W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity

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THE pacemaker activity in the mammalian gut is responsible for generating anally propagating phasic contractions. The cellular basis for this intrinsic activity is unknown. The smooth muscle cells of the external muscle layers and the innervated cellular network of interstitial cells of Cajal, which is closely associated with the external muscle layers of the mammalian gut, have both been proposed to stimulate pacemaker activity[1–5]. The interstitial cells of Cajal were identified in the last century but their developmental origin and function have remained unclear. Here we show that the interstitial cells of Cajal express the Kit receptor tyrosine kinase. Furthermore, mice with mutations in the dominant *white spotting* (*W*) locus, which have cellular defects in haematopoiesis, melanogenesis and gametogenesis[6] as a result of mutations in the *Kit* gene[7,8], also lack the network of intestitial cells of Cajal associated with Auerbach's nerve plexus and intestinal pacemaker activity.

Injection of antibodies directed against the extracellular domain of the Kit receptor tyrosine kinase into newborn mice leads to changes in *in vitro* contraction patterns in the small intestine and absence of Kit messenger RNA in the myenteric plexus area[9]. This observation prompted us to investigate whether the Kit receptor might play a role as a signalling molecule required for the development of the interstitial cells of Cajal (ICC) and therefore be essential for intestinal pacemaker activity. To determine whether mutations at the murine *W/kit* locus might affect normal development of ICC, we first examined the morphology of the intestines of mutant W/W^v mice and their control littermates. Control mice (albino, +/+ and $W/+$) had normal ICC networks in the small intestine, visualized by selective uptake of methylene blue (Fig. 1*a*) and confirmed by electron microscopy (Fig. 2*a*, *b*). By contrast, the network of ICC in the myenteric plexus region was absent in W/W^v mice (Fig. 1*b*), again confirmed by electron microscopy (Fig. 2*c*, *d*). In the mutants, only scattered methylene-blue-positive cells which might be genuine ICC were observed (Fig. 1*b*). Using electron microscopy, we counted 51 ICC along a length of 1,109 cross-sectioned muscle cells bordering the myenteric plexus in $W/+$ control mice; by contrast, only 7 ICC were observed along a length of 3,059 muscle cells in W/W^v mice. Quantitatively, 70% of muscle cells bordering the Auerbach's plexus contacted ICC in control mice, compared with only 1.5% in W/W^v mice. We also observed the direct apposition of large stretches of the circular and longitudinal smooth muscle layers in W/W^v mice in the absence of the intermediary network of ICC found in normal animals (Fig. 2*c*). Light and electron microscopic analysis of duodenal muscularis revealed no differences in the density, morphology or ultrastructural features of enteric neurons or glial cells between +/+ and *W* mutant mice, indicating

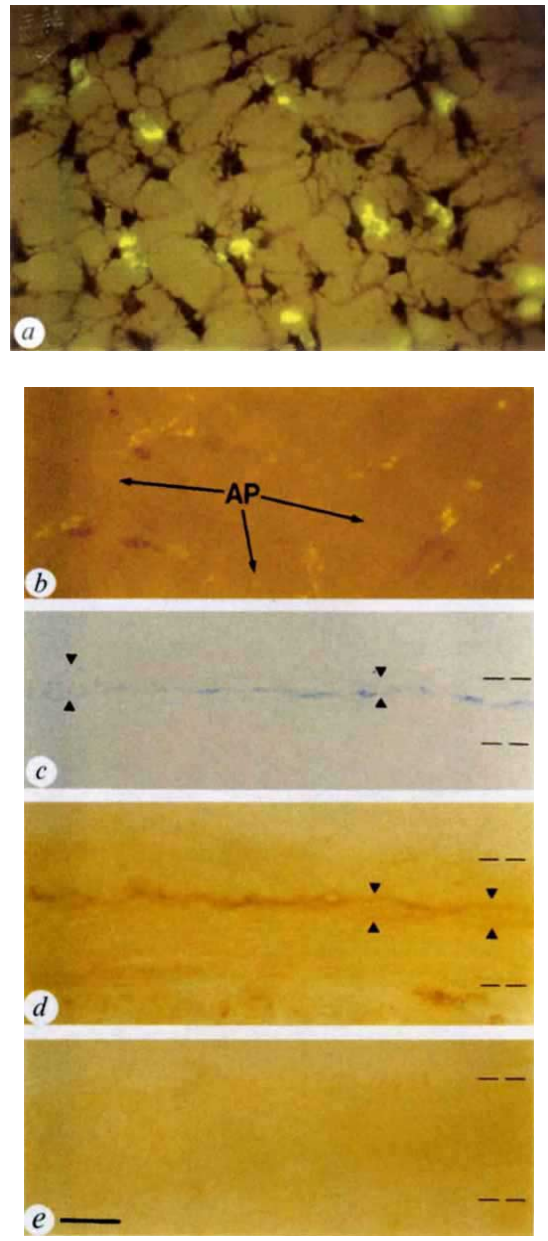

FIG. 1 Histochemical analysis of the small intestine in wild-type and *W* mutant mice. *a*, *b*, Double staining of interstitial cells of Cajal associated with Auerbach's plexus (ICC) (shown as the stained cellular network using vital staining with methylene blue) and macrophages (fluorescence; ingestion of fluorescein isothiocyanate FITC-labelled dextran)[19]. Whole mounts of the isolated duodenal muscularis externa of a control albino mouse (*a*) and W^x/W^v mouse (*b*); *a* is representative of the normal staining pattern of both cell types in albino mice as well as $W^v/+$ mice. Normally, ICC together with macrophages form a partial sheath around ganglia and primary fascicles of Auerbach's plexus (AP) but in *b*, only macrophages are present in normal numbers and normally organized. *c*–*e*, Sectioned jejunal tissue. *c*, 1 μm section of Epon-embedded tissue, processed with preservation of methylene blue, no post-staining. ICC are seen as the blue, broken line between the two layers of the muscularis externa (between dashed lines). Arrowheads point to unstained, partially enveloped elements of Auerbach's plexus (compare with *a*). *d*, *e*, Frozen 8-μm sections, showing Kit immunoreactivity in ICC in a $W^v/+$ mouse and its absence in the W^x/W^v mouse (*e*). Magnification, ×225 (*a*); ×320 (*b*–*e*); scale bar, 25 μm.
METHODS. Methylene blue was preserved through dehydration and embedding by precipitation as a hexachloroplatinate[20]. Immunocytochemistry was performed on unfixed frozen sections of small intestines from W^x/W^v mice and their controls. Both the PAP and the biotin/-streptavidin/Texas-red methods were used[21].

that defects in the Kit signalling pathway do not affect neuronal or glial cells in the Auerbach's plexus region.

These data indicate that a functional Kit receptor is required for the development of ICC in the Auerbach's plexus region of the small intestine. To determine whether the ICC deficiency in *W* mutant mice was a cell-autonomous defect, we analysed Kit RNA and protein expression in *W* mutant mice and control animals. We used a polyclonal antibody directed against the intracellular domain of the Kit receptor tyrosine kinase to localize the protein. In wild type and $W^x/+$, high levels of Kit expression were observed between the longitudinal and the circular muscle layers at the level of Auerbach's plexus (Fig. 1*d*). The stained cells surrounded the ganglia and their distribution in the Auerbach's plexus area was identical to that of the methylene-blue-stained ICC (Fig. 1*c*). By contrast, no Kit immunoreactivity was found in or between the muscle layers of W^x/W^v mice (Fig. 1*e*).

We next performed whole-mount RNA *in situ* experiments on the ileum of wild-type and W/W^v mice. The ileum of +/+ mice contained Kit-positive cells whose organization was identical to that of ICC, as revealed by methylene-blue staining (Fig. 3*a*). These cells were localized between the longitudinal and circular

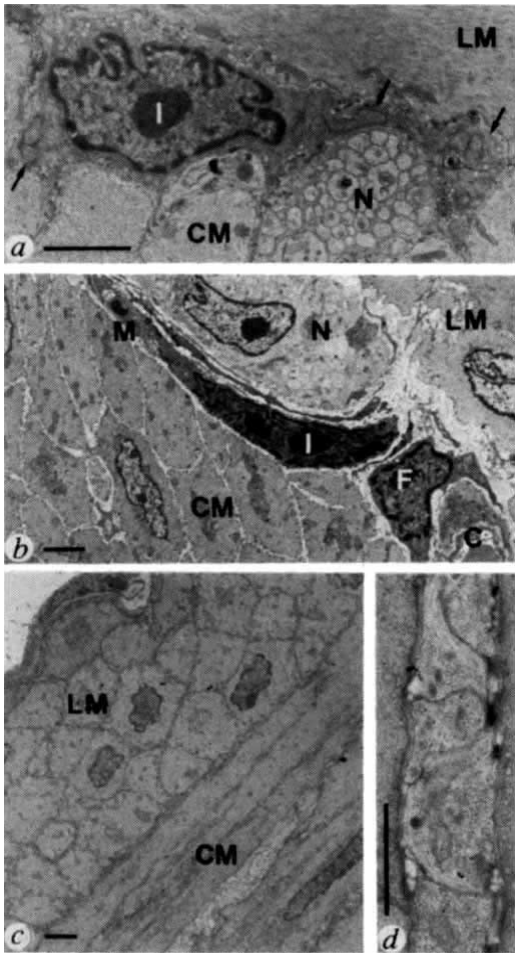

FIG. 2 EM analysis of the small intestine of wild-type and *W* mutant mice. *a–d*, Electron micrographs of the jejunal myenteric plexus area in an albino mouse (*a*), a $W^x/+$ mouse (*b*), and a W^x/W^v mouse (*c*, *d*). *a*, Normal appearance of ICC in the Auerbach's plexus region (I), tightly associated with nerve fascicle (N) of Auerbach's plexus, between the longitudinal (LM) and circular (CM) muscle layers. An abundance of mitochondria (arrows) and caveolae are distinctive ultrastructural features of ICC. *b*, Prestaining with methylene blue assists the differentiation of ICC (I, with increased granularity and electron-density of nuclei and ribosomal areas) from fibroblasts (F), macrophages (M, macrophage process) and pericytes (C, capillary). *c–d*, The overall organization of the muscle layers seemed normal. Although cell types other than ICC were counted in normal numbers in W/W^v, one result of the absence of ICC was a strong increase in the extension of areas of direct apposition of muscle cells of LM and CM (*c*). Sheath cells around nerves were either absent (*d*, small tertiary nerve between the muscle layers) or had fibroblast ultrastructure.

Tissues were fixed and processed for electron microscopy (Philips 300 microscope) by routine methods[5]. Scale bars: 2 μm (*a–c*); 1 μm (*d*).

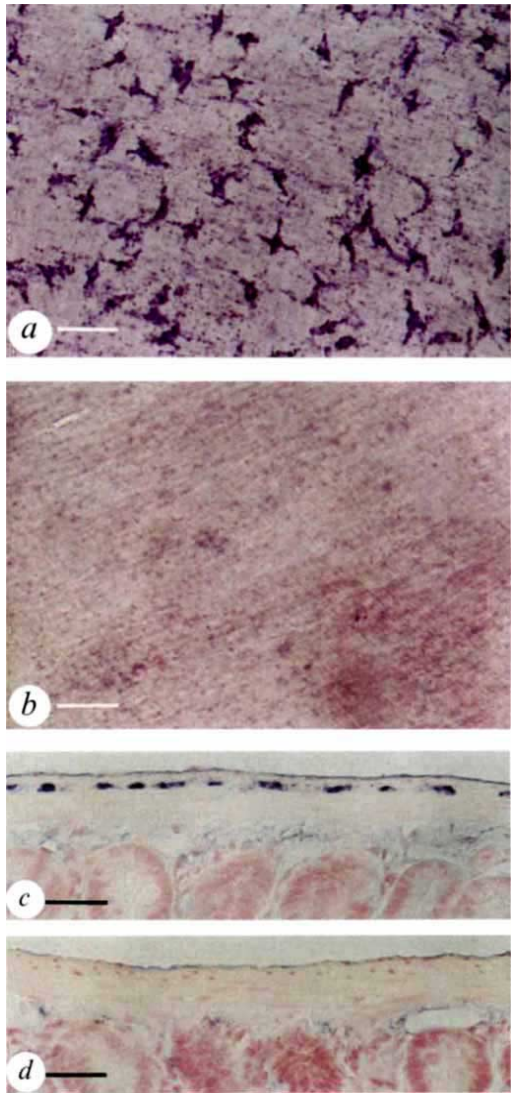

FIG. 3 Whole-mount RNA *in situ* analysis of *kit* expression in the small intestine of wild-type and *W* mutant mice. *a*, A network of Kit-expressing cells is present in the isolated external muscle layers in +/+ mice, identical in appearance to the ICC network (compare Fig. 1*a*). *b*, Kit-positive cells were absent in similar whole mounts of W/W^v mice. Cross-sectioning of the whole-mount tissues localized the Kit-positive cells in +/+ mice between the longitudinal and circular muscle layer (*c*: compare Fig. 1*c* at the same magnification) and confirmed their absence in W/W^v mice (*d*). Scale bars: 50 μm (*a*, *b*); 25 μm (*c*, *d*).

METHODS. Whole-mount RNA *in situ* hybridization was performed according to the method of D. Henrique and D. Ish-Horowisz (personal communication), with the following modifications. Proteinase K digestion was for 30 min at a concentration of 20 μg ml^{-1}. The hybridization mix contained 5 × SSC, 50% formamide, 5 mM EDTA, 50 μg ml^{-1} yeast tRNA, 0.2% Tween-20, 0.5% CHAPS and 100 μg ml^{-1} heparin, pH 6.5 with citric acid. After *in situ* hybridization, tissues were postfixed in 4% paraformaldehyde before wax embedding and sectioning. The *kit* cDNA probe has been described[11].

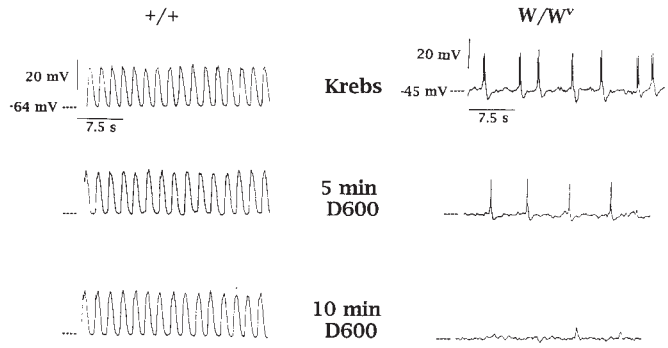

FIG. 4 Action potential generation in wild-type and *W* mutant mice. The left panel depicts the slow-wave-type action potentials generated by +/+ mice. Slow-wave-type action potentials were generated at a constant frequency. L-type calcium channel blockers did not have any influence on the slow-wave component. Occasionally spikes were superimposed on the slow wave and these were abolished by L-type calcium channel blockers. The right panel depicts typical spike-like action potentials generated by the small intestinal smooth muscle cells of W/W^v mice. Fast spikes occurred as single spikes or in groups of 2–3. These spikes were completely abolished by the action of L-type calcium channel blockers.

METHODS. Female mice were killed by cervical dislocation. The small intestine was exposed by a midline abdominal incision and a 3–5-cm segment was removed, 1 cm from the gastroduodenal sphincter. The segment was placed in a dissecting dish filled with oxygenated (95% O_2 and 5% CO_2) Krebs solution and opened flat. It was then pinned to the Sylgard bottom of a dissecting dish and the mucosa removed by sharp dissection. Muscle strips, 15 mm long, were prepared by cutting them parallel to the longitudinal muscle bundles: the tissue was 1.5 mm wide over a length of 10 mm and gradually widened to 3 mm at the proximal end. At the wide end an area of the tissue (3 × 3 mm) was pinned to the Sylgard bottom of a transfer holder. The preparation was mounted by putting insect pins along all four edges. This transfer holder with the tissue attached was then placed in an organ bath at 36.0–37.0 °C. The end of the tissue that was not pinned was tied to a force transducer to record mechanical contraction. Intracellular recordings were made using microelectrodes with 30–60 MΩ tip resistance because microelectrodes with a tip resistance within this range have a tip diameter of ~150 mm, which was appropriate for impaling cells. Microelectrodes were prepared from 1.2 mm outside-diameter glass capillaries (WPI) and filled with 3 M KCl. A microelectrode was inserted into a microelectrode holder (WPI M700P) connected to an electrometer (WPI M-707A) which was a high-impedance probe.

muscle layers in an identical position to the ICC (Fig. 3*c*). By contrast, no Kit-positive cells were observed in the muscle layers of W/W^v mice (Fig. 3*b*, *d*).

To determine whether these morphological and cellular differences between +/+ and *W* mutant mice were associated with functional anomalies, we measured the electrical activity of the small intestinal muscle layers. Normal mice displayed slow-wave-type action potentials with an amplitude of 21.5 ± 5.9 mV, a frequency of 32.4 ± 1.0 cycles per minute (range 30–36 c.p.m.) and a resting membrane potential of −60.0 ± 3.0 mV (Fig. 4; $n = 9$). By contrast, the ilea of W/W^v mice failed to display any slow-wave-type action potentials (Fig. 4). The membrane potential was −44.8 ± 1.3 mV ($n = 14$) and at irregular frequency fast spike-like action potentials arose from it, singly or in groups of 2–6. Their amplitude was 16.4 ± 2.1 mV, the frequency ranged from 4 to 20 c.p.m., and was irregular within one preparation. In 4 out of 14 W/W^v mice, no spontaneous action potentials were observed. The slow-wave component or pacemaker activity of gut smooth muscle is insensitive to L-type calcium channel blockers[10]. In the presence of the blockers nifedipine or D600, the slow-wave component of the action potentials in +/+ mice remained unaltered, whereas the electrical activity of W/W^v mice was completely abolished ($n = 14$; Fig. 4).

These data demonstrate that mutations at the murine *W* locus lead to the absence of the ICC network in the Auerbach's plexus region and of pacemaker activity in the small intestine, demonstrating an essential role for ICC in gut pacemaker activity. Because the ICC express the Kit receptor, we conclude that the absence of these cells in *W* mutant mice reflects a direct role for Kit in ICC development. The phenotype of *W* mutant mice was thought to be restricted to cells of the haematopoietic, germ and melanocyte lineages[6], even though Kit and its ligand Steel factor are contiguously expressed in additional anatomical sites, including the small intestine[11,12]. Thus, our experiments extend the range of cell types affected by *W* mutations to include the interstitial cells of Cajal. Mice with mutations in Steel factor (Sl/Sl^d mutant mice) display abnormalities in the gut similar to those found here in *W* mutant mice (data not shown).

Our data suggest that the functional gut abnormalities and megacolon observed in individuals with piebaldism[13], a hypopigmentation disorder that also results from mutation in the *kit* proto-oncogene[14,15] reflect an identical function of the Kit signalling pathway in the development of the ICC in humans. Mutations in the *RET* proto-oncogene are also associated with gut abnormalities in humans (Hirschprung's disease) and in gene-targeted mutant mice, as the result of a cellular deficit in neural-crest-derived enteric neurons[16 18]. Thus, two members of the receptor tyrosine kinase family, *kit* expressed in ICC and *RET* expressed in neural-crest-derived ganglion cells, are both essential for normal gut function in mammals. □

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