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Loss of fibrinogen in zebrafish results in an asymptomatic embryonic hemostatic defect and synthetic lethality with thrombocytopenia

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Running title: Hemostatic defects in fibrinogen null zebrafish

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ESSENTIALS

- Loss of fibrinogen in zebrafish has been previously shown to result in adult onset hemorrhage
- Hemostatic defects were discovered in early fga$^{-/-}$ embryos but well tolerated until adulthood
- Afibrinogenemia and thrombocytopenia results in synthetic lethality in zebrafish.
- Testing human FGA variants of uncertain significance in zebrafish identified causative mutations

SUMMARY

Background: Mutations in the alpha chain of fibrinogen (FGA), like deficiencies in other fibrinogen subunits, lead to rare inherited autosomal recessive hemostatic disorders. These range from asymptomatic to catastrophic life-threatening bleeds, and the molecular basis of inherited fibrinogen deficiencies is only partially understood. Zinc finger nucleases have been used to produce mutations in zebrafish fga, resulting in overt adult onset hemorrhage and reduced survival. Objectives: To determine the age of onset of hemostatic defects in afibrinogenemic zebrafish, and model human fibrinogen deficiencies. Methods: TALEN genome editing (transcription activator-like effector nucleases) was used to generate a zebrafish fga mutant. Hemostatic defects were assessed through survival, gross anatomical and histological observation, and laser-induced endothelial injury. Human FGA variants with unknown pathologies were engineered into the orthologous positions in zebrafish fga. Results: Loss of Fga decreased survival and resulted in synthetic lethality when combined with thrombocytopenia. Zebrafish fga

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mutants exhibit a severe hemostatic defect by 3 days of life, but without visible hemorrhage. Induced thrombus formation through venous endothelial injury was completely absent in mutant embryos and larvae. This hemostatic defect was restored by microinjection of wild type fga cDNA plasmid or purified human fibrinogen. This system was used to determine whether unknown human variants were pathological by engineering them into fga. Conclusions: These studies confirm that loss of fibrinogen in zebrafish results in the absence of hemostasis from the embryonic period through adulthood. When combined with thrombocytopenia, zebrafish exhibit synthetic lethality, demonstrating that thrombocytes are necessary for survival in response to hemorrhage.

KEYWORDS

fibrinogen, genome editing, hemostasis, thrombocytopenia, zebrafish

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INTRODUCTION

Hemostasis is a critical requirement to maintain blood circulation, and upon injury a cascade of enzymatic reactions is initiated. Platelets adhere to the site of injury and activate, forming the primary plug, after which coagulation factors trigger a burst of thrombin production, driving the conversion of soluble fibrinogen to an insoluble fibrin clot. The regulation of coagulation factor activation and inhibition provides checks and balances that assure proper clot formation and eventual dissolution. Aberrations to these pathways result in both bleeding or clotting disorders which contribute to hemorrhage, ischemia, surgical complications and even death [1].

Fibrinogen is a liver-derived hexameric glycoprotein encoded by paralogous genes FGA, FGB, and FGG (coding for Aα, Bβ and γ chains, respectively) on human chromosome 4 (4q31.3–4q32.1)[2-4]. In the presence of thrombin, fibrinopeptides (A and B) are removed, leaving fibrin monomers, which are stabilized by FXIII cross-linking [3, 5-8] driving insoluble clot formation. Mutations in FGA, FGB, or FGG can all affect the synthesis, assembly, intracellular processing, stability or secretion of fibrinogen. Congenital deficiency of fibrinogen, either quantitatively (hypofibrinogenemia and afibrinogenemia) or qualitatively (dysfibrinogenemia), is an inherited autosomal bleeding disorder with heterogeneous penetrance, although the latter has been shown to result in thrombosis in some cases [9]. Hemorrhaging can occur in various tissues such as skin, soft tissues, muscles, joints, gastrointestinal tract, or genitourinary tract, with intracranial bleeding being a major cause of death [4, 10-12]. Afibrinogenemia is estimated to occur in 1 per 1,000,000 individuals [11, 13] and cumulatively congenital fibrinogen disorders are thought to make up 8% of all rare coagulation disorders [14]. A majority of diagnosed

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dysfibrinogenemia and afibrinogenemia cases have been attributed to mutations and modifications within the FGA sub-chain [15].

Current therapeutic strategies for congenital fibrinogen deficiency include replacement with fresh-frozen plasma, cryoprecipitate, or plasma-derived fibrinogen concentrate. Unfortunately, these treatments have been linked to negative outcomes including transfusion-associated complications, off-target effects on related plasma proteins, and failed virus inactivation [10, 16-18]. Consequently, increased risk and diminished treatment efficiency result in reduced clinical administration [19].

To improve treatments for fibrinogen-related disorders, further interrogation of the disease mechanisms in physiologically relevant model systems are required. In recent years, zebrafish (Danio rerio) has become an increasingly popular model for studying blood pathologies due to high genetic and physiologic homology to humans. Zebrafish are economical, easy to maintain/manipulate, and undergo rapid, external development as transparent embryos/larvae making them amenable to hemostatic assays. We and others have demonstrated a high degree of conservation of the coagulation cascade between zebrafish and humans identifying homologs for nearly all major factors [20-25] with strong data implicating mononuclear thrombocytes as functional equivalents to platelets [26, 27]. However, thrombocytes have not been proven to be required for survival in response to hemorrhage. Recent advancements in genome editing technologies (e.g., TALENs, CRISPR), have enabled the assessment of both individual and multigene knockouts to interrogate the genetic underpinnings of bleeding and clotting phenotypes [28-30].

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We have previously shown that antisense morpholino-mediated knockdown of zebrafish fibrinogen chains results in frequent intracranial and intramuscular hemorrhage at 3 days post fertilization (dpf) [31]. However, this was not observed in a zinc finger nuclease (ZFN)-mediated knockout of fga [28]. Instead, mutant fish survived into adulthood but were found to be underrepresented at 4-5 months of age, with the data suggesting ~40% survival. In this study we used TALEN-mediated genome editing to generate a transmissible mutation in fga similar to and recapitulating the phenotypes of the ZFN mutant. These include survival into adulthood with no gross larval bleeding, but eventual adult lethality due to hemorrhage in various tissues. Despite a lack of overt bleeding, hemostasis was defective as early as 3 dpf. Interestingly, we were able to reverse the hemostatic defect after treatment with an anti-fibrinolytic compound. We also showed that $fga^{-/-}$ fish could be used to discern human FGA variants that abrogate fibrinogen function. Finally, loss of the transcription factor Nfe2 results in severe thrombocytopenia in adult zebrafish [32], similar to mouse [33-35]. Combined deficiency of Fga and Nfe2 results in synthetic adult lethality, which we believe is the first evidence indicating that thrombocytes are required in vivo for prevention of hemorrhage and hemostasis-related survival.

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METHODS

Zebrafish lines and maintenance

Experiments were performed using AB×TL F1 hybrid background zebrafish. Embryos derived from natural spawning were raised at 28.5°C and developmental stages (embryo, larva, juvenile, adult [30]) were determined using age (hours post fertilization (hpf) or dpf) and morphological characteristics [36]. Animal care and usage were in accordance with animal care guidelines approved by the University of Michigan Institutional Animal Care & Use Committee.

TALEN-induced mutagenesis of coagulation factors in zebrafish

TALEN-mediated genome editing was used to generate mutations in exon 2 of zebrafish fga at the target sequence CTTCAAGGCACAAGAC. Engineered TALENs were injected into 1-cell stage zebrafish embryos [30, 37], raised to adulthood, and crossed with AB×TL wild type fish to confirm germline transmission. The resulting fga mutants were used for further studies. nfe2 mutants were previously generated using TALENs [32].

Genotyping

Zebrafish were anesthetized in tricaine (0.16 mg/mL, Western Chemical) and fin biopsies were obtained [38, 39] or fish were humanely killed in high-dose tricaine (1.6 mg/mL) followed by DNA preparation. Tissue was lysed in buffer (10 mM Tris-Cl, pH 8.0; 2 mM EDTA, 2% Triton X-100, and 100 µg/mL Proteinase K) at 55°C overnight [29]. Samples were heated to 95°C for 5 minutes to inactivate Proteinase K, followed by

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PCR (primers in Table S1). PCR products were resolved using agarose gel electrophoresis or a Qiaxcel capillary electrophoresis system (Qiagen).

Laser-induced endothelial injury

Laser-induced thrombi were generated after injury of the endothelium of the posterior cardinal vein (PCV) of larvae as previously described [40-42]. In brief, zebrafish larvae were anesthetized at 3 dpf, embedded in agarose (0.8%), and the endothelium was ablated with a laser at the 5th somite distal to the anal pore (Olympus IX71 and MicroPoint Pulsed Laser System, Andor Technology). Time to occlusion was recorded up to 2 minutes, followed by removal of larvae from agarose and subsequent genotyping.

ε-aminocaproic acid treatment

Mutant offspring were derived from fga$^{+/-}$ and fga$^{-/-}$ intercrosses. At 24 hpf, embryos were treated with 100 mM ε-aminocaproic acid [29, 30]. At 3 dpf, laser injury and genotyping were performed.

Construction and microinjection of expression vectors

Expression constructs and primers used to generate them are listed in Supplemental Tables S1-2. Constructs were made on the pubi:p2A-EGFP backbone [30] with wild type and mutant human and zebrafish fga cDNAs under control of the zebrafish ubiquitin (ubi) promoter [43] with bicistronic expression of egfp using the 2A self-cleaving peptide. Mutations were generated using site-directed mutagenesis [44], verified

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by restriction digestion and sequencing (primers in Table S1), and injected into 1-cell stage embryos. Mutations are numbered according to the human amino acids.

Retro-orbital fibrinogen infusions

Larvae were anesthetized with tricaine at 3 dpf and placed upon an agarose mold for infusions as previously described [29]. In brief, human fibrinogen (Sigma) or bovine serum albumin as a control (BSA, Sigma) were dissolved in 0.9% NaCl to a final concentration of 25 mg/mL and 2 nL were infused into the retro-orbital space. Larvae were allowed to recover in system water for 1 hour before undergoing laser injury and genotyping.

Quantitative (qPCR) and qualitative PCR analysis

Zebrafish embryos and larvae were homogenized with a 21 gauge needle in lysis buffer. Total RNA was isolated with the PureLink® RNA Mini Kit (Life Technologies) or RNeasy Mini Kit (Qiagen) according to the manufacturer protocols. Total RNA was treated with DNase I (Invitrogen) and used for 1st-strand cDNA synthesis with oligo $(dT)_{12\text{-}18}$ primers using Superscript-II (Invitrogen). The cDNA was used for RT-PCR (Eppendorf MasterCycler) and qPCR (Bio-Rad, iCycler) reactions (primers in Table S1). qPCR data were analyzed as previous described [45]. Qualitative PCR analysis was also performed on cDNA derived from 5 dpf larva.

Blood collection and western blot analysis

Adult zebrafish blood was collected and western analysis performed as described [29, 46, 47]. Briefly, blood was collected into EDTA coated microcapillaries

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(ThermoFisher). One microliter of pooled plasma (at least two fish per genotype) in buffer with β-mercaptoethanol was resolved on a 4-20% SDS-PAGE gel (Bio-Rad), and transferred to nitrocellulose (Bio-Rad). Membranes were probed with zebrafish anti-fibrinogen antibody, followed by HRP-conjugated secondary antibody (Santa Cruz), then developed with chemiluminescent substrate (Super Signal West Femto; ThermoFisher) and analyzed on a FluorChem system (Protein Simple).

Histological examination and o-dianisidine staining

Zebrafish from $fga^{+/-}$ incrosses were fixed with 4% PFA/PBST at 4°C overnight, embedded in paraffin, sectioned at 4-5 µm, and stained with hematoxylin and eosin (H&E). Embryos from $fga^{+/-}$ incrosses were stained at 7dpf for hemoglobin with o-dianisidine as previously described [48, 49]. Images were collected on a Leica MZFL III stereoscope or Olympus BX51 and recorded using an Olympus DP70 digital camera.

Assessment of human FGA variants

Variants in human FGA were identified from patients manifesting with hemorrhage and hypofibrinogenemia as documented in the Human Fibrinogen Database [50] and other sources [51]. These were engineered into the orthologous positions of zebrafish wild type fga cDNA constructs under the control of the ubi promoter (Table S2) and injected into 1-cell stage mutant offspring from $fga^{+/-}$ to $fga^{-/-}$ intercrosses, followed by laser injury at 3 dpf.

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Statistical analysis

Statistical analysis was performed using Mann-Whitney U or two-tailed Student t tests. Survival was evaluated by log-rank (Mantel-Cox) testing for significance. Charts and survival curves were generated using Prism (GraphPad Software).

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RESULTS

Targeted mutagenesis of fga using TALENs results in a null allele

Mutations in human FGA result in inherited hypofibrinogenemia with variable manifestations in humans [1, 12, 15]. We inactivated the zebrafish fga gene by TALEN-mediated genome editing in the 2nd exon of fga (Fig. 1A). A mutant line was identified with a net 26 nucleotide insertion, resulting in a frameshift and premature stop codon (Fig. 1B). Frameshifts often result in loss of expression due to nonsense-mediated decay [29, 30] and we tested this possibility using qPCR. We found that transcription of fga mRNA in 3-day-old fga$^{-/-}$ larva was nearly undetectable compared to fga$^{+/+}$, and fga$^{+/-}$ siblings were reduced by ~40% ($p=0.0001$ and $p<0.05$, respectively, Fig. 1C). A qualitative assessment of cDNA isolated from fga$^{-/-}$ embryos showed no detectable fga$^{+/+}$ expression remaining in mutants (Fig. 1D), and no residual Fga was observed in plasma isolated from fga$^{-/-}$ adult fish (Fig. 1E). Furthermore, qPCR data indicate that relative transcription levels of other coagulation factors, prothrombin (f2), factor X (f10) and antithrombin III (at3), are also significantly reduced in fga$^{-/-}$ larva ($p<0.05$, Fig. S1).

fga mutant survival drops off as fish reach breeding age and is exacerbated by loss of Nfe2

Targeted mutation of FGA in mice results in a risk of fatal abdominal hemorrhage, but overall survival is variable and dependent on genetic background [52]. ZFN generated fish mutants produced on a pure AB background demonstrate ~40% survival at 4-5 months of age [28]. We tracked the survival of our fga$^{-/-}$ mutants and their fga$^{+/-}$ siblings daily. fga$^{-/-}$ mutant survival was progressively reduced compared to the fga$^{+/-}$ siblings over the first year of life (Fig. 2A; $p<0.0001$). Loss of fga in zebrafish did not

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manifest with lethality until $fga^{-/-}$ mutants approached breeding age. All $fga^{-/-}$ fish were alive at 45 dpf (Fig. 2A), but survival declined to 90% at 60 dpf, and to 40% shortly after one year of age.

In mammals Nfe2 is required for megakaryocyte maturation and platelet formation [33-35], and zebrafish lacking Nfe2 have severe thrombocytopenia with normal survival [32]. Knockout mice deficient in fibrinogen or Nfe2 have severe hemostatic defects, with variable survival into adulthood. Combined deficiency results in death within 48-72 hours after birth [53]. We intercrossed fga and nfe2 mutant fish and found that loss of both Fga and Nfe2 resulted in synthetic lethality by one year of age (Fig. 2B; $p<0.0001$).

Mutation in zebrafish fga leads to hemorrhage in multiple tissues

In contrast to other coagulation protein deficiencies [29, 30], loss of fga results in a relatively mild hemorrhagic phenotype in zebrafish. As seen previously [28], we also did not detect grossly visible bleeding by o-dianisidine staining in $fga^{-/-}$ larval mutants (data not shown). At 41 dpf juvenile $fga^{-/-}$ and $fga^{+/+}$ siblings were randomly selected for histological analyses. In homozygous mutants, 64% manifested with mild or moderate bleeds in various tissues including brain, jaw, muscle, fin, and abdomen (Fig. 3A). In addition to previously observed bleeding in the eyes, brain, and abdominal cavity [28], massive hemorrhage was observed in muscle, and mild bleeding in the jaw/mouth and fins (Fig. 3A). Hemorrhaging in the brain progressively worsened by 47 dpf (Fig. 3B) and was occasionally accompanied by ocular bleeds. Death was typically preceded by severe intracranial hemorrhage.

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The hemostatic defect of fibrinogen deficiency manifests in the embryonic period

Previously, fga was targeted using ZFNs, resulting in overt hemorrhage and reduced survival [28]. To address whether this affects intravascular physiologic hemostasis, we measured the time to occlusion after laser-induced endothelial injury of the PCV. Normally, venous thrombus formation under these conditions occludes the vessel within 2 minutes, but this did not occur in $fga^{-/-}$ larvae ($p<0.0001$, Fig. 4A). To rescue the hemostatic defect and confirm that it was due to loss of Fga, we injected a Tol2 mediated zebrafish fga cDNA expression plasmid into 1-cell stage $fga^{+/-}$ incross offspring. At 3 dpf, over 50% of the injected $fga^{-/-}$ larvae displayed PCV occlusion times similar to their injected and uninjected $fga^{+/+}$ and $fga^{+/-}$ siblings, which was significantly different than the uninjected homozygous mutants ($p<0.05$ by Mann-Whitney U test, Fig. 4B).

We previously demonstrated that loss of At3 in larvae results in a consumptive coagulopathy that is rescued by infusion of human fibrinogen [29]. To determine whether human fibrinogen could replace zebrafish fibrinogen, we infused human fibrinogen concentrate into $fga^{-/-}$ larvae. Approximately half of the infused $fga^{-/-}$ mutants showed significantly shorter occlusion times in comparison with non-infused $fga^{-/-}$ siblings ($p<0.05$, Fig. 4C). Together, our findings confirm that the fga mutation leads to an intravascular hemostatic defect, similar to that seen in mouse and human pathologies, which can be reversed by transient expression of wild type fga cDNA or infusion of human fibrinogen protein.

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Inhibition of fibrinolysis alleviates the fga$^{-/-}$ hemostatic defect

Fibrinolysis inhibitors are believed to function through stabilization of the fibrin clot. However, we previously showed that ε-aminocaproic acid can reverse the hemostatic defect in our model of consumptive coagulopathy, which is characterized by hypofibrinogenemia [29]. At 24 hpf, fga$^{-/-}$ mutants were incubated in ε-aminocaproic acid and then subjected to laser injury at 3 dpf. No control fga$^{-/-}$ larvae were able to occlude within 2 minutes of injury. In contrast, 33% of ε-aminocaproic acid-treated fga$^{-/-}$ larvae occluded within 60 seconds of laser-induced injury (p=0.0003, Fig. 5).

Evaluation of human FGA variants in fga deficient zebrafish helps link genetic causation to pathology in vivo

We previously showed that we can use zebrafish as an in vivo system for the identification of the causative mutations in coagulation disorders [29, 30]. Since zebrafish Fga deficiency closely parallels human pathology, human FGA variants including C55G, C64Y, Y809C (Human Fibrinogen Database) [50, 54, 55] and M1V [51] were assessed in fga$^{-/-}$ zebrafish. These variants have been associated with hemorrhage in human populations (Table 1), however, they have not been functionally validated in vivo. A multispecies alignment of humans, mice, chickens and zebrafish demonstrates conservation of these amino acids across all species. To evaluate each variant's ability to rescue the hemostatic defect in fga mutants, a series of zebrafish fga cDNA constructs were generated to express the variants at positions orthologous to the human FGA mutations (Table S2). Plasmids were injected into 1-cell stage mutant offspring, and subjected to laser-induced endothelial injury followed by genotyping. M1V and Y809C were not able to reverse the occlusion defects of fga$^{-/-}$ mutant. In contrast, C55G and

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C64Y were able to rescue the mutant hemostatic defect (Fig. 6). By assessing known human gene variants in fga mutant zebrafish, gene regions that convey phenotypic deficiencies can be identified, shedding light on the mechanistic cause of pathologies.

DISCUSSION

We have produced a mutation in exon 2 of the fga gene in zebrafish using TALENs in a nearly identical location as the 3 mutant fga lines generated using ZFNs. The TALENs and ZFN mutations overlap with differences of 8 base pairs or less [28]. In the previous report, the authors identified spontaneous hemorrhage in adult fish, but examination of 3 and 5 dpf larvae found no evidence of bleeding [28]. This suggested the possibility that hemostasis in early development does not require the presence of fibrinogen. However, we have previously demonstrated that induced larval venous thrombi are fibrin rich [31], suggesting an important role in embryonic hemostasis. In this study our endothelial injury experiments confirm that fibrinogen is indeed required for induced thrombus formation, consistent with our previous studies of F10 deficiency, as well as observed hypofibrinogenemia in At3 deficient fish [29, 30]. In all three mutants (fga, f10, at3), embryonic/larval zebrafish tolerate severe coagulopathies into early adulthood before succumbing, although death occurs at a much later timepoint in the fga mutants. Fish et al. [28] found ~40% survival of homozygous mutants at 4-5 months of age, while at a similar age we recovered 80%, with 40% remaining around one year of age. These differences are likely due to variations in housing/aquaculture systems and/or genetic background, since the prior mutant was generated on a pure AB background while we used an AB×TL hybrid. Similar survival variations have been observed with targeted mutation of Fga in mice, which exhibit variable timing of fatal abdominal

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hemorrhage depending on the genetic background. At 2 months of age, 91% of C57BL/6J mice but only 57% of the mixed 129/CF-1 background were found to be alive [52]. The fish fga mutant data are in sharp contrast to F10 deficiency which resulted in death beginning as early as 1 month of age, with complete loss by 4 months [30]. However, this difference is consistent with observations in human patients [1] and mouse knockouts [56-59] demonstrating that common pathway mutations are more severe than defects in fibrinogen.

One of the most intriguing findings of this study was the synthetic lethality observed from combined loss of fibrinogen and Nfe2. Thrombocytes have clearly been shown to express similar genes and function like platelets [26], but definitive proof that they are active in preventing hemorrhage has not been shown. The lethality in $fga^{-/-};nfe2^{-/-}$ mutants bolsters the belief that thrombocytes are the functional equivalent to mammalian platelets and, to our knowledge, are the first in vivo data that they are required for hemostasis-related survival. In mice $fga^{-/-};nfe2^{-/-}$ results in lethality in the immediate neonatal period, but zebrafish initially survive with a steady decline until ~300 dpf. This trend is consistent with coagulation factor knockouts that are embryonic lethal in mice, but exhibit extended survival in zebrafish [29, 30]. We hypothesize this may be due to differential species-specific factors regulating hemostatic balance which could be leveraged to improve our understanding of coagulopathies. Presumably $fga^{-/-};nfe2^{-/-}$ fish die from hemorrhage, although given the broad time span of lethality and low genotypic frequency of double mutants, definitive confirmation will require significantly larger pedigrees.

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Visible hemorrhage was noted after one month of age in fga mutants, most commonly in the brain, muscle, and fins. The timing, location, and extent of hemorrhage are similar to the previous ZFN-induced fga knockout [28], as well as our results for F10 deficiency [30]. In both mutants, this appears to be the cause of lethality, but the timing is significantly different, as all f10 homozygous mutants die by 4 months of age, as opposed to 40-80% survival at that timepoint for fga mutants. Unlike mice, there is great variability within standard zebrafish strains [60]. Even so, genetic background is less likely to be a factor in the difference between these two strains, as the mutant lines were produced from the same AB×TL colony. It seems unlikely that differences in the genetic background of the fga and f10 mutants would cause such a large difference in timing of lethality, suggesting that there may be additional factors beyond the observed hemorrhage.

The reversal of the larval hemostatic defect by ε-aminocaproic acid was surprising. Clinically, ε-aminocaproic acid is thought to stabilize clots by binding to plasmin, preventing fibrinolysis. We have now observed rescue of hypofibrinogenemic [29] and afibrinogenemic zebrafish using ε-aminocaproic acid, which suggests there may be alternate mechanisms for its modulation of hemostasis.

Our previous studies of fibrinogen depletion in zebrafish employed antisense morpholino technology, targeting all three fibrinogen chains, fga, fgb, and fgg [31]. Individual knockdown resulted in 1-5% rates of intramuscular and intracranial hemorrhage in 3 dpf larvae. When all 3 morpholinos were co-injected, the rate increased to nearly 20%. However, in both our TALEN- and the ZFN-induced knockouts, these phenotypes were not present. One possible explanation for this discrepancy is that morpholino and germline targeting yield differential phenotypic effects. There have been

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some reports that suggest there is genetic compensation for germline mutations, but not morpholino knockdowns [61].

Cross regulation of coagulation factors at the mRNA level was identified in the f10 knockout [30]. Loss of F10 resulted in statistically significant increases of fga and at3 mRNA by 75 and 100%, respectively, but no significant effect on f2. We saw the opposite results through loss of Fga, with decreases in f10, f2, and at3 mRNAs by ~50%. These data suggest the possibility of a gene expression feedback loop for the common pathway. In both mutants, fibrin production is reduced, thus the presence or absence of fibrinogen itself may be an important determinant of common pathway mRNA expression. In the fga mutant larvae, we might have expected to see a severe bleeding phenotype, given the decrease in f10 and f2, but this could be balanced by the decrease in at3 expression.

We have previously used zebrafish larvae for qualitative in vivo analysis of human AT3 and F10 coagulation factor mutations. In this work we first showed the ability to rescue the $fga^{-/-}$ defect using zebrafish fga mRNA, followed by human fibrinogen protein. Given the similarities between human and zebrafish fibrinogen deficiencies, we were able to use our model to discriminate the functional consequences of human mutations, finding that M1V and Y809C were unable to rescue the hemostatic defect. M1V is a novel mutation that has been recently identified in patients [51] and here we confirm it in vivo as a pathological mutation. Surprisingly, two cysteine substitutions, C55G (Fibrinogen La Seyne and Fibrinogen Quimper) and C64Y (Fibrinogen Marseilles II), were able to rescue $fga^{-/-}$ mutants despite the fact that both positions are highly conserved and patients with these mutations are hypofibrinogenemic (activity levels down to 20-50% of the lower limit of the normal range [54]). The cysteine at position 55 has been clearly

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shown to be important for disulfide formation with the Bβ subunit of fibrinogen. Its substitution results in impaired hexamer assembly, yet continued secretion of half molecules of fibrinogen in cell culture [62], although not in patients [54]. Hanss et al postulate that this is due to overexpression in culture. This is consistent with our data as we used the ubi promoter which drives robust expression in zebrafish embryos and larvae [43]. The Y809C substitution was originally an incidental finding in a pedigree with Fibrinogen Montreal (D515N), and segregated separately from the affected family member [55]. Y809C is only present in fibrinogen$_{420}$, a variant comprising only ~1% of circulating fibrinogen [63], although it is three-fold higher in fetal plasma [64]. No functional studies had been performed on Y809C until this report in which we demonstrate that this variant abrogates fibrinogen function. One limitation of these experiments is that causative mutations that reduce human FGA expression without affecting function would be expected to rescue in this overexpression system.

These data expand our view of coagulation pathophysiology in aquatic species. Earlier studies demonstrated significant conservation of coagulation physiology in zebrafish [24-26, 65, 66]. With the advent of genome editing, we have now expanded on this foundation and developed genetic models of coagulation disorders and used these to identify novel functional human mutations [29, 30, 66]. The phenotypes align with those seen in mammals, but demonstrate a significant shift in survival. Further understanding of the underlying biology that explains why fish can tolerate such severe coagulation defects, as well as in vivo analysis of clotting factor variants, may lead to novel therapeutic approaches for patients with the associated disorders.

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AUTHORSHIP ADDENDUM

Z. Hu designed and performed research, analyzed data, and wrote the manuscript; K. I. Lavik performed research, analyzed data and wrote the manuscript; Y. Liu, C. E. Richter and A. H. Vo performed research and analyzed data; J. Di Paola provided unpublished reagents and edited the manuscript; and J. A. Shavit designed and supervised research, analyzed data, and wrote the manuscript.

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CONFLICT OF INTEREST DISCLOSURES

J. A. Shavit has been a consultant for Bayer, Shire, CSL Behring, Grifols, and Octapharma in the last 3 years.

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FIGURE LEGENDS

Figure 1：Generation of a null allele in fga using genome editing with TALENs. (A) Schematic of TALENs targeting exon 2 of fga, located on zebrafish chromosome 1. (B) cDNA sequencing reveals a 26 base pair insertion in fga (indicated by red box) which generates a frameshift mutation and subsequent nonsense mutation, resulting in truncation. (C) Transcription of fga is remarkably reduced in $fga^{-/-}$ mutants when compared to $fga^{+/+}$ and/or $fga^{+/-}$ siblings, as evaluated by qPCR (n=25 larvae per genotype at 3 dpf, $p=0.0001$ and $p<0.05$, respectively, unpaired two-tailed Student t test). (D) Qualitative PCR of cDNA isolated from $fga^{-/-}$ embryos reveals that there is no detectable $fga^{+/+}$ transcript. (E) Western blot analysis shows no residual Fga present in plasma isolated and pooled from adult $fga^{-/-}$ mutants in contrast to $fga^{+/+}$ and $fga^{+/-}$ control siblings. Chr1, chromosome 1; C-term, C-terminal domain; DBD, DNA-binding domain; Fok I, Fok I nuclease; NLS, nuclear localization signal; N-term, N-terminal domain.

Figure 2: $fga^{-/-}$ mutants demonstrate synthetic lethality with loss of nfe2. (A) Offspring derived from $fga^{+/-}$ and $fga^{-/-}$ intercrosses were genotyped at 3 dpf and examined daily. Compared to heterozygous siblings (n=29), homozygous mutants (n=20) exhibit a significant decrease in survival over time ($p<0.0001$ by log-rank testing). (B) Offspring derived from $fga^{+/-}$;$nfe2^{+/-}$ intercrosses were genotyped at 86 dpf and tracked for survival. Combined loss of fga and nfe2 results in complete synthetic lethality by one year of age ($p<0.0001$ by log-rank testing).

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Figure 3: fga$^{-/-}$ mutants develop spontaneous hemorrhage in multiple tissues. (A) Fish were fixed, sectioned, and stained with hematoxylin and eosin, demonstrating that fga$^{-/-}$ mutants (n=11) develop mild bleeding in the brain and jaw/mouth, but severe bleeding within the abdomen and abdominal musculature by 41 dpf (compared to wild type siblings, n=3). Insets show magnified regions of interest with sites of hemorrhage as do arrows. (B) Severe bleeds identified in the forebrain (fb), midbrain (mb), and hindbrain (hb) in viable fga$^{-/-}$ mutant fish at 47 dpf contrast with wild type siblings. Arrows indicate sites of bleeding. Anterior is towards the left, and dorsal is at the top. Scale bar: 100 µm.

Figure 4: Laser-mediated injury of the vascular endothelium reveals a hemostatic defect in fga mutant zebrafish. (A-C) Hemostasis was evaluated by measuring time to occlusion up to 120 seconds after laser-induced endothelial injury of the PCV in 3 dpf larvae. Time to occlusion was significantly prolonged in fga$^{-/-}$ (n=50) larvae in comparison with fga$^{+/+}$ (n=10) and fga$^{+/-}$ (n=50) siblings ($p<0.0001$) (A). Injection of wild type fga cDNA into the offspring of fga$^{+/-}$ incrosses rescued the hemostatic defect in fga$^{-/-}$ compared to uninjected fga$^{-/-}$ counterparts (n=19-23 per genotype, $p<0.05$). Hemostasis in injected fga$^{+/+}$ and fga$^{+/-}$ was unaffected compared to uninjected fga$^{+/+}$ and fga$^{+/-}$ (n=6-33 per genotype) (B). (C) Retro-orbital infusion of human fibrinogen into 3 dpf mutant larvae (n=49) rescued time to occlusion compared to BSA infused fga$^{-/-}$ siblings (n=21, $p<0.05$). p-values determined by the Mann-Whitney U test. Horizontal bars represent the median time to occlusion.

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Figure 5: Treatment of fga mutants with ε-aminocaproic acid rescues the hemostatic defect. Mutant offspring were treated with 100 mM ε-aminocaproic acid at 24 hpf. Time to occlusion after endothelial injury was measured at 3 dpf. Treated $fga^{-/-}$ larvae (n=110) exhibited a statistically significant rescue compared to untreated $fga^{-/-}$ (n=29). p=0.0003 by the Mann-Whitney U test.

Figure 6: Functional analysis of human FGA variants in a fibrinogen deficient zebrafish model. Human FGA substitutions M1V, C55G, C64Y, and Y809C were genetically engineered into the orthologous positions of zebrafish fga cDNA in pubi-zfga-p2A-EGFP. Wild type (zfga) or variant cDNAs were injected into 1-cell stage embryos from $fga^{+/-}$ and $fga^{-/-}$ intercrosses and time to occlusion was recorded after laser-induced endothelial injury, up to 120 seconds. Data shown are results from $fga^{-/-}$ larvae. Statistical significance was determined by comparing variant (hM1V n=33, hC55G n= 29, hC64Y n=19, hY809C n=23) and zfga (n=27) injected $fga^{-/-}$ larvae using the Mann-Whitney U test. Horizontal bars represent the median time to occlusion.

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Table 1. Human FGA variants

Human amino acid variant	Human mutation	Orthologous amino acid position (zebrafish)	Hemorrhage	Reference
hM1V	c.1A>G	1	no	[43]
hC55G	c.221T>G	51	yes	[45]
hC64Y	c.249G>A	60	yes	[45]
hY809C	c.2425A>G	640	yes	[46]

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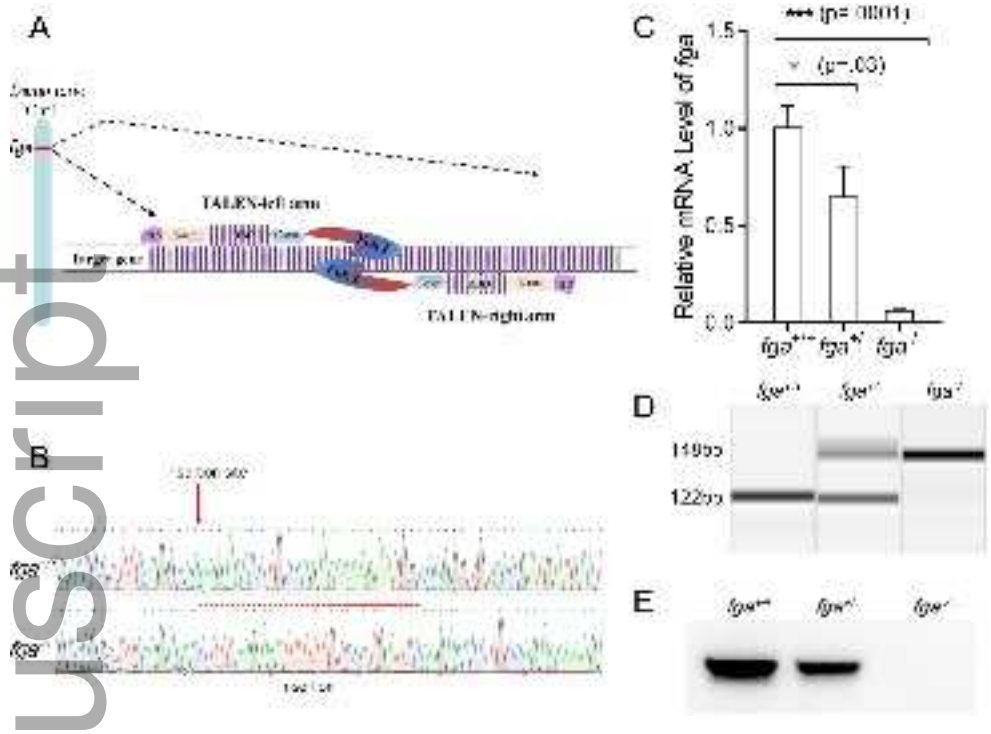

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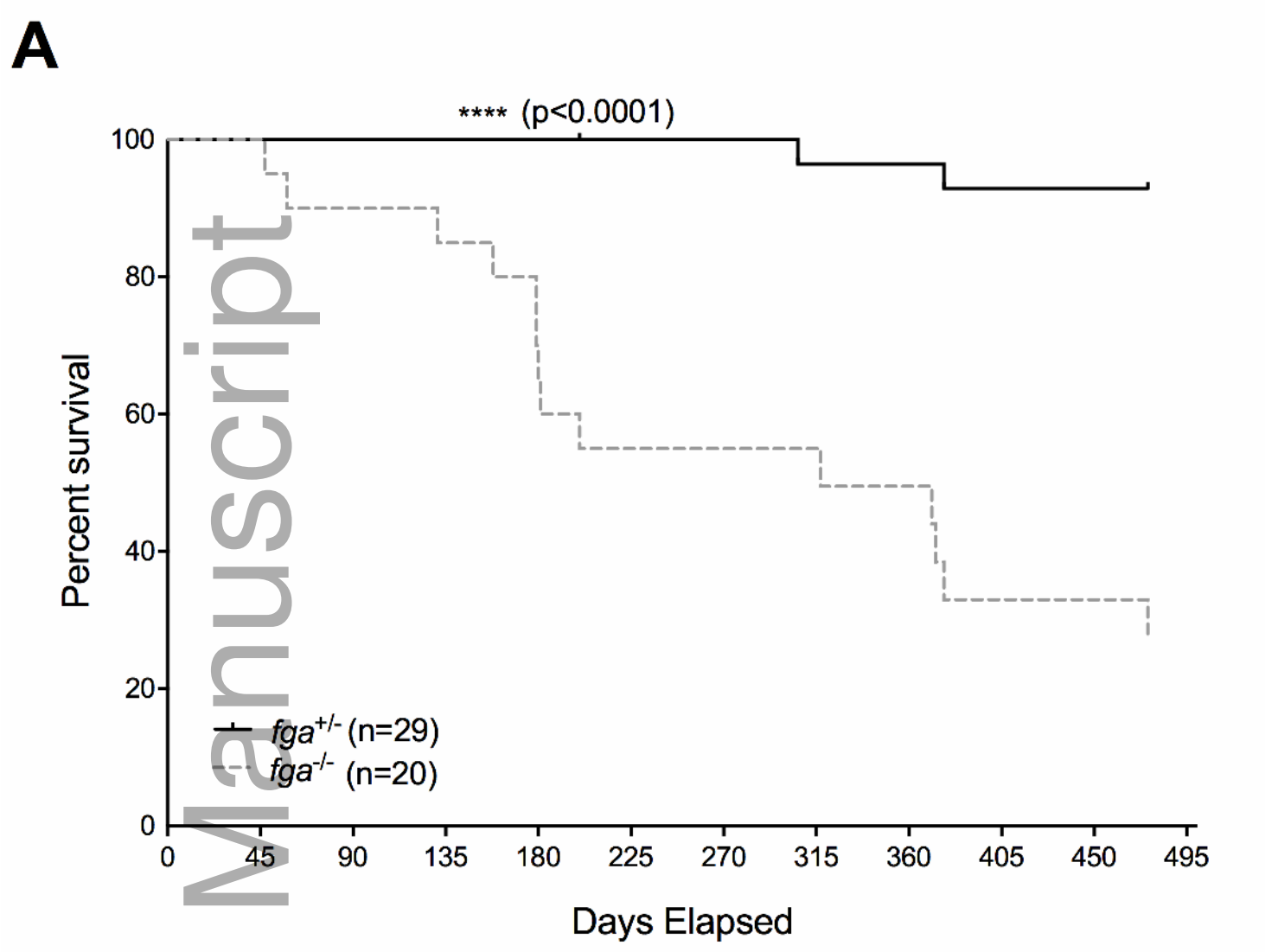

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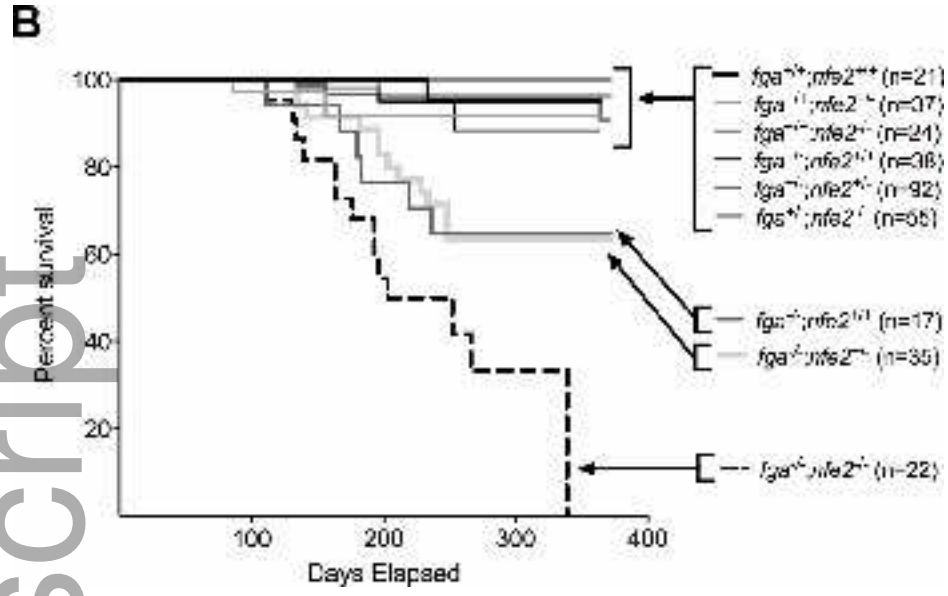

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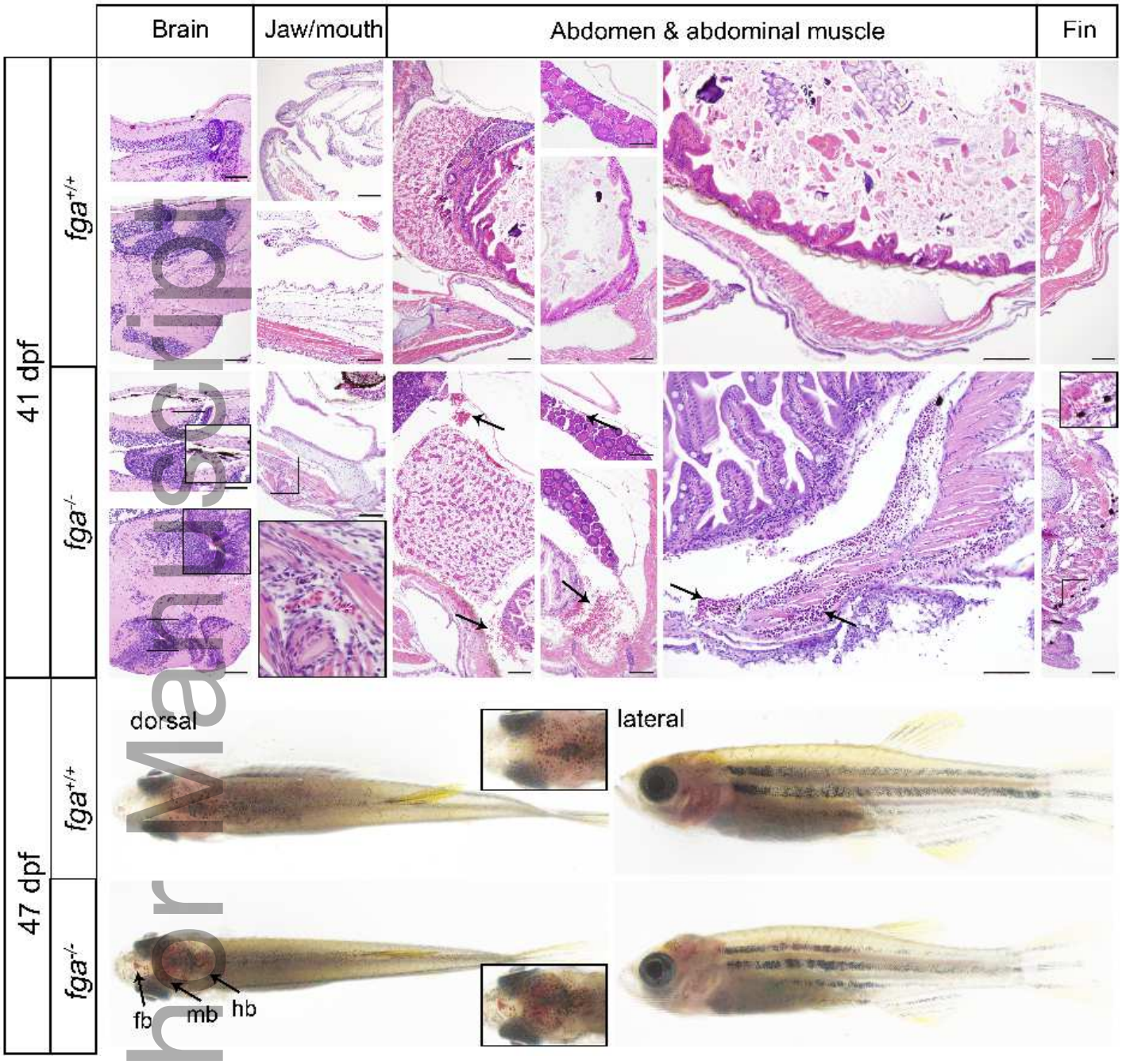

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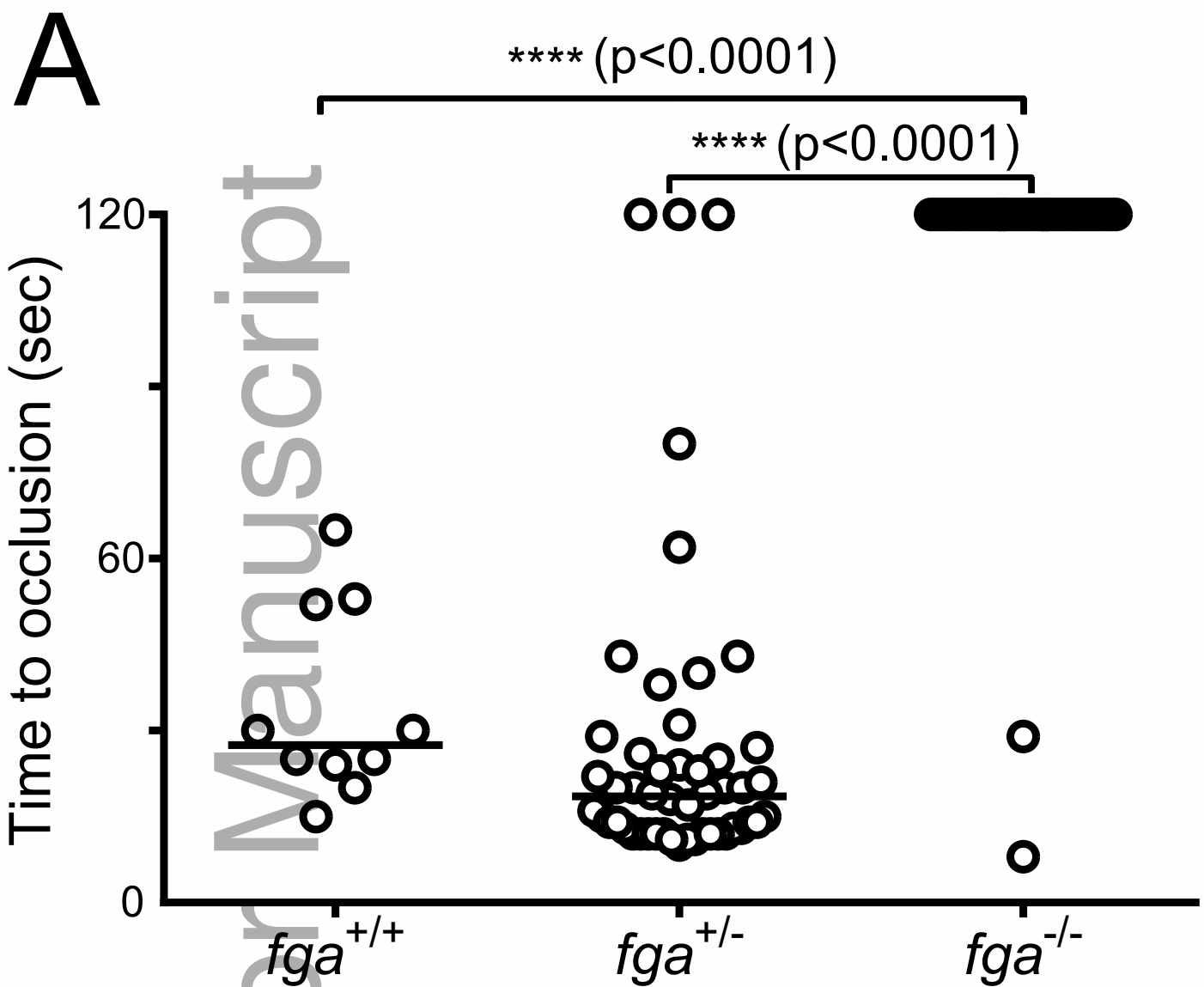

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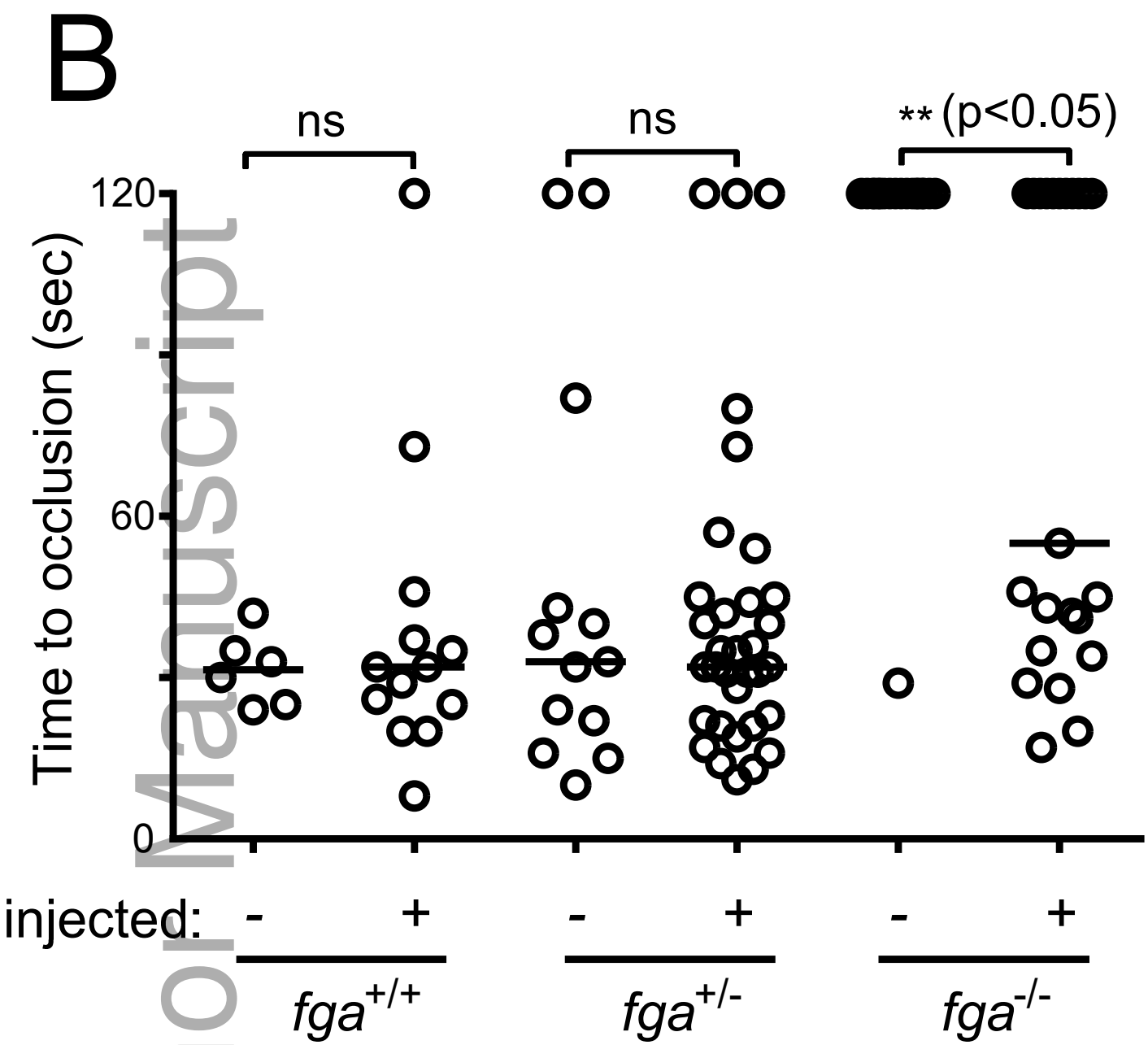

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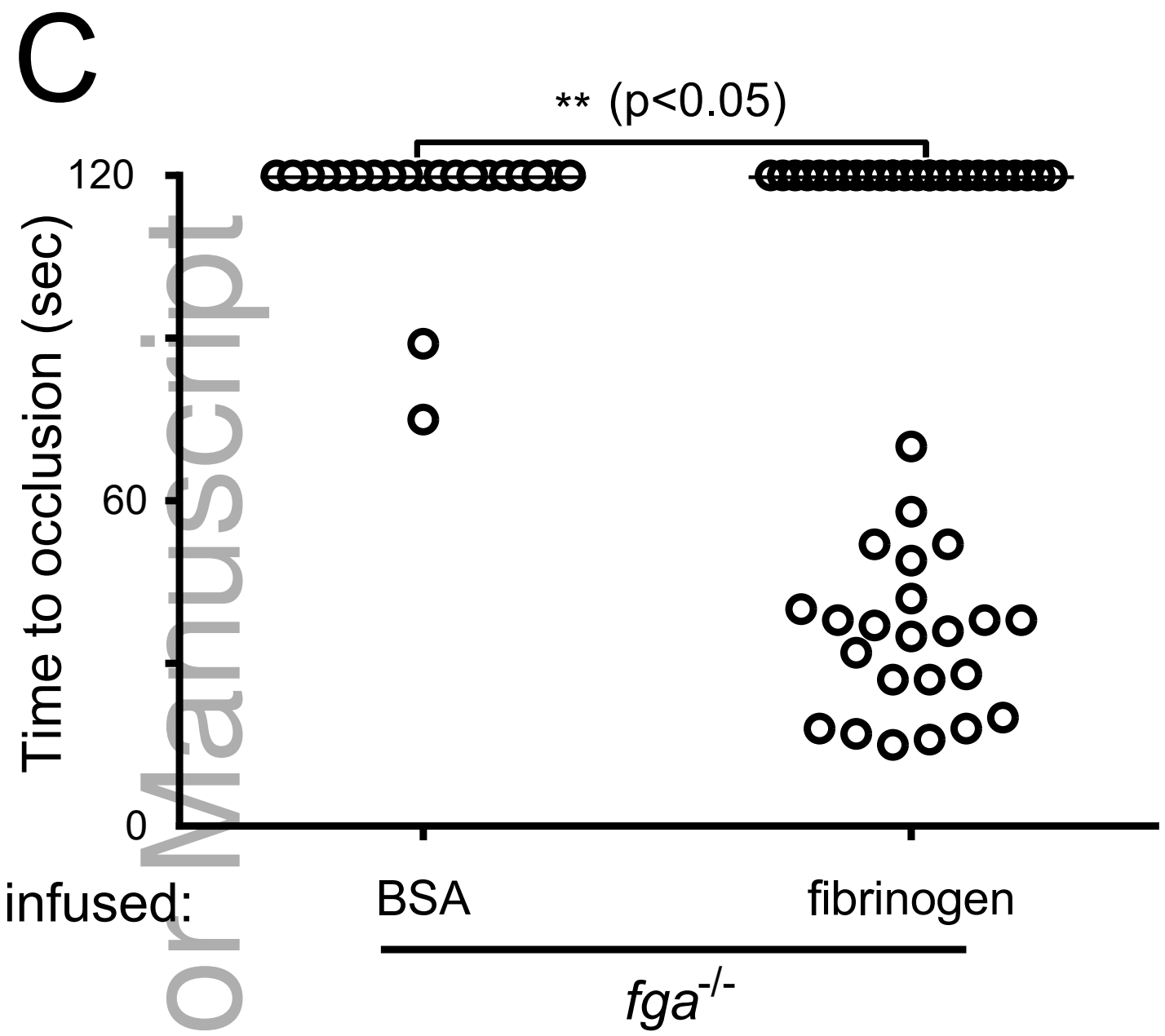

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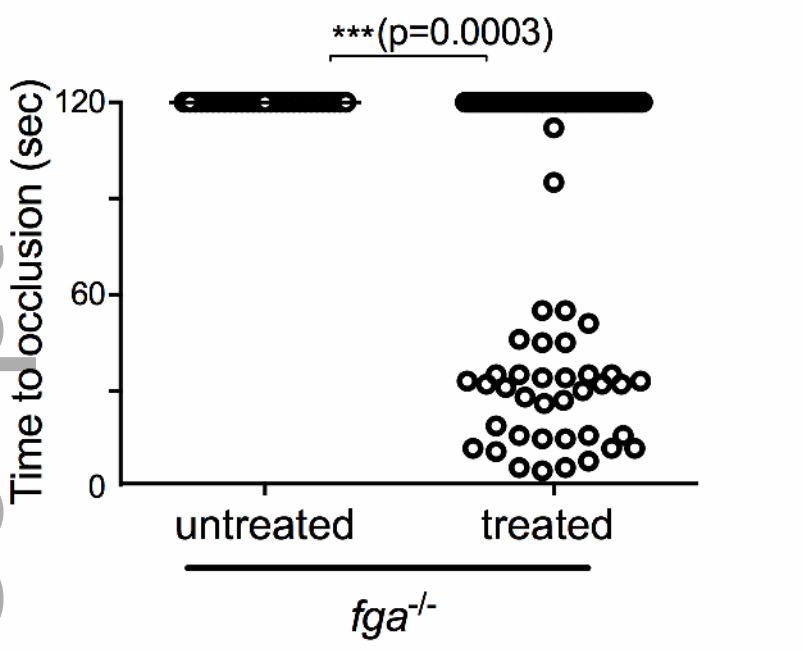

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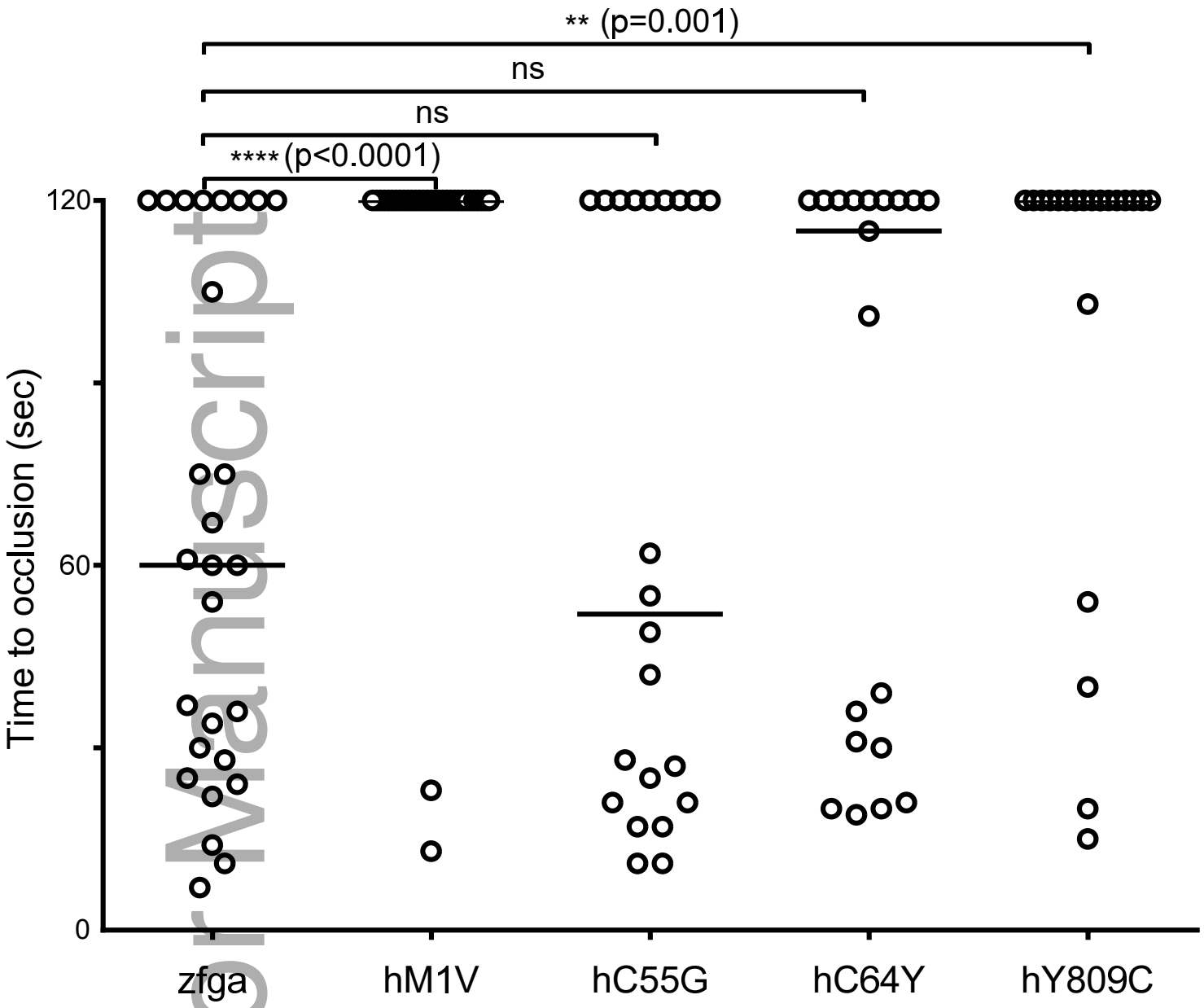

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