Functional Study and Physiological Characterization of Human Lysozyme-Like Protein 6: Postprint

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Abstract

This study investigated the role of human lysozyme-like protein 6 (LYZL6) in the fertilization process and analyzed the physiological characteristics of recombinant LYZL6 protein (rLYZL6) to reveal its physiological functions. Immunofluorescence microscopy localized LYZL6 to the post-acrosomal region of the mature sperm head. Reverse transcription PCR (RT-PCR) analysis demonstrated that LYZL6 protein on the sperm surface originates from testicular and epididymal secretion. Western blot analysis revealed no significant alteration in surface LYZL6 levels before and after sperm capacitation. Hemizona binding assay and sperm penetration assay analyses showed that rabbit anti-LYZL6 serum did not markedly inhibit human sperm-zona pellucida binding but significantly inhibited sperm-egg fusion. The Pichia pastoris expression system was successfully employed to express rLYZL6, and biologically active rLYZL6 could be purified from fermentation supernatant using chitin affinity chromatography and gel filtration chromatography. Enzyme-linked immunosorbent assay (ELISA) analysis indicated that rLYZL6 lacks hyaluronic acid binding capacity, hyaluronic acid hydrolytic activity, and free radical scavenging activity, but exhibits strong peptidoglycan binding capacity and isopeptidase activity. LYZL6, secreted by the testis and epididymis and localized to the post-acrosomal region of the mature sperm head, participates in sperm-egg fusion and possesses peptidoglycan binding capacity and isopeptidase activity, suggesting that LYZL6 may be involved in sperm through multiple mechanisms.

Full Text

Preamble

The Functional Studies of Human Lysozyme-like Protein 6 and Characterization of Its Physiological Properties

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Abstract

This study investigated the role of human lysozyme-like protein 6 (LYZL6) in the fertilization process and analyzed the physiological characteristics of recombinant LYZL6 protein (rLYZL6) to elucidate its physiological functions. Cell immunofluorescence localized LYZL6 to the post-acrosomal region of mature sperm heads. Reverse transcription PCR (RT-PCR) analysis revealed that LYZL6 protein on the sperm surface originates from secretion by the testis and epididymis, while Western blot analysis showed no significant change in the amount of surface LYZL6 before and after sperm capacitation. Hemizona binding assays and sperm penetration assays demonstrated that rabbit anti-LYZL6 serum did not significantly inhibit human sperm binding to the zona pellucida but significantly inhibited sperm-egg fusion. The *Pichia pastoris* expression system was successfully used to express rLYZL6, and bioactive rLYZL6 could be purified from the fermentation supernatant using chitin affinity chromatography combined with gel filtration chromatography. Enzyme-linked immunosorbent assay (ELISA) analysis revealed that rLYZL6 lacked hyaluronic acid binding ability, hyaluronic acid hydrolysis activity, and free radical scavenging activity, but exhibited strong peptidoglycan binding ability and isopeptidase activity. LYZL6 is secreted by the testis and epididymis and localizes to the post-acrosomal region of mature sperm heads, where it can participate in sperm-egg fusion and possesses peptidoglycan binding ability and isopeptidase activity, suggesting that LYZL6 may be involved in sperm function through multiple mechanisms.

Keywords: Human lysozyme-like protein 6; Acrosome; Fertilization; *Pichia pastoris*; Isopeptidase activity
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Introduction

In mammalian testes, undifferentiated germ cells undergo a complex process to develop into mature spermatozoa. Numerous proteins secreted by testicular and epididymal tissues localize to sperm cells, where they directly participate in sperm-egg interactions and play crucial roles in fertilization. Lysozyme-like proteins represent one such group of molecules. Recently discovered in the mammalian reproductive system, lysozyme-like proteins are globulins belonging to the c-type lysozyme/ -lactalbumin family. To date, five lysozyme-like protein-encoding genes have been cloned from humans (LYZL2, LYZL4, LYZL5, LYZL6, and SPACA3) [1]. Preliminary studies have shown that lysozyme-like proteins differ from lysozyme in terms of tissue distribution, existence form, bactericidal activity, and physiological function [2-4]. In mammals, lysozyme is widely distributed in various tissues and body fluids, where it primarily participates in innate immune defense by disrupting bacterial cell walls and causing bacterial lysis and death [5-6]. In contrast, reported lysozyme-like proteins are specifically expressed in the male reproductive systems of mammals (such as mice, rats, and humans), localizing to the testis, epididymis, and sperm, where they are primarily associated with sperm function and play certain roles in fertilization [7-11]. For example, human SPACA3 encodes human sperm lysozyme-like protein 1 (SLLP1), which lacks bactericidal activity and is expressed in the acrosomal region of the sperm head, where it functions during fertilization [7]. Human lysozyme-like protein 4 (LYZL4) also shows no bactericidal activity in vitro; after being expressed by testicular and epididymal tissues, it localizes to the acrosomes of round and elongated spermatids, suggesting its potential involvement in acrosomal structure or function [11].

Our preliminary studies have demonstrated that human lysozyme-like protein 6 (LYZL6) possesses bactericidal activity, is expressed by the testis and epididymis, localizes to the post-acrosomal region of the sperm head, and may play a role in fertilization [8-10]. Compared with SLLP1 and LYZL4, LYZL6 may have additional functions. This study aims to further investigate the role of LYZL6 in the fertilization process and conduct an in-depth analysis of its physiological characteristics to elucidate the physiological functions of LYZL6.

Materials and Methods

1.1.1 Strains and Reagents

The recombinant *Pichia pastoris* strain expressing rLYZL6, rabbit anti-LYZL6 immune serum, and chitin affinity chromatography media were prepared and stored in our laboratory. DNA and protein molecular weight standards, Pyrobest DNA polymerase, and horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG were purchased from TaKaRa. All primers were synthesized by Sangon Biotech. BMGY medium was prepared according to the *Pichia pastoris* experimental manual from Invitrogen. Rhodamine-labeled goat anti-rabbit IgG, fluorescein isothiocyanate-conjugated Pisum sativum agglutinin (FITC-

PSA), trypsin, hyaluronidase (HAase), lysozyme (LYZ) standard, hyaluronic acid, peptidoglycan, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and L- -glutamyl-p-nitroanilide (L- -Glu-pNA) were all purchased from Sigma-Aldrich. The ECL detection kit was purchased from Beyotime. *Micrococcus lysodeikticus* was preserved in our laboratory. The Isolate kit was purchased from Irvine Scientific. Chemical reagents such as 3,5-dinitrosalicylic acid (DNS) and o-phenylenediamine were domestically produced.

1.1.3 Sample Source

Semen samples from healthy adult men and surplus human mature oocytes from in vitro fertilization were provided by the Reproductive Medicine Center of Shanghai Shuguang Hospital East Branch. Written informed consent was obtained from all donors for sample collection and use.

1.2.1 Sperm Immunofluorescence Analysis

Freshly collected semen was used to isolate sperm cells with the Isolate kit, followed by centrifugation at 400× g for 15 min. The cell pellet was collected, washed twice with phosphate-buffered saline (PBS, pH 7.4), and resuspended. Fifteen microliters of the cell suspension were smeared onto polylysine-treated slides and air-dried at room temperature for 15 min. After blocking with 3% bovine serum albumin solution for 10 min, 50 µL of rabbit anti-LYZL6 serum (diluted 1:100) was added and incubated overnight; pre-immune rabbit serum served as a negative control. Following three PBS washes, 50 µL of rhodamine-labeled goat anti-rabbit IgG (diluted 1:200) was added and incubated in the dark for 1 h. After three additional PBS washes, 6% FITC-PSA was added and incubated in the dark for 1 h before coverslips were mounted and images were captured with a fluorescence microscope.

1.2.2 RT-PCR Analysis

cDNA from sperm, epididymis, and testis was prepared in our laboratory. Forward primer RT-LYZL6-F and reverse primer RT-LYZL6-R were designed based on the LYZL6 gene sequence (GenBank accession no. NM_020426.3), yielding a 319 bp amplification product. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) served as an internal control, producing a 247 bp product. Amplification conditions were: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 45 s, and extension at 72 °C for 30 s; followed by a final extension at 72 °C for 5 min.

1.2.3 Preparation of Sperm Protein Extracts

Two 1.5 mL semen samples were centrifuged at 6000× g for 15 min, and the sperm cell pellets were washed twice with PBS and collected by centrifugation at 600× g for 8 min. To one pellet, 250 µL of RIPA buffer containing 1× protease inhibitors was added for resuspension, followed by sonication for 45 s

and lysis at room temperature for 20 min. After centrifugation at 12,000× g for 10 min, the supernatant was collected as the sperm protein extract sample and stored at -80 °C. The other cell pellet was resuspended in Biggers, Whitten, and Whittingham (BWW) medium (containing 30 mg/mL human serum albumin) for overnight capacitation. After centrifugation to collect the pellet, capacitated sperm protein extracts were prepared using the same method. Protein samples were subjected to SDS-PAGE electrophoresis and transferred to nitrocellulose membranes using a Bio-Rad electrotransfer system for Western blot analysis.

Table 1: Primers Used in This Study

Primer Name	Primer Sequence (5 -3)
RT-LYZL6-F	ATGACAAAGGCGCTACTCATC
RT-LYZL6-R	GAAGGTTGGGATTCAGCAGATC
GAPDH-F	CGTGGAAGGACTCATGACC
GAPDH-R	GAGGCAGGGATGATGTTCTG

1.2.4 Sperm Penetration Test

Semen samples were mixed with BWW medium (containing 5 mg/mL human serum albumin) and incubated at 37 °C in a 5% CO incubator for 2 h. Motile sperm cells were collected, centrifuged at 600× g for 8 min, and washed twice with 8 mL of the same medium. The sperm cells were then resuspended in BWW medium (containing 30 mg/mL human serum albumin) for overnight capacitation at a concentration of 2 × 10 cells/mL. Female golden hamsters were injected with 30 IU eCG, followed by hCG injection 72 h later to induce superovulation. Sixteen hours after hCG injection, the animals were sacrificed and cumulus-oocyte complexes were isolated. The complexes were treated with hyaluronidase (1 mg/mL) for 3 min to remove cumulus cells. The obtained oocytes were washed in medium under mineral oil and treated with trypsin (1 mg/mL) for 30 s, followed by three washes. The oocytes were then randomly grouped. Capacitated sperm were mixed with different concentrations of rabbit anti-LYZL6 serum and incubated at 37 °C in a 5% CO incubator for 1 h, with pre-immune rabbit serum serving as a control. Zona-free hamster oocytes were then added and incubation continued for 3 h. After incubation, oocytes were washed to remove unbound sperm, placed on slides with coverslips, and observed for fused sperm. Sperm head swelling was used as the criterion for fusion with the oocyte.

1.2.5 Hemizona Binding Assay

Human oocytes were bisected under a micromanipulator, and the oocyte cytoplasm was aspirated with a micropipette to obtain two identical hemizonae. Paired hemizonae mounted on polylysine-coated slides were treated with rabbit anti-LYZL6 serum (diluted 1:50) or pre-immune rabbit serum as a negative

control, and incubated at 37 °C in a 5% CO incubator for 2 h. After washing, 100 µL of capacitated sperm suspension (concentration 2 × 10 cells/mL) was added to each hemizona, covered with mineral oil, and incubated at 37 °C for 4 h. Hemizonae were rinsed with BWW medium to remove loosely bound sperm, and tightly bound sperm were observed and counted under an inverted microscope.

1.2.6 Recombinant Protein Expression and Purification

A clone of the recombinant *Pichia pastoris* strain expressing rLYZL6 was inoculated into BMGY seed medium and cultured at 30 °C on a shaker for 36 h, then transferred to a fermenter containing 2.5 L BMGY medium for high-density cultivation until glycerol exhaustion. Methanol was added at 0.5% every 12 h to induce expression, and fermentation was terminated after 96 h. The fermentation broth was centrifuged at 5000× g for 30 min, and the supernatant was collected and mixed with chitin affinity medium. After stirring and settling at room temperature, the chitin medium was packed into a chromatography column. The column was washed with PBS, and elution was performed with 0.01 mol/L acetic acid solution. The eluate was collected, dialyzed against PBS, and concentrated using Amicon® Ultra centrifugal filter units (3 kDa molecular weight cutoff). The concentrated solution was loaded onto a Sephadex G-75 column, and independent elution peaks were monitored at A280nm, collected, and concentrated for SDS-PAGE analysis.

1.2.7 Assay for Peptidoglycan and Hyaluronic Acid Binding Ability

Ninety-six-well plates were coated with 40 g/mL peptidoglycan solution or 100 L of 100 g/mL hyaluronic acid solution and incubated overnight at 37 °C. After blocking with 200 L of 1 mg/mL bovine serum albumin for 2 h, 100 L of rLYZL6 solution at concentrations of 0.25 mol/L, 0.5 mol/L, and 1 mol/L prepared in acetate buffer (pH 5.0) was added and incubated at room temperature for 3 h. One hundred microliters of rabbit anti-LYZL6 immune serum was then added to each well and incubated at 37 °C for 1 h, followed by the addition of 100 µL of HRP-labeled goat anti-rabbit IgG (diluted 1:1000) and incubation at 37 °C for 1 h. All steps were followed by three washes with PBS containing 0.05% Tween-20. Finally, o-phenylenediamine chromogenic substrate was added for color development, and the ELISA index was calculated by measuring A450nm with a microplate reader. ELISA Index = average A450nm of test sample / average A450nm of blank control. HAase and LYZ were used as controls, and experiments were repeated twice.

1.2.8 Assay for Hyaluronic Acid Hydrolysis Activity

To 0.5 mL of hyaluronic acid solution (0.15%, w/v), 0.5 mL of rLYZL6 solution at concentrations of 1 mol/L, 5 mol/L, and 10 mol/L was added and incubated at 37 °C for 24 h. The reaction was terminated by boiling for 10 min, and denatured proteins were precipitated by centrifugation at 5000× g for 10 min.

The supernatant (0.5 mL) was mixed with 1 mL of DNS solution and boiled for 10 min for complete color development. After cooling, A540nm was immediately measured. HAase, LYZ, and PBS served as controls, and experiments were repeated twice.

1.2.9 Assay for Free Radical Scavenging Activity

A 0.05% DPPH solution was prepared in methanol. To 2 mL of DPPH solution, 0.5 mL of rLYZL6 solution at concentrations of 1 mol/L, 5 mol/L, and 10 mol/L was added and incubated in the dark at room temperature for 30 min. The absorbance at 519 nm was recorded after the reaction. Free radical scavenging rate (%) = (A - A min) / A × 100%, where A and A min represent the absorbance values measured immediately after sample addition and after 30 min, respectively. LYZ and PBS served as controls, and experiments were repeated twice.

1.2.10 Assay for Isopeptidase Activity

A substrate solution of 0.0175 mol/L L- -Glu-pNA was prepared in 0.05 mol/L 3-(N-morpholino)propanesulfonic acid buffer (MOPS, pH 7.0, containing 0.01 mol/L NaCl). One hundred microliters of rLYZL6 solution was mixed with 2.5 mL of substrate solution and reacted at room temperature for 1 h. The increase in A405nm (ΔA405nm) was recorded every 10 min. LYZ and PBS served as controls, and an absorbance curve was plotted.

1.2.11 Statistical Analysis

All data were analyzed using SPSS 17.0 statistical software. Comparisons between groups were performed using paired sample t-tests, with $P < 0.05$ considered statistically significant.

Results

2.1 Sperm Immunofluorescence Detection

After counting sperm cells, immunofluorescence was detected in over 95% of sperm cells, with fluorescence localized to the post-acrosomal region of the sperm head (Fig. 1 [Figure 1: see original paper]).

2.2 RT-PCR Analysis

To determine the origin of LYZL6 on sperm, RT-PCR was used to analyze LYZL6 gene expression in the male reproductive system. Results showed that LYZL6 gene expression was detected in both testis and epididymis, with higher expression levels in the testis than in the epididymis. No expression was detected in sperm, indicating that LYZL6 protein on the sperm surface originates from secretion by the testis and epididymis (Fig. 2 [Figure 2: see original paper]).

2.3 Immunodetection of LYZL6 in Sperm

This study examined changes in LYZL6 in sperm protein extracts before and after capacitation by Western blot. The results indicated that the amount of LYZL6 on the sperm surface did not change significantly after capacitation (Fig. 3 [Figure 3: see original paper]).

2.4 Hamster Egg Sperm Penetration Test

After capacitated sperm were incubated with rabbit anti-LYZL6 serum at dilutions of 1:100, 1:200, and 1:400, the number of sperm fused with oocytes decreased significantly by 65.4%, 61.9%, and 51.5% compared with the control group, respectively. Anti-serum at dilutions of 1:800 and 1:1600 did not cause a significant decrease. Therefore, rabbit anti-LYZL6 serum exhibited a dose-dependent inhibitory effect on sperm-egg fusion (Fig. 4 [Figure 4: see original paper]).

2.5 Hemizona Binding Test

The experiment showed that after sperm were incubated with anti-LYZL6 serum at dilutions of 1:10, 1:50, and 1:200, an average of 62, 46, and 56 sperm bound to the hemizona, respectively. In paired hemizona tests with sperm incubated in pre-immune rabbit serum, the average numbers of bound sperm were 54, 52, and 49, respectively. Statistical analysis indicated that anti-LYZL6 serum at various dilutions could not significantly inhibit sperm binding to the zona pellucida (Fig. 5 [Figure 5: see original paper]).

2.6 Recombinant Protein Expression and Purification

SDS-PAGE detected expression of a target protein of approximately 14.8 kDa in the fermentation supernatant, consistent with the expected molecular weight of LYZL6 (Fig. 6 [Figure 6: see original paper]). Purification using chitin affinity chromatography and molecular sieve chromatography yielded a protein of the same molecular weight, which was confirmed as rLYZL6 by immunoblotting (Fig. 7 [Figure 7: see original paper]).

2.7 Determination of Peptidoglycan Binding Ability

The results showed that under pH 5.0 conditions, the peptidoglycan binding ability of rLYZL6 was significantly higher than that of LYZ (Fig. 8 [Figure 8: see original paper]).

2.8 Determination of Hyaluronic Acid Binding Ability

The results indicated that HAase had strong hyaluronic acid binding ability, LYZ had weak hyaluronic acid binding ability, and rLYZL6 had the weakest hyaluronic acid binding ability, significantly lower than both HAase and LYZ (Fig. 9 [Figure 9: see original paper]).

2.9 Determination of Hyaluronic Acid Hydrolysis Activity

The results showed that hyaluronidase exhibited concentration-dependent hyaluronic acid hydrolysis activity, while neither rLYZL6 nor LYZ showed detectable hyaluronic acid hydrolysis activity, with no significant difference between the two (Fig. 10 [Figure 10: see original paper]).

2.10 Determination of Free Radical Scavenging Activity

The results demonstrated that LYZ exhibited concentration-dependent free radical scavenging activity, whereas no significant free radical scavenging activity was observed for rLYZL6 (Fig. 11 [Figure 11: see original paper]).

2.11 Determination of Isopeptidase Activity

The results showed that LYZ exhibited weak isopeptidase activity, while rLYZL6 displayed strong concentration-dependent isopeptidase activity, significantly higher than LYZ (Fig. 12 [Figure 12: see original paper]).

Discussion

Our previous studies using immunohistochemistry detected LYZL6 localization in late spermatocytes and round spermatids in the testis, and immunofluorescence staining further confirmed its localization to the post-acrosomal region of the sperm head [10]. Combined with existing research, it is evident that lysozyme-like proteins localize to sperm cells after secretion, but their specific subcellular localizations are not identical. For example, SLLP1 localizes to the acrosomal membrane of the sperm head after secretion, mouse LYZL4 localizes to both the acrosome and tail of the sperm head, while LYZL6 shows a different localization pattern, suggesting that various lysozyme-like proteins may have distinct functions [3, 7]. Although LYZL6 could be detected in protein extracts from testis, epididymis, and sperm by Western blot, the exact origin of LYZL6 on sperm could not be determined. This study used RT-PCR analysis to demonstrate that LYZL6 is not expressed by sperm itself but is secreted by testicular and epididymal tissues and subsequently attaches to the post-acrosomal region of the sperm head. As sperm undergo capacitation after entering the female reproductive tract, various changes occur in surface molecules, and some proteins that initially attach to sperm, such as the glycoprotein Glycodelin-S, are lost [12-13]. If LYZL6 were subject to such loss, it might not function in subsequent fertilization processes. This study confirmed that LYZL6 was not lost after capacitation by detecting LYZL6 in sperm protein extracts before and after capacitation, suggesting that LYZL6 may still play a role in fertilization.

For successful fertilization, sperm must sequentially undergo a series of steps including capacitation, cumulus penetration, zona pellucida (ZP) protein binding, acrosome reaction, zona pellucida penetration, and oolemma binding, with various molecules playing critical roles at each stage [14-15]. This study em-

ployed hamster egg sperm penetration tests and hemizona binding assays to preliminarily investigate whether LYZL6 participates in the fertilization process. Incubation of sperm with anti-LYZL6 serum significantly reduced the number of sperm fused with the oolemma, consistent with previous findings [10]; however, anti-LYZL6 serum did not affect sperm binding to the zona pellucida. This suggests that LYZL6 may function only in specific stages of fertilization (such as sperm-egg fusion). Previous studies have shown that SLLP1 can function in sperm-egg binding during fertilization [7], and our group has also found similar functions for LYZL4 (unpublished results), leading us to speculate that lysozyme-like family proteins can function at different stages of fertilization. Furthermore, since SLLP1 functions in sperm-egg binding by binding to ligand proteins on the oolemma, it is possible that LYZL6 may function through a similar ligand-binding mechanism [16].

Studies have shown that some proteins from the testis and epididymis bind to sperm and influence sperm function at multiple levels, most importantly by regulating spermatogenesis and sperm maturation [17-18]. In addition to these functions, some molecules possess antimicrobial activity and thus play important roles in innate immunity of the male reproductive system, such as HE2, EPPIN, and PATE family proteins [19-22]. LYZ is one of the abundant molecules in male reproductive tract secretions, and besides its bactericidal activity, it can also regulate semen viscosity [23]. Current research indicates that the functions of lysozyme-like family proteins differ from those of LYZ. For example, SLLP1 and LYZL4 lack significant muramidase activity and therefore cannot hydrolyze the -1,4-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in bacterial cell walls, thus losing bactericidal activity [7]. Our previous studies have shown that LYZL6 possesses bactericidal activity under acidic conditions. This study demonstrates that LYZL6 has strong peptidoglycan binding ability under low pH conditions compared with LYZ, which is a prerequisite for its strong bactericidal activity. Moreover, since the pH conditions for maximal LYZL6 activity are similar to those of the female vagina, LYZL6 may provide certain protective effects for sperm after entering the female reproductive tract.

This study also found that LYZL6 possesses strong isopeptidase activity. To date, only a few invertebrate-type (i-type) lysozymes have been found to possess isopeptidase activity, such as i-type lysozymes isolated from clams and eastern oysters [24-25]. The impact of LYZL6' s isopeptidase activity on sperm function remains to be further investigated, but it is known that fibrin is cross-linked through isopeptide bonds between lysine and glutamic acid side chains, and isopeptidases can cleave these bonds to dissolve fibrin and thrombi. This suggests that LYZL6 provides a potential option for thrombosis therapy. Notably, lysozyme-like family proteins appear to have different physiological characteristics; for example, LYZL4 does not possess isopeptidase activity (unpublished results). Additionally, although LYZL6 cannot bind hyaluronic acid or scavenge free radicals, our group has found that LYZL4 possesses strong hyaluronic acid binding ability and free radical scavenging capacity, indicating significant functional differences among different lysozyme-like proteins.

In summary, this study further confirms that LYZL6 is secreted by the testis and epididymis, localizes to the post-acrosomal region of mature sperm heads, may function in the sperm-egg fusion stage of fertilization, and possesses both bactericidal and isopeptidase activities, suggesting that LYZL6 may be involved in sperm function through multiple mechanisms. Our group will further investigate the mechanism of LYZL6 action in fertilization and the physiological significance of its enzymatic activities to promote understanding of lysozyme-like family protein functions.

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Note: Figure translations are in progress. See original paper for figures.