y[+t7.7]=CaryP}attP2* (Bloomington stock 8622), in accordance with methods in the previous reference [18]. For HA-NELF-B experiments in imaginal wing discs protein extract, stock F003904 from FlyORF was used for expression under the UAS/Gal4 system.

Lifespan assay

For every replicate, between 80-100 flies were collected no longer than 48 hours from pupae eclosion and separated into males and females. Three replicates for every condition were used, and dead and live flies were counted every day. The control condition was at 29°C, with the flies changed from the tubes every two days. The heat shock condition was at 37°C for 30 minutes every day.

Immunofluorescence in polytene chromosomes

The squash protocol and antibody staining for polytene chromosomes were performed according to the Johanssen protocol [79]. The GFP antibody used was Cell Signaling (code D5.1), dilution 1:250, and the RNA Polymerase I α antibody (full phosphorylated CTD) used was Abcam (ab5408), dilution 1:1000. DAPI stain was used at 1:10000. The glands were dissected from larvae with *nub>Gal4; UAS-dAxud1::GFP* genotype.

TaDa-seq

In total, 100 to 120 imaginal wing discs per sample were dissected from third instar larvae (*nub>Gal4; UAS-mCherry.Dam* & *nub>Gal4; UAS-mCherry.Dam::dAxud1*). The larvae grew for 7 days at 17°C and were then placed in 29°C for 24 hours, to the third instar stage. The discs were dissected and placed in PBS 1X, and the DNA was extracted according to the Southall protocol [41]. Accordingly, two biological replicates

for every condition (Dam and Dam-DAxud1) were used to generate libraries, sequenced in Illumina HiSeq-2500, single end 100 bp. The data were processed with the pipeline designed and published by Marshall [80] using the dm6 *Drosophila* genome version (release 6.22). The replicates were merged and data was generated in bedgraph format, according with the pipeline. The GFF files with the coordinates of significant peaks were visualized with the WashU epigenome browser. These data were processed with HOMER [35] for motif discovery and finding. The annotation was done with T-Gene from Meme-Suite [81]. The metagene and heatmap profiles were generated with the deeptools toolbox platform [82].

RNA extraction and qPCR

RNA extraction was carried out with the TriZol reagent following manufacturer instructions. The RNA was resuspended in water and the RT reaction was performed with the iScript® kit from BioRad. The qPCR mix reactions were done with Brilliant II SYBR® reaction solution with 10 minutes of initial denaturation and 40 cycles in the following sequence: 30 seconds at 90°C; 30 seconds at 60°C; and 30 seconds at 72°C. The primers used were as follows: *dAxud1* Fw 5'-AGGGGACCACCAGCCTAAC-3', Rv 5'-GGTTCGCTCTGATTATCCTTGTG-3'; *hsp26* Fw 5'-ATGCCACGATCTGTTCCATC-3', Rv 5'-GTACGCGAATAACGACGAC; *hsp67Bc* Fw 5'-GACTCCCCGGACTCCATGTA, Rv 5'-GACCCAGGGTGTGCAATCAA-3'; *hsp70* for B paralogs (for Ba, Bb and Bc) Fw 5'-AAGAACCTCAAGGGTGAGCG-3', Rv 5'-CGAACAGAGATCCCTCGTCG-3'. For *hsp70B* 5'-UTR Fw 5'-GCTAAGCAAATAACAAGCGCAG-3', Rv 5'-CAGTTGATTTACTTGGTTGCTGGT-3'; *hsp70B* 3'-UTR Fw 5'-GAGGATTTGGCGGCTACTCT-3', Rv 5'-TTTAAAACTTAAGCCAGGAAGTGA-3'; *actin-42A* (as normalizer) Fw 5'-GCGTCGGTCAATTCAATCTT-3', Rv 5'-AAGCTGCAACCTCTTCGTCA-3'

Chip-PCR protocol

Chromatin immunoprecipitation was performed from salivary glands expressing DAxud1-GFP or *dAxud1* RNAi in third instar larvae growth at 25°C using the driver *nubbin>Gal4* (X). Larvae from the heat shock condition were placed at 37°C for twenty minutes. The glands were dissected and processed according to Ghosh et al.'s protocol [54] for three biological replicates per condition. Ten pairs of salivary glands from third-instar larvae were incubated for five minutes on ice in 100 µl of 1% formaldehyde in PBS 1X and then at RT for 7 minutes. The cross-linking reaction was quenched by adding 2.5 M glycine to a final concentration of 125 mM; then the glands were placed on ice for two minutes. They were centrifuged at 900 × *g* for two minutes at 4°C, and the supernatant was removed. A 100-µl volume of sonication buffer (20 mM Tris [pH 8.0], 0.5% SDS, 2 mM EDTA, 0.5 mM EGTA, 0.5 mM phenylmethylsulphonyl fluoride [PMSF]) and 1 µl Halt protease inhibitor cocktail (Thermo Fisher cat. 78430) were added to the glands to incubate at room temperature for ten minutes and then on ice for 10 minutes. The glands were vigorously shaken for ten minutes and homogenized with a small pestle. Lysates were sonicated at 4°C in Omniruptor-4000 at 100% power, 90% pulse for fifteen minutes to shear the DNA to an average fragment size of 400 bp. The lysate was clarified by centrifugation at 14,000 × *g* for 7 minutes. In total, 45 µl per lysate were used for each immunoprecipitation assay (mock and IP), with 10 µl for input. Immunoprecipitation was performed using 1 µl of anti-GFP antibody (Abcam ab290, 5µg for chromatin

from ten pairs of salivary glands) or 30 μ l of RNA Polymerase II antibody (Santa Cruz, 8WG16, 6 μ g for chromatin from ten pairs of salivary glands), diluted half in IP Buffer (50mM Tris-HCl pH 8; 100mM NaCl; 2mM EDTA; 1mM EDTA, 1% NP40, 1X Thermo Fisher protease cocktail), and immunoprecipitated with 30 μ l of protein-A Dynabeads® solution, with the same quantity for the mock assay. Beads were washed twice in sequence with CHIP 1 Buffer (IP buffer + 1% Na-deoxycholate), CHIP 2 Buffer (IP Elution buffer + 1% Na-deoxycholate + 500mM NaCl), and CHIP 3 Buffer (IP Elution buffer + 1% Na-deoxycholate + 270mM LiCl), then washed twice in Tris-HCl 10 mM pH 8. Finally, elution was performed with elution buffer (NaHCO₃ pH 8.8 0.1M; SDS 1%), added to the precipitated samples until 200 μ l was reached (same as input).

Decrosslinking was performed by adding 10 μ l of NaCl 4M and 0.5 μ l of fungal proteinase K (Thermo Fisher), with six hours of incubation at 65°C. The samples were purified and eluted with MicroChip Diapure Columns. Samples were analyzed with qPCR. The primers used were as follows: *hsp70Aa* TSS (-800,-680) Fw 5'-AACAAACAATTCCAAGTTTGAC-3', Rv 5'-CACGGTTTTTGCATATGCTTT-3'; *hsp70B* paralogs 5'-UTR Fw 5'- GCTAAGCAAATAAACAAGCGCAG-3', Rv 5'- CAGTTGATTTACTTGTTGCTGGT-3'; *hsp70* common AGAGTG motif Fw 5'-TTCTCTGGCCGTTATTCGTT-3', Rv 5'-TCGAACCAACGAGAGCAGTA-3'; *hsp70* TSS Fw 5'-CGACATACTGCTCTCGTTGG-3', Rv 5'-CAGCTGCGCTTGTATTTG-3'; *hsp70B* 3' end Fw 5'-AATGGAATCCTGAACGTCAGC-3', Rv 5'-CACATTGAAGACGTAGCTCTCC-3'. Additional primers for 5' and 3' were extracted from reference [16] and were named CHIP-A and CHIP-B, respectively.

Co-immunoprecipitation

Coexpression of DAXud1-GFP and NELF-B-HA on the imaginal wing disc was achieved with driver *nub>Gal4*. In total, 150 wing discs were dissected on PBS 1X, then precipitated and resuspended in RIPA buffer. Protein extracts were immunoprecipitated with GFP antibody (Abcam ab290) with O.N incubation at 4°C, then isolated with Dynabeads® Protein-A. Western blot assay was performed in cells with INPUT (total protein), NB (non bound fraction, supernatant post Dynabeads isolation), and protein precipitated with Dynabeads. Page ruler 2166 (thermoscientific) was used as a weight marker.

Results

Distribution of DAXud1 across the genome

According to results from previous reports, vertebrate DAXud1 orthologs possess transcription factor-like features, including DNA binding, acidic and transcription transactivation domains [20, 21, 28]. They have also been detected bound to specific promoters related with neural crest differentiation [28]. Further, vertebrate and invertebrate orthologs of DAXud1 are related to the stress response and cancer through induction of apoptosis [21, 23], although, until now, this process has not been linked to its putative transcriptional function. Considering this information, we asked which groups or categories of genes are transcriptionally regulated and/or bind DAXud1 in *Drosophila*, using this information as a proxy to get a

better perspective on the function of *dAxud1*. For this purpose, we performed a TaDa-Seq (DamID-seq) experiment using the method described by Southall et al. [29], expressing the Dam-DAXud1 fusion protein to explore the *loci* in which DAXud1 could reside. This was performed in imaginal wing disc tissue using the *nub>Gal4* driver. Two replicates were generated per condition (third instar larvae imaginal wing disc, with Dam as a control or Dam-DAXud1). The aligned sequences were analyzed with the *findpeaks* script, generated by Marshall Owen [30]. Using this method, we identified 1811 significant peaks across the *Drosophila* genome, representing zones where Dam-DAXud1 has a stronger, more stable positioning than the Dam signal. As shown in Fig. 1A, the Dam-DAXud1 distribution was mainly intronic, with 52.3% of peaks localizing to these gene regions. The first intron accounted for more than half of all instances of intron localization, representing 25.7% of overall genome occupancy. The other main significant locations were the intergenic and the proximal promoter regions (-1000 to +1). These regions are also the most enriched in transcription factor binding sites [31, 32]. To better understand the distribution of DAXud1 across the genome and in specific genes, we used the information from significant peaks to create a metagene profile, using gene bodies as a reference scaled to 1000 bp. The resulting metagene profile (Fig. 1B) shows a robust average signal at the promoter zone and a maximum within the 5'-half of gene bodies, confirming the previously described distribution (Fig. 1A) and suggesting a role in activating or poised gene transcription.

Several reports describe DAXud1 vertebrate orthologs as stress response genes [24, 26, 33, 34]. Therefore, we searched for significant DAXud1 binding on stress response gene *loci* (Additional Table S1) and found heat shock response genes as recurrent targets. Further, we generated alignment charts to visualize the distribution of peaks within this group of genes (Fig. 1C). These graphics clearly reveal that the distribution of Dam-DAXud1 specifically on *hsp* genes has a highly similar pattern to that observed in the metagene profile.

The intergenic, promoter, and gene-body (intron and exon) peaks were sorted and annotated for nearby genes using HOMER [35]. Additional Table S2 shows the most relevant enrichment analysis results. Within the “biological processes” category, the two major enriched groups are related to neural development, but no groups show a significant score for stress response or tolerance. One pathway category identified involves Wnt signaling, concordant with reports that DAXud1 orthologs in mammals are linked to this signaling pathway [19, 36, 37] and apoptosis [21, 38].

Potential DAXud1 DNA binding motifs

After having found genes in which Dam-DAXud1 is enriched, we searched for recurrent binding motifs within the Dam-DAXud1 peaks. The sequence peaks had a size between 50-5000 bp and were extracted for *motif find analysis* from HOMER [39]. As shown in Fig. 1D, the most recurrent DNA motif (TACATACATA), present in 1019 sequence peaks out of 1811, is novel compared with a previous report on Axud1 vertebrate orthologs [28], possibly due to the wide-range of sequences that the TaDa-seq experiment provides [40–42], but this information could provide insights from the chromatin context surrounding DAXud1. Using the matrix sequence, we conducted another analysis using this data and the

GOMO platform, which provides information about the gene ontology (GO) of the promoter regions, using a range between -1000 and +200 bp from the TSS for each gene in the *Drosophila melanogaster* genome. For the TACATACATA motif, the most related GO found is “Heat shock mediated polytene chromosome puffing” (GO:0035080). This motif also found a match in the *Drosophila* Topoisomerase 2 gene, which encodes a protein that removes supercoils in chromatin to facilitate transcription, with an essential role in RNA polymerase pausing-release in *hsp*s genes in the fast stress response [43, 44]. These analyses suggest that DAxud1 might play a role in the transcription of heat shock and other stress-induced genes. To confirm this last hypothesis, we performed chromatin immunoprecipitation on the *hsp70* promoter with DAxud1-GFP as bait, followed by a CHIP-PCR with primers flanking the TACATACATA motif. The result confirms the presence of DAxud1 at this *locus* (Additional Fig. S9), reinforcing the idea that it regulates the stress response by modulating *hsp* gene expression and also that the regions discovered using TaDa-seq are *bona fide* DAxud1 binding sites representing potentially functional interactions.

DAxud1 regulates thermotolerance and lifespan in *Drosophila*

Our results reveal the presence of DAxud1 bound to *hsp* genes (Fig. 1; Additional Fig. S9), suggesting a possible role in stress tolerance, a hypothesis supported by studies on Axud1 orthologs in mammals [23, 45]. Although “stress response” is not the major GO category of DAxud1 genome occupancy (Additional Table S2), we tested whether flies with a global knockdown of this gene exhibit an altered stress response or aberrant *hsp*s expression, given its presence in this class of genes. For this, we used flies expressing a DAxud1 RNAi construct (Vienna stock 26479, UAS-IR-DAxud1) in all tissues using the Tubulin-Gal4 driver. Flies overexpressing DAxud1 cannot be assessed in this type of experiment since ubiquitous expression of this protein is lethal [21]. We established adult flies at 29°C for an optimal expression of the UAS RNAi construct; then, control and experimental adult flies were exposed to a heat shock of 37°C for a half-hour every day as the thermal stress condition, and their survival was measured. Knockdown of DAxud1 leads to a diminished lifespan of adult flies compared to the control genotype under the same stress condition (Fig. 2A-2B). Curiously, under control conditions (no heat shock), DAxud1 knockdown animals have a longer lifespan compared to control animals for both males and females (Figs. 2A and 2B). To confirm whether there is a fluctuation in *hsp* gene expression, and to relate this to the observed phenotypes, we performed qPCR analysis for different *hsp* genes in imaginal wing discs and salivary glands, expressing the RNAi construct or DAxud1-GFP version using the *nubbin*-Gal4 driver. This allowed us to study the effects of diminished DAxud1 levels in salivary glands and imaginal wing discs, tissues that have cells in an endo replication state (salivary glands) and in a mitotic state (imaginal wing discs). Figs. 2C, 2D, and 2E show the qPCR results for three *hsp* genes (*hsp70*, *hsp26*, *hsp67*). In these experiments, we can appreciate that DAxud1 knockdown increases the expression of *hsp* genes in control conditions, but does not potentiate the transcriptional heat shock response in either tissue. Notably, the rise of *hsp* mRNA expression at the control temperature can explain the longer lifespan since there is evidence that *hsp* overexpression generates this effect [46]. On the other hand, DAxud1 overexpression reduces *hsp* basal expression at the control temperature, perhaps not inhibiting the *hsp* stress response but generating a milder induction (Figs. 2C-E). These results, especially those observed after DAxud1 knockdown, are in

conflict with our first hypothesis in which DAXud1 could function as a transcription factor that facilitates *hsp* expression. Therefore, we then set out to further clarify the role of DAXud1 on the *hsp70 locus*.

DAXud1 exhibits a widespread presence across the genome, whereas heat shock induces its recruitment to the *hsp70 locus*

Further examination of the results of *hsp* gene expression in DAXud1 knockdown conditions (Fig. 2), suggests that DAXud1 may participate directly in *hsp* transcription, possibly in a repressive manner. Previous studies have reported that DAXud1 orthologs (CSRNP in mouse) have transcription factor features, including a C-terminal trans-activating domain and a DNA binding motif [47]. However, there is no evidence indicating which type of regulatory factor it is, and its dynamics within the nuclear structure. *Drosophila melanogaster's* polytene chromosomes represent a suitable model to answer these questions.

As there is no available antibody against DAXud1, we used the GFP-tagged version of DAXud1 [21] (DAXud1-GFP) to perform immunofluorescence using an anti-GFP antibody. RNA Polymerase Ilo (Hyperphosphorylated) was used as a positive control for a chromosome attached protein and for detecting transcriptionally active *loci*. As seen in Fig. 3A, DAXud1-GFP on the polytene chromosome exhibits a pattern with widespread RNA Pol Ilo co-distribution, which means the specific function on *hsp* genes may be distinct from other functions of DAXud1 in gene expression. The *hsp70 locus* (Fig. 3B) has been extensively analyzed with regards to its chromatin rearrangements under stress conditions. Its documented that during stress conditions such as heat shock, cytological zones known as *chromatin puffs* appear in *hsp70* region, clearly visible with confocal microscopy, a reflection of chromatin opening for transcription and transcription factor recruitment [48, 49]. We evaluated chromatin and DAXud1-GFP dynamics on this locus (at both 17°C and 37°C, Fig. 3C) and observed that DAXud1-GFP localizes to the 87A-87B *locus* together with RNA Polymerase Ilo after heat shock treatment. Transcription factors that relocate in this way are identified mostly as activators, for instance HSF, p-TEFb [2], and DSIF [16]. From these results, we reasoned that DAXud1 is a chromatin element that relocates to the *hsp70 locus* during heat stress, but it does not interfere with the transcriptional induction since *hsps* mRNA levels rise by the same magnitude as in control, under a DAXud1 overexpression condition (Fig. 2C-E).

To confirm these results, we performed chromatin immunoprecipitation for DAXud1-GFP (Fig. 3D) to detect the presence of this factor on the *hsp70* paralogs *hsp70Ab* and *hsp70Bb/Bc*, or *loci* 87A and 87C, respectively. According to those results, DAXud1-GFP has a clear presence on *hsp70* promoters as do other heat shock transcription factors [4]. Interestingly, DAXud1-GFP did not exhibit enrichment after oxidative stress induction by peroxide (Fig. 3D), a condition that increases *hsp* mRNA levels [50], indicating that the involvement of DAXud1 in the heat shock response is different to the mechanism evoked during oxidative stress.

Together, the data obtained here, have described the genome-wide distribution of DAXud1, its influence on *hsps* transcription, and its effects on lifespan. However, how does DAXud1 exert its function and what is its relevance for cell physiology? In the *hsp70* gene, there is a special chromatin configuration, known as the pausing complex, in which the activated RNA Polymerase II pauses the transcription process at

positions +30 to +50 bp of the gene, and remains stalled as a “poised polymerase” [44]. This poised polymerase forms a complex with two main components, NELF and DSIF, whose role is to maintain the polymerase in the paused state. Transcription resumes when the p-TEFb complex phosphorylates NELF and DSIF, releasing the active polymerase for resumption of elongation [44]. *hsp70* genes have been used as transcriptional pausing models because the resumption of transcription occurs quickly when heat shock or stress signals reach the cell [44]. Considering the data on the position and behavior of DAXud1 on the *hsp70* gene after heat shock and that elements of the pausing complex on *hsp70* exhibit a similar pattern of reorganization on chromatin during heat shock [4, 18], we conjectured that DAXud1 might interact with the pausing complex.

This hypothesis is also supported by RNA-seq evidence (Additional Figs. S3 and S4), in which DAXud1 overexpression increases *hsp70* RNA synthesis only at the 5' end of the gene, with an incomplete synthesis of *hsp70* mRNAs, specifically on *hsp70B* paralogs, which display high reads only between +1 and +100 region. This effect was not detected in qPCRs performed in Figs. 2C-E because the primers were designed to detect the 3' side. In addition, this role in pausing is also supported by the evidence of double-hybrid experiments with *Drosophila* proteins, which demonstrated a physical interaction between DAXud1 and Spt5 [51], a member of the DSIF complex, with a pivotal role in the RNA Polymerase transcriptional pausing and elongation complex [52]. Notably, the DAXud1 overexpression wing phenotype is reversed in a heterozygous *spt5* mutant background (Additional Fig. S6). Another component of the pausing machinery is the NELF complex (NELF-A, NELF-B, NELF-C/D, NELF-E), a stabilizer factor of the stalled polymerase in basal conditions. These components dissociate from the polymerase when transcription is resumed after heat shock [18], and the loss of function of some of its components generates higher levels of *hsp70* [53], which is similar to our observations in the case of DAXud1 knockdown (Fig. 2C). Considering this information, we tested whether DAXud1 influences RNA Polymerase II location within the *hsp70* gene body [53]. Chromatin immunoprecipitation (CHIP) for RNA Pol II was performed under different DAXud1 levels of expression (Fig. 4A). In the RNA Pol II CHIP, we find that, in the case of DAXud1 knockdown, its levels decrease uniformly in the *hsp70* gene locus, and there is no change in the 5'/3' ratio of RNA Pol II compared to the control and heat shock conditions (Fig. 4B). On the other hand, DAXud1 overexpression generates an increase of this ratio, more so than in the control condition, due to an increase of RNA Pol II on 5' end of the *hsp70* gene body (Fig 4A).

To find out whether DAXud1 interacts with pausing factors, we performed a co-immunoprecipitation assay in imaginal wing disc expressing NELF-B-HA and DAXud1-GFP. In this assay (Fig. 4C), a fraction of NELF-B-HA was co-immunoprecipitated with DAXud1-GFP, indicating a physical interaction between these two proteins, suggesting they could act in the same process in *hsp70* expression.

The information provided by the physical and genetic interaction of DAXud1 with NELF-B and Spt5, respectively, strongly suggests that it could be influencing RNA Polymerase II dynamics on the *hsp70* gene. To confirm this, we performed a CHIP for RNA Polymerase on the 5' and 3' regions of the *hsp70* gene, under DAXud1 overexpression and knockdown conditions. The results, presented in Fig. 4, reveal

that DAXud1 overexpression generates an apparent pausing effect on RNA Polymerase II distribution (Fig. 4), coincident with extensive upregulation of the 5' RNA levels of *hsp70* (Additional Figs. S3 and S4).

Promoter-proximal pausing in *hsp* genes allows a rapid expression after heat shock induction due to pausing elements as DSIF/NELF complexes. Also, this rapid expression has a shut-off mechanism to restore *hsp70* mRNA levels after the heat shock condition is reverted (heat shock recovery), and there is evidence that supports the role of NELF complex in the shut-off mechanism [54]. We tested the physical interaction between NELF-B and DAXud1 (Fig. 4C) as well as its genetic interaction (Additional Fig. S7), allowing us to further clarify the role of DAXud1 in *hsp70* shut-down levels during heat shock recovery. To evaluate the effect of DAXud1 in this process, we performed qPCR for *hsp70* mRNA 40' after a 20' heat shock (37°), as shown in Fig. 4D, in DAXud1 knockdown salivary glands. It is possible to observe that *hsp70* induction has an apparently slower turn-over than in control animals (Fig. 4D), suggesting that DAXud1 plays a role in shutting-down *hsp70 locus* induction, and confirming DAXud1 has a synergistic function with NELF-B in the transcriptional pausing of *hsp70* as well as the complex stabilization for further heat shock stimulus.

Discussion

Role of DAXud1 in tissue homeostasis through *hsp* regulation

In the first part of this study, we searched for the most frequent sites where DAXud1 locates in the *Drosophila* genome, and upon finding *hsp*-type genes in these sites we performed thermotolerance analysis under DAXud1 knockdown in order to test the physiological relevance of this gene. We further studied *hsp* expression during DAXud1 knockdown to mechanistically link these cellular players. In lifespan assays with DAXud1 knockdown (Figs. 2A-B), we found it generates adults with extended lifespan in control conditions, but reduced thermal resistance compared with control genotype animals, with no other change in developmental timing or apparent alterations (Fig. 1; Additional Fig. S2). Interestingly, in DAXud1 knockdown larvae raised at 29°C (no heat shock), the organisms exhibit an increase in the level of *hsp70* expression in salivary glands and imaginal wing discs, compared to the control animals subjected to the same temperature. In *Drosophila*, the effect of *hsps* overexpression is well documented, in that one of its effects is precisely an extended lifespan due to its cytoprotective and anti-apoptotic effect [46, 55], explaining why DAXud1 knockdown extends lifespan in control temperature, compared with animals only expressing the Gal4 driver (Figs. 2A-B). On the other hand, when animals with DAXud1 knockdown are exposed to daily thermal stress, lifespan shortens, situation that did not occur with control animals in which lifespan remained within the same range, as in control temperature, during daily thermal stress (Figs. 2A-B). This seems paradoxical since animals with higher levels of Hsps would be expected to maintain enhanced thermotolerance. However, the diminished tolerance to stress in DAXud1 knockdown could be explained alternatively by the reported pro-apoptotic DAXud1 function through an activating role on JNK signaling in a DAXud1 overexpression background [21, 26]. Thus, in the context of DAXud1 knockdown, the JNK signaling pathway could be less strongly activated, allowing damaged cells to survive though interfering with development and regenerative processes, which are

known consequences of impaired JNK signaling [56, 57]. This last suggestion is supported by the occupancy of Dam-DAxud1 on the *loci* of genes associated with the GO- KEEG apoptosis pathway, related to JNK (Additional Tables S2 and S5). Regarding this point, the pro-apoptotic activity of DAxud1 is associated with control of tumorigenesis [19, 21] and cancers with poor prognosis [58], which may be due not only to decreased pro-apoptotic activity resulting from lower Axud1 levels, but also to increased *hsp* gene expression. *hsp* overexpression strongly correlates with cancer cell progression and poor prognosis [59, 60], so this condition of certain cancer cells may be due to low levels of Axud1, a condition also associated with aggressive cancers, as previously described [19, 21, 58]. This function in tissue homeostasis through transcription modulation could be one of the reasons DAxud1 is conserved among metazoans. However, the reduced thermotolerance phenotype would not be directly related to the expression of *hsps*, despite the fact that DAxud1 exhibits recurrence in these genes (Fig. 1) and at the same time the strong effect on their expression generated by the knockdown of DAxud1. These observations prompted us to investigate the relationship between *hsps*, widely conserved in all kingdoms, and DAxud1, which only has orthologs in metazoans, probably participating in a type of modulation on *hsps* expression exclusive to metazoans. This led us to focus on its effects at the chromatin level.

DAxud1 function in chromatin as pausing factor on the *hsp70* gene

Although *dAxud1* mRNA levels do not change significantly during heat shock (Additional Fig. S8), the DAxud1 protein relocates to the *hsp70 loci*. The localization of DAxud1 to chromatin, observed in Fig. 3C, can be mediated by putative phosphorylation sites [21] or by the DAxud1 cysteine-rich region [20, 21]. These cysteine-rich regions have been shown to act as modulators of protein conformation, in which heat or oxidative stress can alter the disulfide bonds, changing the conformational state of the protein. This phenomenon is well documented [61, 62] in proteins with cysteine-rich regions, including the DAxud1 CSRNP family [20]. Thus, changes in DAxud1 protein conformation induced by post-translational modifications could be key in the regulation of its localization and its effect on gene expression.

Transcriptional pausing is a state in which the RNA Polymerase initiates transcription but remains stalled on the first 30-50 base pairs of the gene, forming a complex with the DSIF (Spt5/Stp4 proteins) and NELF complexes [16, 48, 63]. The pausing is released when internal or external cell signals activate p-TEFb, which phosphorylates RNA Polymerase II itself as well as the DSIF, and NELF complexes which, in turn, disengage from the RNA Polymerase [18, 64, 65]. Also, there is evidence that pausing relies on the chromatin state, in which NELF exerts its pausing effect by depleting H3K4me3 histones and, therefore, when NELF dissociates from the pausing complex, the polymerase elongates in a favorable transcriptional context [53]. This mechanism is found in fast-response genes like *hsp* genes or in innate immune response genes [66]. Strikingly, overexpression of DAxud1 generates an increase in *hsp70B* transcription only on its 5' end of the transcript sequence (Additional Figs. S3 and S4). This increment of 5' sequences of the *hsp70B* genes indicates that there is an increase of mRNA synthesis, from approximately +1 to +80 bp. We interpret that DAxud1 retains the pausing complex completely stalled, generating an enrichment of 5' *hsp70* mRNA. Whatever the explanation is at this level, the effect of

DAXud1 on this particular *hsp* gene expression could involve the transcriptional pausing mechanism, in which DAXud1 might interact with the pausing complex.

The possible role of DAXud1 in the pausing complex is also supported by the co-immunoprecipitation of DAXud1-GFP with NELF-B (Fig. 4C). NELF-B acts as a component of the pausing complex, stabilizing and pausing RNA Polymerase II in a hypo-phosphorylated CTD state, and dissociates from the complex under heat shock or another signal [54]. Additionally, data concerning Spt5, another component of the pausing complex (DSIF complex), further supports this observation: Biogrid [51], a database that documents physical interactions between proteins, reported an interaction of DAXud1 with Spt5 [4] and, importantly, the DAXud1 overexpression phenotype is partially reverted in a *spt5* heterozygous mutant background (Additional Fig. S6). Furthermore, Spt5 interacts with NELF-B in the pausing complex [67], and stabilizes it synergistically with the NELF complex, maintaining the RNA Polymerase stalled. This pausing complex keeps stable until p-TEFb kinase phosphorylates both complexes, with dissociation of NELF complex [68] and DSIF (Spt5/6) remaining as a component of RNA Polymerase holoenzyme, acting as an elongation factor. In this context, DAXud1 may play a role in pausing the stabilization complex, since DAXud1 knockdown causes an increase in the transcription rate of *hsp70* mRNA, while its overexpression causes stalled transcription at the 5' region, perhaps instigating the formation of the pausing complex (Fig. 4) in the same way as NELF does [53], but not interfering with RNA Polymerase II pausing release in heat shock conditions. This interaction could be conserved in other instances, such as in the regulation of the expression of the MMP1 gene in humans, which requires the ortholog of DAXud1 (CSRNP1) for its transcription upon cytokine activation [69]. More importantly, there is detailed evidence that MMP1 is a transcriptionally paused gene, in which the NELF/DSIF pausing complex is necessary for its appropriate expression in response to immune-activating signals [66]. Also, in myeloid cells, the NELF complex and Spt5 are essential for maintaining the repression of pro-apoptotic genes during myeloid development in zebrafish. In this case, they rely on their role in RNA Pol II pausing of pro-apoptotic genes [70] and, accordingly, Spt5's deficiency generates a loss of the myeloid line, due to early apoptosis of hematopoietic stem cells, the same phenotype observed after *Csrnp1* reduction in morphant zebrafish larvae [71]. Curiously, in imaginal wing discs and, subsequently, in the adult wing, the overexpression of NELF-B reverts the DAXud1 overexpression phenotype, while NELF-B knockdown exacerbates the DAXud1 overexpression phenotype (Additional Fig. S7). These results suggest an antagonistic role of these two proteins on gene expression, though not necessarily related to transcription of *hsp* genes, because the knockdown of both genes separately provokes a similar effect in the increase of *hsp* transcription. Instead, such an effect could be found in genes related to apoptosis, in which NELF-B functions as a repressive element, as previously described [53]. This is also similar to the interaction between DSIF and NELF, both are necessary for RNA Pol II pausing, but after p-TEFb activation (through heat or morphogen signals) their roles are quite opposite, in which the DSIF elements are necessary for RNA Pol II elongation while NELF dissociates completely from the transcription complex [18].

The relation between pausing and DAXud1 motif founded with TaDa-seq

In support of the pausing-release hypothesis, we found that the most enriched motif found in TaDa-seq peaks is “TACATACATACA.” This sequence strongly matches the binding site of Topoisomerase 2 (Top2), a chromatin modifying protein (Fig. 1D). There is evidence that Top2 plays a role in pausing-release and in the *hsp70* fast transcriptional response in *Drosophila* Kc cells [72]. In addition, GOMO analysis shows that this motif is present in promoters of genes that belong to the “heat shock-mediated polytene chromosome puff” (GO:0035080) and “response to hypoxia” (GO:0001666) GO categories. Additionally, there is evidence that Topoisomerase 2, due to its structural chromatin function, plays a role in the cell cycle, in which inhibition or missteps in the arrangement of this protein lead to cell cycle arrest in G2-M [73]. The same cellular phenotype was previously reported when DAXud1 was overexpressed [21], raising the possibility that these proteins interact at the chromatin topology level, likely interfering with Topoisomerase 2 positioning before the G2-M checkpoint. However, more analysis is required to support this conjecture.

In previous studies, the vertebrate orthologues of DAXud1 were associated with the binding motif AGAGTS [28, 69, 74]. Curiously, with HOMER (*denovo motif discovery* function), no similar sequence was found. We thus used HOMER to search for the occurrence of the AGAGTS motif among the peak sequences; we found AGATGS 4657 times in 1407 peaks among the 1811 significant sequences. In total, 895 genes were identified (Additional Table S4). Again, pathways like Wnt and Apoptosis are present in this group (Additional Tables S2 and S5), most of those belonging to the apoptosis group are part of JNK signaling, including *msn*, *puc*, *rpr*, and *hid*, a finding which is in agreement with *bsk* and *puc* upregulation upon DAXud1 overexpression (Additional Fig. S5, [21]). This result indicates that DAXud1 acts at different levels on transcriptional regulation to balance cell physiology.

With regard to its physiological role, low levels of human Axud1 expression are related to tumor development [19]. Considering the role of Axud1 in the stress response, this condition could predispose cells to undergo more damage in the first stage of tumor development due to the loss of *hsp* gene regulation, leading to higher levels of Hsp proteins, thereby buffering damage signals and likely inhibiting the apoptotic process and extending the lifespan of impaired cells. Later on, in the next stage, Axud1 could be necessary at higher expression levels to initiate apoptosis through JNK signaling [21], though this Axud1 upregulation does not occur in tumorigenesis [19].

Developmental role of Axud1

A previous study showed that the DAXud1 vertebrate ortholog, Axud1, acts as a transcription factor, located only in the promoter region and related to the positive transcriptional activity of the Pax7 and Msx1 transcription factors, driving the development of neural crest by Wnt signaling [28]. According to this study, the presence of Axud1 on these promoters depends on the AGAGTS binding site, previously described for human and mouse orthologs [11], from *in vitro* experiments. The AGAGTS motif was found in the TaDa-seq data, with a frequency of 4657 times out of 1407 unique peak sequences. The AGAGTS sequence is present 88,167 times in the whole *Drosophila* genome and, therefore, the Dam-DAXud1 peak sequences comprise 5.28% of the whole genome motif's abundance. Out of those 1400 peaks, gene

ontology analysis mainly points towards morphogenesis, axon guidance, and Wnt signaling (Additional Tables S2 and S5). This information is in line with previous work on vertebrate Axud1 [19, 28, 75] and our data in which Axin and Wnt are downregulated upon DAXud1 overexpression (Additional Fig. S5), which could indicate that DAXud1 is part of a feedback loop in the Wnt pathway. Mainly, the data from other studies supports the role of Axud1 in neural development as previously described, in which vertebrate homologs of DAXud1 could exert a pro-neural function in both CNS and neural crest development, and this depends on wnt signaling [28, 76]. Therefore, this regulatory network is conserved in animals with different roles in development. This function in neural structures is a hallmark of DAXud1 orthologs because our previous data showed that zebrafish Csrnp1a is highly expressed in the growing brain [77] and in the ventral nerve cord in *Drosophila* during germ band elongation stage [21], making this protein a regulator of a specific process more than a general factor of animal development, and not classifiable in a binary category of transcription activator/repressor.

Overall, our study raises new insights about DAXud1 function, demonstrating new chromatin features related to the stress response. We propose this is mainly supported on the stabilization of the pausing complex, maintaining a chromatin configuration for rapid *hsp* expression under stress conditions in the first stage of the thermal stress response. This function might not represent the complete chromatin regulatory function of DAXud1, which could play a role in JNK signaling in long-term stress to promote apoptosis, and an additional role in neurogenesis as a mediator of Wnt signaling, as described in vertebrates [78].

Declarations

Ethics approval:

Our laboratory and the manage of animals were approved to perform the experiments exposed in this article. This was approved by Bioethics committee of the Faculty of Sciences (Universidad de Chile), chaired by Dr. Marco Méndez from 2013.

Competing interests:

No potential competing interest was reported by the authors.

Author's contributions:

JM Zúñiga-Hernández was the main executor, writer of the original draft, designer of the experiments, as well as the data analysis, including NGS data. Also, JM Zúñiga-Hernández is the corresponding author. Meneses C contributed to facilitate the access to Illumina sequencing platform, and support experimental design. Bastías M was the technician in charge to process the libraries for the Illumina sequencing. Allende ML contributed to the editing and review of the manuscript, funding and provider of resources. Glavic A was the supervisor and reviewer of the manuscript, funding provider, as well as the manager of the initial idea for this research.

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TaDa-seq are available in Sequence Read Archive (SRA), ID: PRJNA776616

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Figures

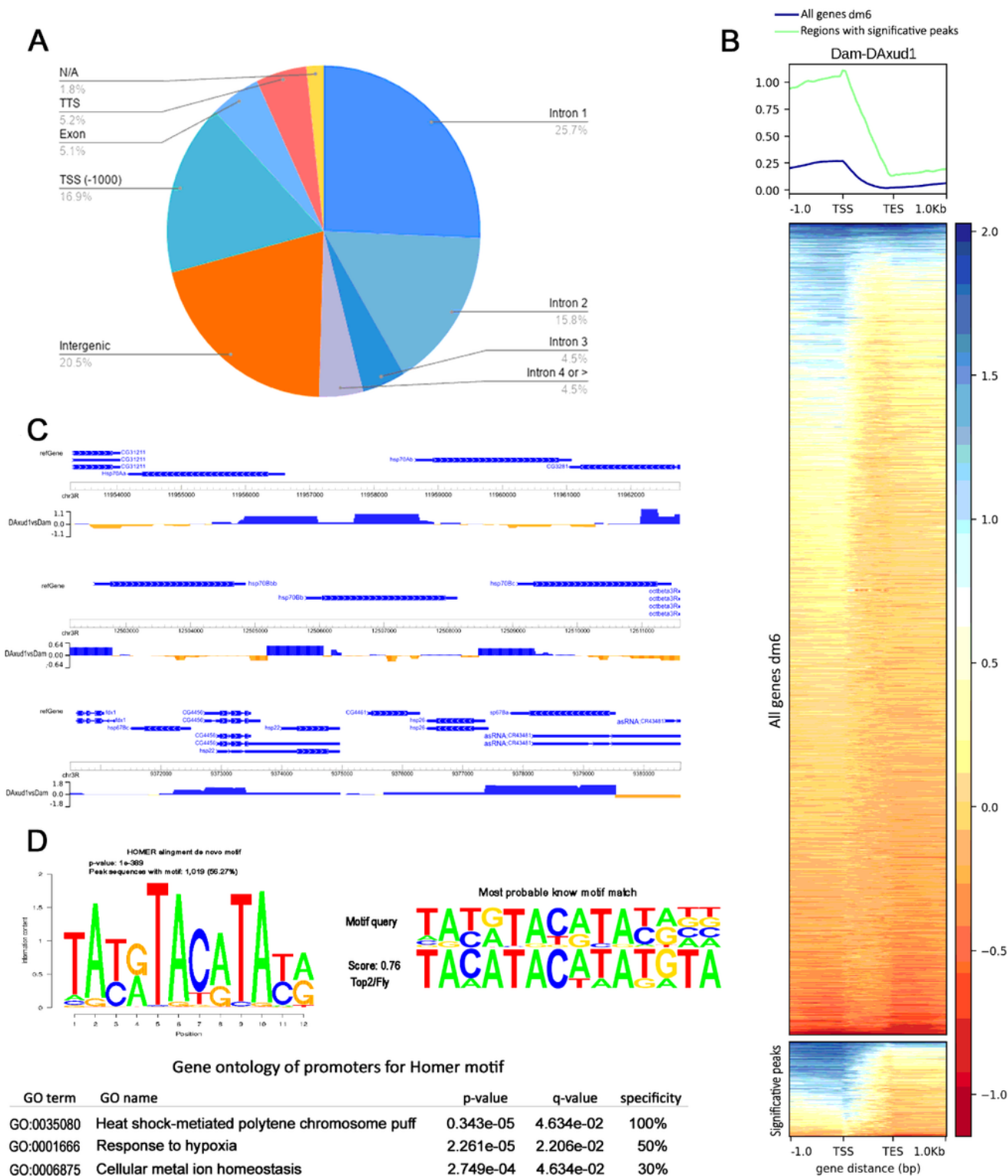


Figure 1

TaDa-seq from Dam-DAXud1/Dam shows the genome-wide distribution and main motif in significant binding regions. 1811 peaks were identified as significant over the background from the subtraction of signals (Dam-DAXud1/Dam (no fusion) using the Dam/TaDa pipeline and annotated with HOMER. (A) Annotation of significant peaks reveals that Dam-DAXud1 is prevalent in the first intron, intergenic, and promoter zones. (B) The metagene profile and heatmap show the main signals on the TSS surrounding

zone with a low signal at the gene body and TTS (transcription termination sites). Gene bodies of all genes were scaled to 1000 bp. (C) Gene tracks for *hsp* genes confirm the presence of Dam-DAxud1 in these sites, concordantly with the genome distribution in (A). Positive average scores are in blue and negative are in orange. (D) The main motif identified with HOMER using the denovo application, matching with the Topoisomerase 2 binding site motif, whose search using GOMO (Gene Ontology of Motif using promoter zones) matches the biological process related to heat stress and hypoxia.

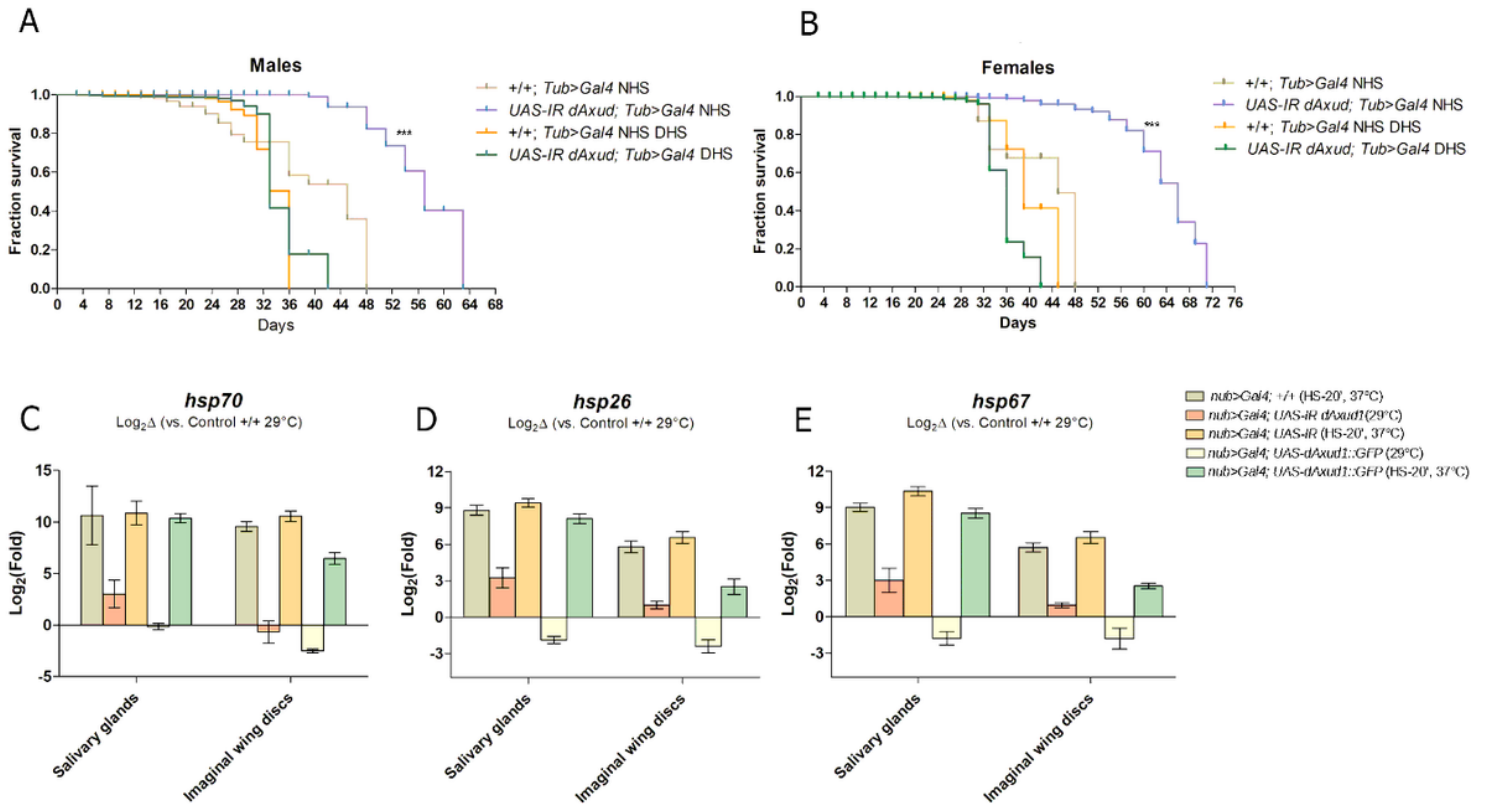


Figure 2

Effect of dAxud1 knockdown in pupation and lifespan. (A) and (B) represent the survival chart from dAxud1 knockdown (*yw; UAS-IRdAxud1/+; Tub>Gal4/+*) genotypes under heat shock episodes of 37° for 30 minutes every day (DHS) and compared with flies in stable temperature at 29°C (NHS). $p < 0.0001$ with Log-rank statistical analysis. Every assay was performed with 25-35 flies with 3 replicates. Figs. C, D, and E represent the gene expression of two different tissues for three heat shock genes, *hsp70*, *hsp26*, and *hsp67*, respectively. The expression was tested on 5 different conditions and compared to control conditions, including dAxud knockdown (*nubbin Gal4/+; UAS-IRdAxud1*) and dAxud overexpression (*nubbin Gal4/+; UAS-dAxud1-GFP*). The control genotype was *nubbin Gal4/+* (Driver only).

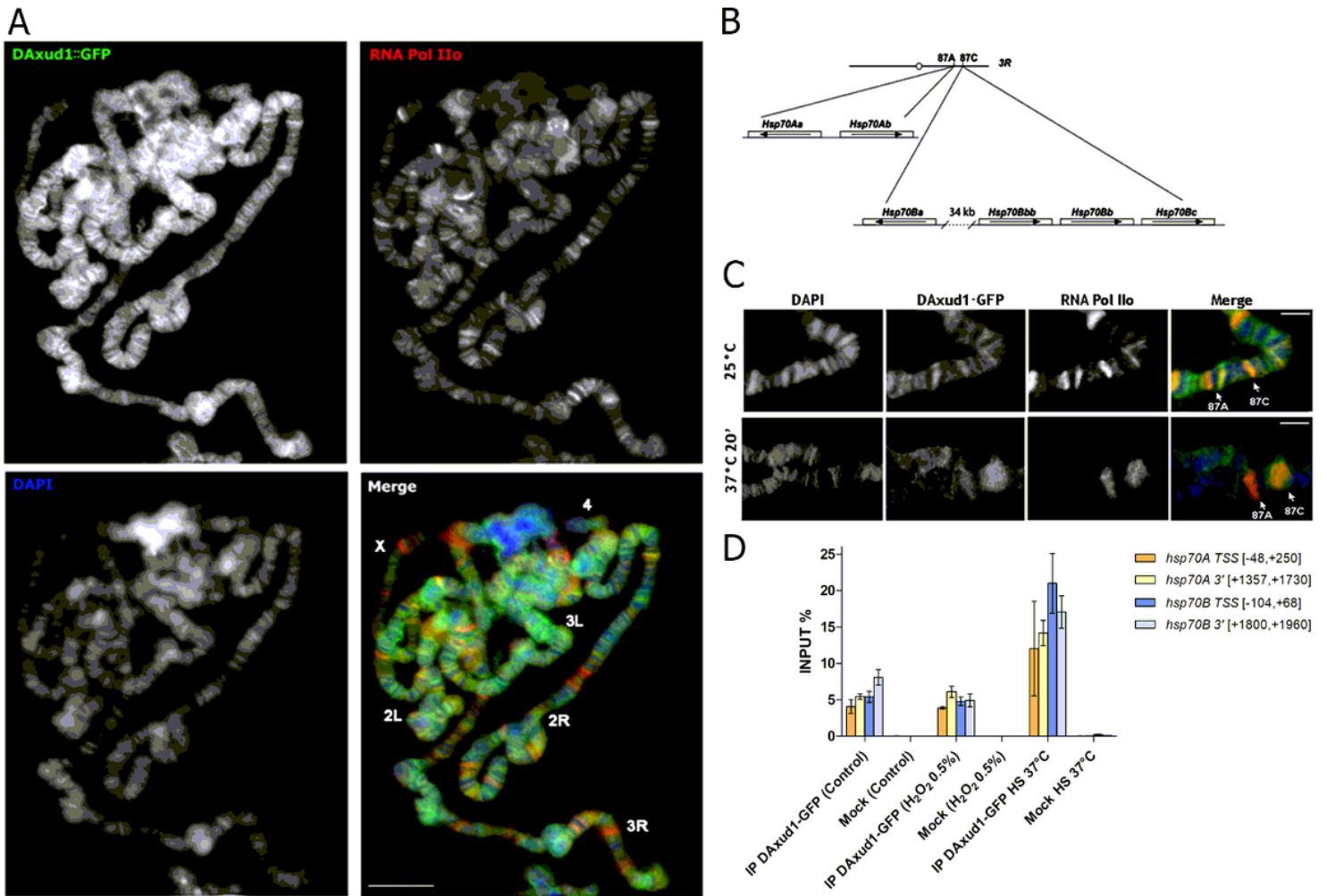


Figure 3

DAXud1-GFP has an extensive distribution on polytene chromosomes. In accordance with Johanssen's protocol [30], immunostaining was performed for RNA polymerase Ilo and GFP for DAXud1-GFP protein localization; DAPI was added to visualize condensed DNA. A) 20X picture from 17°C growth third instar larvae. Bar 20 μ m. B) Scheme with *hsp70* genes cytolocation at 87A-87C. C) Changes in *hsp70* locus during heat shock (37°C at 20 minutes) reveal the relocation of DAXud1-GFP in the same way as RNA Pol Ilo. Bar: 5 μ m. D) CHIP analysis from third instar larvae salivary glands for DAXud1-GFP on *hsp70* locus for 5' and 3' gene zones. Control conditions were defined as 25°C. The oxidative stress condition was induced by feeding larvae H₂O₂ 0.5% v/v for 24 hours (late 2nd to 3rd instar larvae, then dissected). Heat shock was performed by exposing 3rd instar larvae to 37°C for 20 minutes; then the salivary glands were dissected for chromatin extraction and immunoprecipitation.

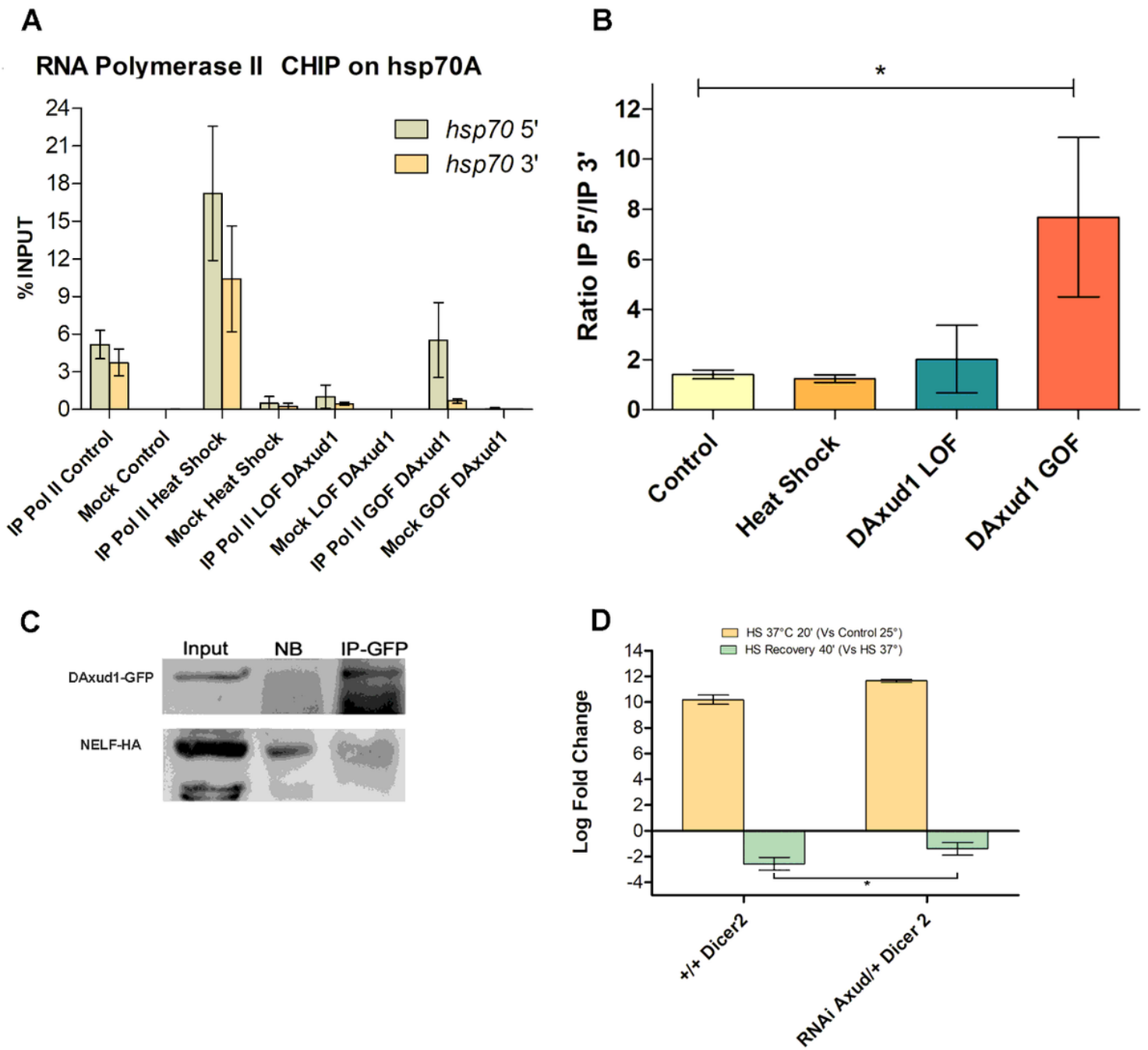


Figure 4

CHIP analysis of RNA Polymerase II occupancy on hsp70 locus under DAXud1 overexpression and DAXud1 knockdown. A) CHIP analysis RNA Pol II (RPB1 subunit) occupancy on hsp70A locus. Each condition was examined in triplicate B) Comparison of the 5'/3' occupancy ratio between conditions. Statistical analysis was performed as a Tukey test, * $p < 0.05$. D) Western blot from the co-immunoprecipitation of DAXud1-GFP with NELF-B-HA. D) qPCR for hsp70 from salivary glands (3rd instar) expressing drive nubbin>Gal4 and Dicer2 (to improve RNAi silencing activity). The hsp70 levels were measured at 20' heat shock exposure (37°C) and heat shock recovery (40' at 25°C after 20' heat shock) to see if there were differences between levels of hsp70 at the same recovery time. Statistical analysis was performed as a t-test, * $p < 0.05$.

Supplementary Files

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