SUPPLEMENTAL MATERIAL:

Methods

***In Situ* Hybridization:** Procedures were as described (Novak et al., 2006) (Pineda et al., 2006). Briefly, fixed embryos (4% paraformaldehyde in 4.0% sucrose, 0.15 mM $CaCl_2$, 0.1 M PO_4, pH 7.3) were hybridized to digoxygenin labeled sense or antisense probes. Detection of the Fab antibody was performed using the Fast Red chromagen (Sigma, St Louis, MO).

Electrophysiology: For two-electrode voltage clamp experiments, *scn8aa* (Fein et al., 2007), *scn1bb*, and *Scn1b* cRNAs were synthesized using the T3 (for *scn8aa*), SP6 (for *scn1bb*), or T7 (for *Scn1b*) mMessage Machine kits (Ambion, Austin TX) according to the manufacturer's instructions. The *scn8aa* and *scn1bb* cRNAs were derived from the zebrafish genes, while the *Scn1b* cRNA corresponded to the mammalian gene. Total cRNA yields were estimated through the comparison of ethidium bromide stained bands with the intensity of known RNA standards. *Xenopus* laevis oocytes where harvested and maintained as previously described (McPhee et al., 1995; Fein et al., 2007). Briefly, oocytes were injected with 50 nl of cRNA (50-300 ng/μl). An approximate 5-fold greater concentration of *scn8aa* was used in comparison to *Scn1b*, as described (Fein et al., 2007), and the concentration of *scn8aa* mRNA was approximately 100-fold greater than *scn1bb* mRNA.

Na^+ currents were recorded using two electrode voltage clamp and a TEV-200A amplifier (Dagan Corporation, Minneapolis, MN). Voltage pulses were applied and data collected on an IBM PC using Clampex software (Axon Instruments, Foster City, CA). Residual linear currents were subtracted using the P/4 technique (Armstrong and Benzanilla, 1977). Signals were low pass filtered at 2 kHz using internal voltage clamp circuitry and data sampled at 20 kHz. During recording, the bath was continuously

perfused with Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM Hepes, pH 7.2). Intracellular pipettes were filled with 3 M KCl.

Voltage dependence of channel inactivation was determined from currents recorded during a 90 msec-long prepulse followed by test pulses ranging from -100 mV to 55 mV in increments of 5 mV from a holding potential of -80 mV. Conductance (G) was calculated from the peak current amplitude (I) according to $G=I/(V-V_{rev})$ where V corresponds to test potential and V_{rev} corresponds the measured reversal potential. Voltage dependence of channel inactivation was also measured using a 90 msec-long prepulse and pulses ranging from -100 mV to 55 mV in increments of 5mV from a -80 mV holding potential, followed by a test pulse to 0 mV. Conductance-voltage curves and inactivation curves were fit with the Boltzman relationship, $G=1/(1+\exp(V-V_{1/2})/k)$ where $V_{1/2}$ corresponds to the midpoint of the curve and k is the slope factor. To determine the time course of recovery from inactivation, Na^+ currents were inactivated by a 100 msec long prepulse to 0 mV, followed by a recovery period of variable duration at -80 mV, and a test pulse to 0 mV to determine the fraction of recovered channels. Recovery curves were fit with single or double exponentials to determine the time constant(s) for recovery from inactivation. Statistical significance between groups was accessed using one way ANOVA followed by post hoc Tukey analysis. Differences were considered significant when $p < 0.05$. Electrophysiological data were analyzed using pCLAMP software (Axon Instruments, Foster City, CA) and plotted with Origin (Micrococal, Northampton, MA).

Results:

Characterization of the anti-Scn1bb antibody

A polyclonal antibody was generated against the Scn1bb peptide epitope indicated in the sequence alignment (Fig. 1, underlined in green). Anti-Scn1bb recognized a band at

36 kD in Chinese hamster lung 1610 cells transfected with *scn1bb* cDNA (Supplemental Fig. 1 A, arrow). No signal was detected in untransfected cells, cells transfected with *scn1ba_tv1* cDNA, or in mouse brain. Importantly, the anti-Scn1bb antibody recognized a band at 40 kD in membranes prepared from whole zebrafish embryos, but did not recognize a band in mammalian brain membranes (Supplemental Fig. 1 B). We further tested the specificity of anti-Scn1bb immunohistochemically by performing peptide blocking experiments. Anti-Scn1bb antibody was pre-absorbed for 1 h at room temperature with the peptide to which it was raised. As shown in Supplemental Fig. 1 C, pre-absorption with the immunizing peptide resulted in a complete abolishment of the immune signal.

Analysis of scn1bb mRNA expression

We examined the expression of *scn1bb* mRNA in zebrafish embryos by performing *in situ* hybridization with a probe specific for *scn1bb*. The developing spinal cord expressed *scn1bb* mRNA at 24, 48, and 72 hpf (Supplemental Fig. 2 A). At 24 hpf, *scn1bb* mRNA was detected throughout the spinal cord, along both the dorsal-ventral and medial-lateral axes. This pattern of expression is consistent with the location of early-born post-mitotic primary spinal neurons. At 48 hpf, *scn1bb* mRNA was present dorso-medially in a position characteristic of the large RB cells that have migrated towards the midline by this stage. Additionally, abundant expression was detected in intermediate to lateral regions consistent with the position of newly differentiating secondary spinal neurons. At 72 hpf, *scn1bb* mRNA was found throughout the spinal cord, extending through the entire medial-lateral axis and from the middle to the dorsal spinal cord, but not in the ventral spinal cord. At more anterior levels, 72 hpf embryos also expressed *scn1bb* mRNA in structures surrounding the eye, consistent with the location of lateral line neuromasts and the trigeminal ganglion (Supplemental Fig. 2 BI).

At intermediate positions along the anterior-posterior axis, *scn1bb* was present in the nervous system, developing otic vesicle and lateral line (Supplemental Fig. 2 BII).

Scn1bb modulates Na^+ *currents*

To investigate the effect of Scn1bb on Na^+ currents, we co-expressed *scn1bb* mRNA with *scn8aa* mRNA, encoding the tetrodotoxin sensitive α subunit $Na_v1.6a$, in *Xenopus* oocytes. *scn8aa* mRNA induces the expression of robust Na^+ currents in oocytes (Fein et al., 2007). For comparison, we co-expressed *scn8aa* and rat *Scn1b* mRNAs. We showed previously that *Scn1b* modulates Na^+ currents induced by *scn8aa* similar to its effects on Na^+ currents induced by mammalian *Scn8a*, resulting in increased current amplitude, hyperpolarizing shifts in the voltage dependence of activation and inactivation, and acceleration of the rate of channel recovery from inactivation (Kohrman et al., 1996; Fein et al., 2007).

As demonstrated in Supplemental Fig. 3 A, co-expression of *scn8aa* and *scn1bb* mRNAs resulted in Na^+ currents that inactivated rapidly, with a time course that was indistinguishable from *scn8aa* and *Scn1b*. Similar to *Scn1b*, co-expression of *scn1bb* mRNA resulted in a 5-fold increase in peak current amplitude when currents were normalized to mean peak current expressed by *scn8aa* mRNA alone (Supplemental Fig. 3B). As expected, *Scn1b* mRNA produced hyperpolarizing shifts in the voltage dependence of steady-state channel activation and inactivation ($p < 0.001$; Student's t-test) (Supplemental Fig. 3 C and 3 D and Supplemental Table 1) (Kohrman et al., 1996; Fein et al., 2007). Coexpression of *scn8aa* with *scn1bb* also produced significant ($p < 0.001$; Student's t-test) shifts that were similar in magnitude and direction to those induced by *Scn1b*. These values were similar to those reported for co-expression of mammalian *Scn8a* and *Scn1b* mRNAs in oocytes (Kohrman et al., 1996). Hyperpolarizing shifts in the voltage dependence of activation in the presence of β1-like

subunits are predicted to allow a larger percentage of channels to open in response to small depolarizations in membrane potential. On the other hand, hyperpolarizing shifts in the voltage dependence of channel inactivation caused by β1-like subunits are predicted to cause a percentage of channels to be functionally removed at rest by inactivation. The net effect of β1-like subunit coexpression is that Na^+ channels may be able to respond more robustly to smaller input depolarizations *in vivo* without prolonging channel open time.

To measure the rate of channel recovery from inactivation in the presence and absence of *scn1bb* mRNA, channels were first inactivated using a 100 msec conditioning pulse to 0 mV and then allowed to recover for increasing periods of time before being reactivated by a 0 mV test pulse. Expression of *scn8aa* in the absence of β subunits produced currents with a very slow rate of recovery, which never fully recovered over the time course of the experiment (Supplemental Fig. 3 E). This recovery curve was best fit with two exponentials, reflecting the population of channels that recovered slowly from inactivation (Supplemental Table 2). In contrast, coexpression of *Scn1b* or *scn1bb* with *scn8aa* resulted in currents that recovered almost completely (97% for *Scn1b* and 98% for *scn1bb*), exhibiting a fast time constant that was best fit with a single exponential (Supplemental Fig. 3 E and Supplemental Table1). Interestingly, the extent of channel recovery in the presence of *scn1bb* was different from that induced by *scn1ba_tv1* or *scn1ba_tv2* (Fein et al., 2007), which produced incomplete recovery of *scn8aa* expressed currents. Acceleration in the rate of channel recovery from inactivation produced by expression of *Scn1b* or *scn1bb* mRNA is predicted to shorten the refractory period, allowing more channels to become available to undergo a second round of activation on a shorter time scale than in the absence of β subunits.

In sum, co-expression of Scn1bb with a pore-forming tetrodotoxin-sensitive Na^+ channel α-subunit in *Xenopus* oocytes increases the current amplitude, shifts the range of channel activation and inactivation to more negative membrane potentials, and speeds the rate of channel recovery from inactivation, effects that are predicted to promote more efficient conduction of action potentials in a physiologically relevant range of membrane potentials *in vivo*.

Tables:

	Voltage dependence of activation			Voltage-dependence of inactivation		
	V ½ (mV)	k	n	V ½ (mV)	k	n
scn8aa	-11.73 ± .52	-6.16	5	-33.42 ± .99	8.23	5
scn8aa + *Scn1B*	-34.68 ± 1.58	-3.13	8	-48.98 ± 1.15	4.96	7
scn8aa + *scn1bb*	-27.12 ± 1.27	-3.97	18	-43.35 ± .70	5.61	21

Supplemental Table 1. Co-expression of *scn8aa* with *scn1bb* results in shifts in the voltage dependence of channel activation and inactivation. *scn1bb*-induces shifts in the half voltages of activation and inactivation are comparable to the shifts produced by co-expression of *scn8aa* with *Scn1b*.

	τ_{fast}, msec	%	τ_{slow}, msec	%	n
scn8aa	5.996 ± 1.265	37.4 ± .08	448.69 ± 132.63	32.0 ±. 09	5
scn8aa + *Scn1b*	4.46 ± 2.018	97.2 ± .60			11
scn8aa + *scn1bb*	3.33 ± 1.39	97.5 ± .23			31

Supplemental Table 2. Modulation of *scn8aa* by *Scn1b* and *scn1bb*. *scn8aa* expresses two populations of channels, one that exhibits fast recovery from inactivation, and one that recovers slowly from inactivation. Co-expression of *scn8aa* with *Scn1b* or *scn1bb* results in a single population of channels that recover rapidly from inactivation.

Figure Legends:

Supplemental Figure 1. Specificity of anti-Scn1bb antibody. A. Western blot performed with anti-Scn1bb (diluted 1:500) on equal protein aliquots (50 μg) of untransfected Chinese hamster lung 1610 cells, 1610 cells transiently transfected with *scn1bb* or *scn1ba_tv1* cDNA, or mouse brain membranes. Arrow indicates position of Scn1bb specific band. Molecular weight markers shown in kDa. B. Western blot performed on whole fish membrane preps (5 dpf) and rat brain membranes (5 μg). Arrow indicates position of Scn1bb specific band. Molecular weight markers shown in kDa.

Supplemental Figure 2. *scn1bb* mRNA expression. A. Expression of *scn1bb* mRNA in medial, intermediate, and lateral regions of the spinal cord at 24, 48, and 72 hpf as indicated. At 24 hpf, *scn1bb* mRNA is found throughout the spinal cord, extending through all regions along the dorsal-ventral and medial-lateral axes. At 48 hpf, *scn1bb* mRNA is found throughout the spinal cord, extending throughout the entire medial-lateral axis and through the middle and dorsal regions, but is absent from the ventral spinal cord. At 72 hpf, *scn1bb* mRNA is found throughout the spinal cord, extending throughout the entire medial-lateral axis and from the middle to the dorsal spinal cord, but not in the ventral spinal cord. Scale Bars, 50 μm. B. I. 72 hpf embryo showing *scn1bb* mRNA expression in neuromasts surrounding the eye. II. 72 hpf embryo showing *scn1bb* mRNA expression in the otic vesicle and the anterior pronephros. For all images (A and B), embryo is mounted with dorsal at the top and anterior to the left. Scale Bar, 100 μm.

Supplemental Figure 3. Na^+ current modulation by *scn1bb*. A. *scn8aa*-expressed Na^+ current traces demonstrating shifts in gating mode with co-expression of *Scn1b* or *scn1bb*. B. Co-expression of *Scn1b* or *scn1bb* results in increased Na^+ current

amplitude. C. Co-expression of *Scn1b* (σ) or *scn1bb* (□) with *scn8aa* versus expression of *scn8aa* alone (■) results in hyperpolarizing shifts in the voltage dependence of activation. D. Co-expression of *Scn1b* (σ) or *scn1bb* (□) with *scn8aa* versus expression of *scn8aa* alone (■) results in hyperpolarizing shifts in the voltage dependence of inactivation. E. Co-expression of *Scn1b* (σ) or *scn1bb* (□) with *scn8aa* versus expression of *scn8aa* alone (■) speeds the rate of channel recovery from inactivation.