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A Large-scale Mutagenesis Screen to Identify Seizure-resistant Zebrafish

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Summary

Purpose—Zebrafish are a vertebrate organism ideally suited to mutagenesis screening strategies. Although a genetic basis for seizure susceptibility and epilepsy is well established, no efforts have been made to study seizure resistance. Here we describe a novel strategy to isolate seizure-resistant zebrafish mutants from a large-scale mutagenesis screen.

Methods—Seizures were induced with pentylenetetrazole (PTZ). Zebrafish were analyzed between 3 and 7 days postfertilization (dpf). Genome mutations were induced in founders by using *N*-ethyl-nitrosourea (ENU). Seizure behavior was monitored by using a high-speed camera and quantified by locomotion-tracking software. Electrographic activity was monitored by using a field-recording electrode placed in the optic tectum of agar-immobilized zebrafish.

Results—Short-term PTZ exposure elicited a burst-suppression seizure pattern in 3-dpf zebrafish and more complex activity consisting of interictal- and ictal-like discharges at 7 dpf. Prolonged exposure to PTZ induced status epilepticus-like seizure activity and fatality in wild-type zebrafish larvae. With a PTZ survival assay at 6–7 dpf, we identified six zebrafish mutants in a forward-genetic screen covering nearly 2,000 F₂ families. One mutant (s334) also was shown to exhibit reduced behavioral activity on short-term PTZ exposure and an inability to generate long-duration ictal-like discharge.

Conclusions—Zebrafish offers a powerful tool for the identification and study of a genetic basis for seizure resistance.

Keywords

Seizure; PTZ; Mutagenesis; Electrophysiology; Forward genetics; Screen; Behavior

Genetically tractable organisms present a distinct advantage in studying the fundamental basis of neurologic disease. In epilepsy, a neurologic disorder afflicting nearly 2% of the populations, a variety of disease-causing mutations are already known (for review, see Noebels, 2003). Unfortunately, strategies to identify single-gene mutations conferring disease resistance have not been widely considered. We recently demonstrated that seizures can be generated in

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Supplemental Videos. Examples of seizure-like behaviors in zebrafish larvae. (1) Baseline recording of aWT zebrafish larvae (7 dpf) in embryo medium. Note the overall lack of movement. (2) Sample WT sibling zebrafish ~15 min after exposure to bathing medium containing 15 mM PTZ. Note the rapid whole-body convulsions. This activity was designated a stage III seizure. (3) Sample s334 putant zebrafish ~15 min after exposure to bathing medium containing 15mM PTZ. Note the rapid whirlpool-like swim behavior designated as a stage II seizure.

developing zebrafish (*Danio rerio*) (Baraban et al. 2005; Baraban, 2006). At 6 or 7 days postfertilization (dpf), zebrafish larvae exposed to a common convulsant agent, pentylenetetrazole (PTZ; Yonekawa et al., 1980), exhibit a stereotyped and concentration-dependent sequence of behavioral changes culminating in whole-body clonus-like convulsions. Electrophysiology revealed long-duration “ictal-like” and high-frequency, short-duration “interictal-like” electrographic discharges after application of PTZ. These were blocked by glutamate-receptor antagonists and suppressed by antiepileptic drugs (AEDs). This zebrafish seizure model serves as the basis for the high-throughput mutagenesis screen described here.

A genetic screening approach, wherein mutagen treatment introduces random mutations into the genome, and resultant mutants are recovered in subsequent generations (based on their abnormal phenotypes; Brockerhoff et al., 1995; Haffter et al., 1996; Patton and Zon, 2001), is a powerful and unbiased method for identification of epilepsy genes. This approach requires simple organisms possessing an intact nervous system, and recent studies suggest that these organisms can provide important insights into epilepsy and AED efficacy. Indeed, “seizure susceptibility” genes and the seizure-inhibiting profiles of common AEDs were identified in *Drosophila melanogaster* (Kuebler et al., 2001; Stilwell et al., 2006). Here we describe the first forward-genetic screen to identify seizure-resistant zebrafish mutants. In a focused large-scale mutagenesis screen encompassing >500,000 F₃ larvae, we isolated six mutant families by using a PTZ assay. We also present evidence for reduced PTZ-induced seizure behavior and an inability to generate ictal-like electrographic discharge in one mutant.

MATERIALS AND METHODS

Animals and maintenance

Zebrafish of the TL and WIK strains were maintained according to standard procedures (Westerfield, 1995) and by following guidelines approved by the University of California, San Francisco, Institutional Animal Care and Use Committee. For all experiments, zebrafish larvae were maintained in “embryo medium” consisting of 0.03% Instant Ocean (Aquarium Systems, Inc., Mentor, OH, U.S.A.) in deionized water containing 0.002% methylene blue as a fungicide.

We used zebrafish from the TL strain for mutagenesis (for additional details, see Muto et al., 2005). In brief, exposing the spermatogonia of 41 founder males (F₀ generation) to *N*-ethyl-nitrosourea (ENU, 3 mM for 1 h, three to five treatments at weekly intervals) induced random mutations in the zebrafish genome. Approximately 5,000 F₁ ENU-mutagenized offspring were then bred to homozygosity over two generations. The mutagenized males were bred several times with females to determine the efficiency of mutagenesis (Solnica-Krezel et al., 1994). Adult F₁ fish were mated with other F₁ fish [or wild types (WTs)] to generate >2,000 F₂ families. For each F₂ family, ≤25 single pair crosses were set up in breeding traps, eggs were harvested into Petri dishes, and parents of successful crosses (i.e., those yielding healthy F₃ offspring) were transferred pairwise into holding containers. Each pair was given a unique label (s100, s111, s112, etc.) for later identification. In this screen, 3,171 F₁ adult fish were used to generate 1,896 F₂ families that yielded at least one healthy clutch of F₃ embryos in a subsequent generation. F₃ larvae were obtained by random crosses between siblings from F₂ families (6,468 F₃ clutches in total). The screen was designed as a two-generation breeding screen with analysis of mutant phenotypes in F₃ larvae (Driever et al., 1996). A large-scale forward-genetic screen for seizure-resistant (SR) zebrafish mutants was performed in which 25 to 50 larvae (F₃) were tested in a PTZ survival assay. As soon as an SR clutch was identified in one PTZ survival assay, the parents were designated as putants and crossed again 2 weeks later for subsequent clutch testing. Although >100 families were identified on a first-pass PTZ assay, only mutant families testing positive on three independent clutch trials were designated as SR and maintained. Including retests, >500,000 individual fish were screened; clutches

contained F₃ progeny that were WT, heterozygous, and homozygous for a recessive mutation. For preservation of mutations, usually three sperm samples were frozen from the heterozygous F₂ male. In addition, heterozygous fish were outcrossed with a TL or WIK strain, and segregation of the mutation was confirmed in subsequent PTZ survival assays from F₃ (or later) generations.

Behavioral monitoring

For PTZ survival assays, zebrafish larvae were maintained in Petri dishes. Visual observation of PTZ-exposed larvae freely swimming in Petri dishes was initially performed by three investigators; for logistic purposes of large-scale screening, this was later reduced to one investigator. Given that one or two fish in a 50-clutch sample may be identified falsely (a number empirically obtained from the three investigator trials), we set a margin of error for the survival assay at 5%. For locomotion tracking, single zebrafish larvae were placed individually in 96-well Falcon culture dishes (BD Biosciences, Franklin Lakes, NJ, U.S.A.). Each well contained ~50 μ l embryo medium. Swimming behavior was monitored by using a CCD camera (Hamamatsu C-2400, Hamamatsu City, Japan) and EthoVision 3.0 locomotion tracking software (Noldus Information, Inc., Leesburg, VA, U.S.A.).

Electrophysiology

To obtain stable physiological recordings, zebrafish larvae (3 to 7 dpf) were immobilized in 1.2% low-melting-temperature agarose. Zebrafish were placed in agarose so that the dorsal aspect of the fish is exposed to the agarose gel surface and is accessible for electrode placement. With this approach, zebrafish are completely immobilized in an agar block, and anesthetic agents, with their potential for interfering with synaptic function and convulsant activity, were not necessary. Fish immobilized in agar are maintained in normal bathing medium and remain viable for ≤ 24 h (data not shown). Under direct visual guidance, a glass microelectrode (~1- μ m tip diameter; 2–7 M Ω) was placed in the optic tectum, the largest midbrain structure in the zebrafish central nervous system; in some experiments, electrodes were placed in telencephalic fore-brain structures. Electrodes were filled with 2 M NaCl, and electrical activity recorded by using an Axopatch 1D amplifier (Axon Instruments, Union City, CA, U.S.A.). Voltage records were low-pass filtered at 1 kHz (–3 dB, eight-pole Bessel), high-pass filtered at 0.1–0.2 Hz, digitized at 5–10 kHz by using a Digidata 1300 A/D interface, and stored on a PC computer running pClamp software (Axon). Recording sessions between 30 and 90 min were performed. Standard hippocampal slice electrophysiology studies were performed on P30 mice, as described previously (Marsh et al., 1999).

Drugs

Stock solutions of PTZ (Sigma-Aldrich, St. Louis, MO, U.S.A.) were prepared at a concentration of 75 mM in embryo medium. Working concentrations (15 mM) were made fresh in embryo medium (pH 6.5). Compounds dissolved in embryo medium are readily absorbed through the gastrointestinal tract or across the skin of developing zebrafish larvae (Peterson and Fishman, 2004)

RESULTS

A survival assay to identify seizure-resistant mutants

A seizure-induction protocol using PTZ was recently established and validated as exhibiting behavioral, molecular, pharmacologic, and electrophysiologic aspects of mammalian seizures (Baraban et al., 2005; Baraban, 2006). Field recordings obtained from the forebrain or optic tectum confirmed electrographic seizures and status epilepticus (SE)-like activity in PTZ-exposed fish. Early in development, at 3 dpf, these waveforms consisted of large long-duration

burst discharges followed by periods of quiescence (e.g., a burst-suppression pattern) (Fig. 1A and B). At 7 dpf, a more complex electrographic discharge pattern comprising high-frequency small-amplitude ictal-like events building to large-amplitude long-duration ictal-like discharge and subsequent burst-suppression activity was observed (Fig. 1A; 7 dpf). All agar-immobilized WT zebrafish exposed to 15 mM PTZ exhibit this pattern of electrical activity ($n = 195$). Ictal-like discharges in 7 dpf intact zebrafish exposed to 15 mM PTZ closely resemble discharge patterns elicited in mouse hippocampal slices bath exposed to 1.5 mM PTZ ($n = 10$). Representative burst discharges are shown in Fig. 1B for comparison.

To select for seizure-resistant fish, we first developed a simple “survival” assay with WT larvae. The principle underlying our assay is that high concentrations of PTZ reliably elicit electrographic and convulsive seizure activity in zebrafish larvae. Further, continuous exposure to a convulsant agent results in uncontrolled status-like epileptic activity that will ultimately lead to death (Fig. 2A). Our hypothesis is based on clinical observations that uncontrolled SE is invariably fatal (Simon, 1985; Sahin et al., 2001; Bassin et al., 2002). First, we exposed WT zebrafish larvae to three different PTZ concentrations and monitored the number of zebrafish that were able to respond with movement after a “tap” on the Petri plate at 1-h intervals. Fish that move in response to a gentle tap were classified as alive and “responsive.” In a concentration-dependent manner, PTZ exposure induced stage III (convulsive) seizures and death in WT larvae (Fig. 2B). This assay was repeated in triplicate. At the highest concentration, 15 mM PTZ, seizure-induced death was observed in 99.5% of all WT zebrafish starting at 15 h after drug exposure. Based on this PTZ-survival assay, we designated larvae that were responsive between 16 and 19 h after prolonged exposure to 15 mM PTZ as “seizure resistant” (SR). For high-throughput screening, we assumed a mendelian inheritance rate of one in four (as predicted for a fully penetrant recessive trait) and a 5% margin of error (allowing for variability associated with a behavioral assay). With these constraints, we screened >2,500 F₃ larvae per week.

Mutants detected on two separate PTZ tests in the primary screen were identified. Putative F₂ carriers were then mated again for confirmation of a seizure-resistance phenotype in their progeny and, if positive, designated for outcrossing to a WIK background strain. The SR screen initially identified 27 putative mutant families on a TL background (i.e., “putants”). Outcrossed putants were retested in the PTZ assay ≥ 3 times, resulting in the reidentification of six stable SR putant families that we currently maintain. PTZ-survival data for all putant clutches is shown in Fig. 2C. Putant families were otherwise healthy in the absence of PTZ exposure and have been maintained for more than three generations. Behavioral and electrographic monitoring studies were performed on F₃ (or later) clutches from all six putant SR families (50 to 75 larvae per family). In the following section, one such mutant is described.

Characterization of one seizure-resistant mutant (s334)

Zebrafish larvae freely swimming in 15 mM PTZ progress through three stages of seizure behavior: (a) increased swim activity, (b) whirlpool-like circling, and (c) clonus-like whole-body convulsions followed by a brief loss of posture (see Supplemental Videos). With a locomotion-tracking system, we can reliably identify these seizure stages in WT fish (Baraban, 2006). Here putative F₂ carriers from mutant s334 were mated, and the resulting offspring placed in one well of a 96-well Falcon plate. Baseline locomotion plots were obtained (Fig. 3A1; 2 min in duration), and solution was then replaced with 15 mM PTZ. Zebrafish larvae were exposed to convulsant for 15 min, and a second locomotion-tracking plot was obtained (Fig. 3A2). Identified sibling WT zebrafish (22 of 30) exposed to 15 mM PTZ progressed to stage III seizures, as expected. In contrast, putative s334 mutants (26.6% of all fish tested: eight of 30) exposed to the same concentration of PTZ progressed only to stage II (whirlpool-like circling) behaviors. Representative locomotion plots for two F₃ larval zebrafish are shown in

Fig. 3A. Analysis of the percentage of time each fish moved (an indirect measure of seizure activity) indicates a significant PTZ-induced increase over baseline activity for sibling WT zebrafish; stage II circling behavior in putant s334 fish was not associated with a significant increase (Fig. 3A3). WT siblings and s334 putants identified in tracking studies could not be distinguished based on their appearance at 7 dpf (Fig. 3B). Further to quantify convulsive seizure behavior in these locomotion plots, we also counted the number of dashed lines; stage III convulsions are too rapid to be followed by the PC computer-based locomotion-tracking system and are extrapolated as dashed lines. With this measurement, a decrease in seizure behavior was further confirmed for s334 putant compared with sibling WT zebrafish (Fig. 3C; 25% of all fish tested: five of 25).

Electrophysiology data on mutant s334 revealed another interesting phenotype. A bipolar stimulating electrode was placed on the retina, and an extracellular field recording electrode was placed in the contralateral optic tectum (Fig. 4A). In WT fish at low stimulus intensities, a small negative-going field excitatory postsynaptic potential (fEPSP) reached peak amplitude within ~15 ms; higher stimulus intensities elicited larger fEPSP responses. Evoked activity was abolished by addition of 1 mM kynurenic acid, a non-specific glutamate receptor antagonist, to the bathing medium (Fig. 4B; $n = 4$). In WT sibling and putant s334 zebrafish larvae (7 dpf), retinal stimulation at 1.4 mV elicited a simple fEPSP that decayed back to baseline within ~300 ms. No differences in evoked field EPSP responses were noted (Fig. 4C). As expected, WT siblings exposed to 15 mM PTZ (45–60 min) exhibited an electrical pattern characterized by interictal-and ictal-like forms of epileptiform discharge. Interestingly, putative s334 mutants (26.8% of all fish tested; 11 of 41) exposed to the same concentration of PTZ and for the same (or longer) recording periods, exhibited only interictal-like activity (Fig. 4D and E). Further to confirm these findings, larvae from additional s334 clutches ($n = 6$) were exposed to a convulsant agent with an independent mechanism of action [e.g., A-type potassium channel blocker 4-aminopyridine (4-AP)]. Similar to the PTZ data, 4-AP failed to elicit long-duration, high-amplitude electrographic discharge in s334 putant zebrafish (Fig. 5).

DISCUSSION

We demonstrated that seizures can be elicited in developing zebrafish. As early as 7 dpf, a stage late in larval development when most morphogenesis and primary neurogenesis is complete (Haffter et al., 1996; Chitnis and Dawid, 1999), zebrafish exposed to PTZ exhibit complex electrographic seizure patterns reminiscent of those in mammals. Recent anatomic studies described networks of glutamatergic and GABAergic neurons in the immature zebrafish brain (Higashijima et al., 2004). Although it is not yet clear how these circuits govern the generation of epileptiform activities, we have shown that PTZ-induced seizures in zebrafish respond to glutamate-receptor antagonists and certain antiepileptic drugs (AEDs) (Baraban et al., 2005). Moreover, the work presented here clearly demonstrates that large-scale genetic screening strategies in zebrafish can be applied to disease-based research (e.g., the isolation and characterization of SR zebrafish mutants).

The success of forward-genetic screens, specifically those in simple organisms, lies in the rapid identification of detrimental phenotypes (i.e., poor development or impaired visual behavior) that permit the isolation of mutants and subsequent identification of defect-causing genetic mutations. In these screens, the ENU-induced allele provides novel information about the role of a given gene in normal development or vision (Brockerhoff et al., 1995; Haffter et al., 1996). To design similar screening strategies by using a seizure phenotype could be of considerable value to the epilepsy community. Because >70 gene mutations result in an epileptic phenotype (Noebels, 2003), we did not systematically search for epileptic zebrafish mutants in our screen. Instead, we focused on “resistance” and a large-scale unbiased screening strategy by using zebrafish and PTZ. Mutants isolated and described here for the first time (a)

demonstrate the power of this genetic approach and (b) will serve as starting points for the discovery of new molecular targets to prevent epilepsy

We isolated six viable and stable zebrafish families resistant to prolonged exposure to a convulsant agent. Admittedly, the lack of available pharmacokinetic data on PTZ absorption and distribution in zebrafish suggests that some mutants may be “false positives” capable of excluding the drug. By using picrotoxin, we have confirmed seizure resistance in the six mutants described here (M. Dinday and S.C. Baraban, unpublished observations). Because the SR zebrafish families and underlying gene mutations are likely to be diverse, we focused our initial efforts on analysis of only one mutant. The interesting phenotype characterized in the s334 mutant family provides important insights into biologic mechanisms conferring seizure resistance, even before the gene mutation is identified. A molecular target for the cellular or synaptic events mediating a transition from brief interictal-like epileptic discharge patterns to a more complex pattern incorporating large-amplitude, long-duration ictal-like events is likely to emerge from further analysis of mutant s334. Although brain-slice work has implicated depolarizing GABA responses (Dzhala and Staley, 2003) and GABA-B receptors (D’Antuono et al., 2004) in this process of ictogenesis, the precise mechanism for generation of these events in a PTZ assay based on blockade of inhibition remains unclear. Activation of a metabotropic glutamate receptor is an intriguing cellular mechanism to convert interictal into ictal discharge patterns (Merlin and Wong, 1997; Sayin and Rutecki, 2003), leading to speculation that an mGluR1 subunit is lacking in these fish. Given the evolutionary conservation of glutamate receptors between zebrafish and humans (Bjarnadóttir et al., 2005), such a loss-of-function mutation is entirely possible. From our characterization studies, it is interesting to note that s334 mutants exhibited “normal” synaptic transmission and interictal-like discharge activity but failed to progress to full clonus-like convulsions. A biologic link between ictogenesis and behavioral seizure state is suggested by these results.

Given the ease with which seizures can be induced in zebrafish and the powerful genetic approaches that this organism offers, it is clear that focused screens will provide valuable insights into epilepsy (and other neurologic disorders). Although our results represent a very small step in this process, they demonstrate that the tools to identify genetic mechanisms for disease resistance are now within our grasp.

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FIG. 1.

Electrographic seizure activity in immature zebrafish. **A:** Representative gap-free field recording from an electrode placed in the optic tectum of an agar-immobilized zebrafish larvae at 3 and 7 days postfertilization (dpf). Fish were exposed to 15 mM PTZ for 40 min. **B:** Individual burst-discharge waveforms from zebrafish exposed to 15 mM PTZ at 3 and 7 dpf. For comparison, a representative field recording from an electrode placed in CA3 stratum pyramidale mouse hippocampus maintained in vitro and perfused with artificial cerebrospinal fluid containing 1.5 mM PTZ is shown at right. Note the similarities in burst-discharge patterns between 7-dpf zebrafish larvae and mouse.

FIG. 2.

A screen for seizure-resistant zebrafish. **A:** Sample tectal recording in agar-embedded zebrafish larvae (7 dpf) exposed to 15 mM PTZ for 10 h. Flat EEG, a lack of heart beat, and death were noted several hours later. Traces are clipped by the data-acquisition program. *Arrow*, Initiation of status-like electrographic seizure activity. **B:** Plot of responsive WT zebrafish larvae at various time points during prolonged exposure to PTZ; fish are freely swimming in a Petri dish, 30–50 fish per plate. Three different PTZ concentrations are shown. Note that 15 mM PTZ resulted in clonus-like convulsions and fatalities starting at ~15 h of continuous drug exposure, confirmed by visual observation. Seizure resistant (SR) mutants were identified as a dish containing 20–30% responsive fish at 16–19 h PTZ exposure. **C:** Summary of PTZ

survival assay data for six mutant strains. SR assays were performed in duplicate on all positive mutants (first and second PTZ) and then, in triplicate, on all outcrossed zebrafish (OX first, second, third). Results from each of these assays, presented as a percentage of zebrafish responsive per clutch, are shown for six putants. All other fish exhibited <5% responsiveness (not shown). Mendelian ratio of 25% responsiveness was taken as a “positive hit,” with a 5% margin of error (*dashed lines* in plot).

FIG. 3.

Behavioral characterization of mutant s334. **A:** Sample locomotion plots from 2-min recording epochs obtained in embryo medium (A1) and 10 min after exposure to medium containing 15 mM pentylenetetrazole (PTZ) (A2). Sibling WT fish exhibited many clonus-like convulsions; confirmed by simultaneous monitoring of a live video feed. Putant s334 fish exhibited only stage II whirlpool-like circling behavior and were identified based on observation of their behavior in response to PTZ. Plots of the percentage of time spent moving during the 2-min recording epoch are shown at right (A3); $n = 22$ siblings; $n = 8$ s334 putants. Data are presented as mean $\pm$ SEM. $*p < 0.001$ with a Student's t test. Note that baseline movement is fairly minimal, and PTZ exposure significantly increases this measurement for sibling WT zebrafish (A3, top plot) but not putant s334 fish (A3, bottom plot). **B:** WT sibling and s334 putant fish are indistinguishable in their appearance at 7 dpf. Fish shown here were first sorted by using locomotion tracking. Scale bar, 1 mm. **C:** Box plot illustrating the absence of stage III convulsions in putant s334 zebrafish (s334) and siblings (sib) sorted by using locomotion tracking. In this plot, the median value is illustrated as a vertical line in the box. Inner quartiles are shown as the edges of the box, and the outer quartiles, as lines extending from the box. Outliers are shown as black circles.

FIG. 4.

Electrophysiologic characterization of mutant s334. **A:** Schematic showing the recording configuration for agar-immobilized zebrafish; a stimulating electrode was placed on one eye, and a recording electrode was placed in contralateral optic tectum (**top**). Representative input–output responses for stimulations at 0.7, 1.4, and 2.1 mV in wild-type larvae are shown below. **B:** Input–output plot for field EPSP responses obtained in a representative WT zebrafish during perfusion with normal bathing medium (*blue circles*) and 40 min after perfusion with medium containing 1 mM kynurenic acid (*red circles*). **C:** Stimulation-evoked tectal response from an s334 putant zebrafish and WT sibling embedded in agar. Fish were bathed in normal medium. Stimulation artifacts were clipped (*open circle*). **D:** Sample field recordings from the same fish as in **C**. Fish were exposed to 15 mM PTZ for 45 min. Note the presence of interictal and ictal-like discharge in WT fish, as expected (*red arrows* and *inset*) and the absence of such discharge in s334 putant (*inset*). **E:** Box plot illustrating the frequency of large-duration ictal-like discharges in PTZ-exposed zebrafish. Note the complete absence of ictal-like activity (putant = 0) in potential s334 homozygote mutants. Values as described in Fig. 3C.

FIG. 5. Additional electrophysiologic characterization of mutant s334. Tectal field recordings from sibling WT and putant s334 zebrafish exposed to 4-aminopyridine. Note the absence of large-duration ictal-like discharge activity in 334 putant fish (*arrows* in WT).