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KBP interacts with SCG10, linking Goldberg–Shprintzen syndrome to microtubule dynamics and neuronal differentiation

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Goldberg–Shprintzen syndrome (GOSHS) is a rare clinical disorder characterized by central and enteric nervous system defects. This syndrome is caused by inactivating mutations in the Kinesin Binding Protein (KBP) gene, which encodes a protein of which the precise function is largely unclear. We show that KBP expression is upregulated during neuronal development in mouse cortical neurons. Moreover, KBP-depleted PC12 cells were defective in nerve growth factor-induced differentiation and neurite outgrowth, suggesting that KBP is required for cell differentiation and neurite development. To identify KBP interacting proteins, we performed a yeast two-hybrid screen and found that KBP binds almost exclusively to microtubule associated or related proteins, specifically SCG10 and several kinesins. We confirmed these results by validating KBP interaction with one of these proteins: SCG10, a microtubule destabilizing protein. Zebrafish studies further demonstrated an epistatic interaction between KBP and SCG10 in vivo. To investigate the possibility of direct interaction between KBP and microtubules, we undertook co-localization and in vitro binding assays, but found no evidence of direct binding. Thus, our data indicate that KBP is involved in neuronal differentiation and that the central and enteric nervous system defects seen in GOSHS are likely caused by microtubule-related defects.

INTRODUCTION

Goldberg–Shprintzen syndrome (GOSHS) is an autosomal recessive disorder characterized by polymicrogyria, mental retardation, microcephaly, facial dysmorphisms and, in most cases, by Hirschsprung disease (1,2). Homozygosity mapping studies showed linkage to chromosome 10 and subsequent sequence analysis of all 35 genes in this region led to the identification of truncating mutations in the KIAA1279 gene (2). KIAA1279 encodes a protein with two tetrastric
peptide repeats whose function is still uncertain. Based on the GOSHS clinical phenotype, it is clear that mutations in KIAA1279 are associated with both central and enteric nervous system defects. Subsequent studies by Wozniak et al. (3) led to KIAA1279 being named kinesin binding protein (KBP) due to its interaction with the motor domain of two related kinesin-like proteins, KIF1C and KIF1Bα. The interaction with KIF1Bα suggested that KBP plays a role in mitochondria localization and distribution (3). More recently, KBP has been implicated in the regulation of neuronal microtubules organization, and in axonal growth and maintenance (4). This finding stemmed from the characterization of a kbp mutant zebrafish, the first animal model to be described for GOSHS (4). However, the mechanism by which KBP exerts its effect on microtubules is still unknown.

Microtubules are dynamic structures that provide mechanical support for the shape of cells, and a track along which molecular motors, kinesins and dyneins move organelles from one part of the cell to another (5). To perform these functions, a cell must carefully control the assembly and orientation of its microtubule cytoskeleton. Several proteins have been described that modulate microtubule dynamics and they can be divided into two groups: microtubule stabilizing proteins (MAPs) and microtubule destabilizing proteins (6). Although much is known about the role of MAPs, relatively little is known about the molecular machinery that controls the microtubule destabilizing family of proteins, especially with respect to neuronal development and regulation (7).

Here we describe the identification of several KBP interacting proteins, all of which are implicated in microtubule transport and microtubule dynamics, and show that KBP is necessary for proper neuronal differentiation and neuronal development.

**RESULTS**

KBP plays a role in neuronal development and neuronal differentiation

GOSHS is a developmental disorder in which both the central and enteric nervous systems are affected. To determine KBP expression levels in neuronal cells, we isolated E15 mouse cortical neurons and cultured them for 6 days. Cells were lysed each day from the second day of the experiment onwards, and protein levels were analyzed by western blotting (Fig. 1A). Our data show that there is an up-regulation of KBP expression levels in primary cortical neurons that seem to go hand-in-hand with neuronal differentiation.

In situ hybridization performed in zebrafish confirmed the expression of KBP both in the central and enteric nervous systems (Fig. 1B). A detailed examination of the expression pattern in the developing gut revealed that KBP is only expressed at low levels in migrating enteric neural crest cells (encc) express only low levels of KBP (C2 and C2').

**Figure 1.** KBP expression during neuronal development and neuronal maturation. (A) Western blot analysis of mouse primary cortical neurons cultured for 6 days showed that KBP expression levels increase during neuronal development. (B) Whole-mount in situ hybridization of KBP in zebrafish. At 20–somite stage KBP is expressed ubiquitously (B'). At 24 hpf KBP expression is restricted to the anterior CNS and to the anterior gut (B''). This pattern of expression is maintained at 48 hpf with cranial ganglia (cg) also expressing KBP (B'''). (C) KBP expression in the hindbrain, cranial ganglia and the gut. Transverse sections of whole-mount KBP in situ hybridization/GFP immunohistochemistry performed in Tg(foxd3:gfp) zebrafish at 48 hpf showed a strong KBP expression in the ventral neural tube (nt), the posterior lateral line ganglia (pllg), the developing liver (L) and gut (g) (C1 and C1'). A magnified view of the gut shows that GFP/Foxd3 positive enteric neural crest cells (encc) express only low levels of KBP (C2 and C2').
any extension that could be seen from the cellular body, a neurite, and the presence of one small extension was enough to consider the cell as differentiated. Our results show that the number of PC12 cells that differentiate and produce neurites when KBP expression was impaired was significantly lower (P, 0.05) than for the control (Fig. 2C). These data indicate that KBP strongly contributes to the NGF-induced neuronal differentiation of PC12 cells.

Yeast two-hybrid interactions of KBP

KBP contains two tetratricopeptide repeats which mediate protein–protein interactions (9), suggesting that KBP likely binds to other proteins. To identify KBP interacting partners, a pre-transformed E11 mouse cDNA library was screened using a yeast two-hybrid screen. The E11 mouse library was used, as this is a critical stage in brain development and coincides with the stage at which the colon is being colonized by neural crest cells.

From our yeast two-hybrid screen, we selected 69 positive clones which we isolated and sequenced. Nineteen of these 69 clones encoded for the same protein, SCG10, a stathmin-like protein. In addition, six kinesins were found among these inserts, namely KIF5B, KIF5C, KIF7, KIF2C, KIF3A and, also, KIFC1. A blast search using the cDNA of the kinesins found revealed that they all interact with KBP via their motor domain. None of the kinesins previously described to interact with KBP (3), KIF1C and KIF1B, were found in our screen. Additionally, three of these 69 clones encoded for tubulin, the main constituent of microtubules (Table 1). Taken together, these results strongly suggest a role for KBP in microtubule organization and stability. Moreover, the fact that all these kinesins were obtained in this screen reinforced the idea that KBP is involved in kinesin-mediated microtubular transport and is indeed a kinesin binding protein.

Since the majority of clones obtained encoded for microtubule associated or microtubule interacting proteins, we conclude that KBP might be involved in microtubule-related events. In order to confirm the validity of this result, we decided to further study KBP interaction with the most frequently found clone, SCG10.

KBP interacts with SCG10

SCG10 is a member of the stathmin family of proteins, all of which bind to tubulin to act as a sequestering agent and promote microtubule disassembly (10). SCG10 is a membrane-associated neuronal protein that is largely expressed during development and whose expression correlates with neurite outgrowth (10). It possesses a unique N-terminal domain that is critical for membrane binding, is responsible for SCG10 localization to the Golgi complex and is important in the targeting of SCG10 to the growth cones (11,12).

To confirm the specificity of the KBP interaction with SCG10, we performed a co-immunoprecipitation assay. HA-KBP was expressed in Human Embryonic Kidney cells (HEK293) alone or in combination with Myc-tagged SCG10. Expression of exogenous Myc-SCG10 was necessary, as HEK293 cells do not endogenously express SCG10. Precipitation of Myc-SCG10 was only observed in the presence of HA-KBP (Fig. 3A), confirming an interaction between KBP and SCG10. This interaction was further supported by co-localization studies in a mouse neuroblastoma cell line, N1E-115. This cell line was chosen due to its ability to differentiate in neuronal-like
cells when incubated in the presence of low serum concentrations. Using this cell line, we evaluated GFP–KBP and SCG10 cellular distribution in the cell. SCG10 is enriched in the Golgi complex and in the growth cones as expected (12), while KBP shows a cytoplasmic distribution in the cell that overlaps with SCG10 specifically in the Golgi complex (Fig. 3B). To confirm KBP and SCG10 co-localization in the Golgi complex, we used a specific antibody against a Golgi protein, giantin (13), as a Golgi marker (Fig. 3B).

These results support our yeast two-hybrid data and confirm the cellular interaction between KBP and SCG10.

**Epistatic interaction between KBP and SCG10 in zebrafish**

To further validate the KBP–SCG10 interaction, the distribution of the mRNA transcripts for both these proteins was evaluated and epistasis was determined using a zebrafish model.

The zebrafish genome contains one KBP gene and two SCG10 orthologs (4,14). To determine the spatial expression pattern of the zebrafish scg10a and scg10b and to compare it with kbp orthologs during embryogenesis, matched whole-mount *in situ* hybridizations were performed using riboprobes.
for all three genes. Discrete patterns of expression were detectable as early as 16 h post-fertilization (hpf). At this stage, expression of both \textit{scg10a} and \textit{scg10b} genes is restricted to the posterior lateral line (PLL) ganglia and Rohon-Beard sensory neurons in the spinal cord (Fig. 4A). In contrast, expression of \textit{kbp} is initially rather diffused and ubiquitous in the whole embryo (Fig. 1B'). From 24 hpf, \textit{kbp}’s expression becomes predominantly restricted to the anterior central nervous system (CNS) and this pattern of expression persists throughout all the stages examined in the present study. \textit{kbp} transcript is present in all brain regions, as well as in the anterior lateral line (ALL) and PLL ganglia (Figs 1C and 4A). SCG10 expression at 24 hpf can be detected within the anterior CNS regions including ventral telencephalon and diencephalon, all hindbrain rhombomeres, the ALL and PLL and primary sensory neurons of the spinal cord (Fig. 4A). At 48 hpf, expression of the SCG10 genes becomes more abundant but like the pattern of \textit{kbp} expression, they are primarily restricted to the anterior CNS and cranial ganglia. Further in development (72 and 96 hpf), SCG10 transcripts remain expressed in the same CNS regions and are also expressed in the enteric neurons (14). The pattern of expression of both SCG10 orthologs in zebrafish is very similar, although \textit{scg10a} transcript is much more abundant (14). At 48 hpf expression of \textit{scg10a}, \textit{scg10b} and \textit{kbp} overlap in the forebrain region, retina, optic tectum, trigeminal, vagal ganglia, ALL and PLL ganglia, and in the hindbrain (Fig. 4A).

To determine whether there is an epistatic interaction between KBP and SCG10, 1–2-cell stage zebrafish embryos were injected with splice blocking morpholinos (SBMO) targeted against \textit{scg10a}, \textit{scg10b} and \textit{kbp} transcripts (15). Injections were carried out either separately or in combination.

In addition, combined injections were performed with a \textit{p53} morpholino (MO) to exclude potential cytotoxic side effects of the morpholinos used (16). The concentration of \textit{scg10} and \textit{kbp} MOs was titrated to the sub-threshold doses so that when injected separately they resulted in no apparent phenotype (Fig. 4B). In contrast, when the MOs were injected in combination, perturbation in development became apparent. In the case of \textit{scg10a} and \textit{scg10b} double injections, morphants displayed a general delay in development, smaller heads and eyes and axial defects (Fig. 4B). This result suggests that the \textit{scg10} genes are functionally redundant and this is consistent with their almost identical patterns of expression. Injections of \textit{scg10a}, \textit{scg10b} and \textit{kbp} MOs together resulted in a much more severe phenotype. Triple morphants were significantly smaller with severely malformed body axis, very small head and eyes, necrotic areas in the brain and heart edema. Injections with \textit{p53} MO reduced the cell death in the retina and optic tectum areas but did not change the general morphology of the morphants, confirming that the observed morphant phenotype is not the result of offsite effects of the MOs (Fig. 4B) (16). These results strongly suggest an epistatic interaction between \textit{kbp} and \textit{scg10} genes and support the results of the yeast two-hybrid screen and co-immunoprecipitation assays.

**KBP interaction with microtubules**

Based on the yeast two-hybrid results and on the direct interaction between KBP and SCG10, a microtubule destabilizing protein, we hypothesize that KBP plays a role in microtubule organization or microtubule stability and dynamics. Furthermore, the recent study characterizing a \textit{kbp} mutant zebrafish showed that the loss of KBP function leads to a disorganization...
of axonal microtubules and to an improper orientation of microtubules along the axonal axis (4). In an attempt to further explain this microtubule effect, we investigated whether there is a direct interaction between KBP and microtubules by determining the co-localization of GFP–KBP with tubulin in mouse fibroblast cells (NIH-3T3). We detected no co-localization between KBP and tubulin (Fig. 5A). To rule out the possibility that the interaction of KBP with microtubules is very transient and thus difficult to detect in co-localization studies, in vitro microtubule binding assays were performed. Taxol-stabilized microtubules were purified and mixed with cytosolic cell extracts of NIH-3T3 cells expressing KBP. Microtubules were pelleted by high-speed centrifugation and the binding of KBP to the microtubules was determined by co-sedimentation. We found that KBP remains in the supernatant both in the absence and presence of microtubules, suggesting that KBP does not directly associate with microtubules (Fig. 5B).

**KBP has a cytoplasmic localization**

During the co-localization studies performed, we noticed that KBP was distributed throughout the cytoplasm. Previous studies performed in NIH-3T3 cells reported that KBP co-localizes with mitochondria and that it plays a role in mitochondrial distribution by interaction with KIF1Bα (3). To reassure ourselves that the GFP tag was not influencing KBP localization in the cell, we expressed HA-human KBP in HeLa cells. Using an anti-HA antibody and a specific marker for mitochondria (MitoTracker), we repeatedly observed that KBP is distributed throughout the cytoplasm and is not specifically localized to mitochondria (Fig. 6A).

To further support this observation, we co-expressed a modified Bicaudal D2 (BICD2-N) construct in HeLa cells with the HA-human KBP construct (Fig. 6B). BICD2 is a motor-adaptor protein involved in the transport of various cargoes by dynein-mediated transport in *Drosophila* and mammals. Previous studies have shown that in the absence of its C-terminal, BICD2-N-terminal strongly binds to dynein and impairs its normal function leading to disruption of the retrograde distribution of membranous organelles (17). When we over-expressed BICD2-N in HeLa cells, mitochondria became aggregated; however, we saw no change in KBP localization (Fig. 6B). Since NIH-3T3 cells were also used by us with no change in the diffuse localization of KBP in the cell (Fig. 5A), maybe a difference in the experimental procedure used could explain the differences in co-localization seen by us and by the previous study (3). However, based on our results, we conclude that KBP has a cytoplasmic localization and does not co-localize with mitochondria.

**DISCUSSION**

Homozygous nonsense mutations in the *KIAA1279/KBP* gene encoding KBP lead to GOSHS. Affected patients have undetectable levels of KBP and show both central and enteric nervous system defects (2). Based on the clinical phenotype, KBP is required for normal neuronal development, but its precise function is largely unclear. Subsequent to our initial identification of *KBP* mutations underlying the GOSHS phenotype, two independent studies have investigated the function of the KBP protein. The first study showed that KBP has a mitochondrial localization and that it interacts with the α isoform of KIF1B, increasing its motility. The
same study showed that a reduction in the expression levels of KBP led to mitochondrial aggregation, suggesting that KBP regulates mitochondria distribution in the cell by controlling KIF1Bα activity (3). The second study characterized a zebrafish kbp mutant, which had reduced axonal growth and disruption of axonal microtubules (4). Axonal degeneration and mislocalization of mitochondria were also observed in the neurons at later stages of development, suggesting that KBP is a regulator of the neuronal cytoskeleton (4). Although both studies shed some light on KBP function, they only partially explain the role of KBP in GOSHS.

Our results show that KBP plays a role in neuronal development and is necessary for neuronal differentiation. Using zebrafish as a vertebrate model, we clearly show that KBP is strongly expressed in the CNS during embryonic development. KBP is also expressed in some peripheral nervous system structures, such as the cranial ganglia, although its expression is low in the migrating enteric precursors. Our study also shows that KBP interacts with several microtubule-related proteins, including the stathmin SCG10, a microtubule destabilizing protein. Based on this interaction, we suggest that KBP potentially modulates the proper organization of microtubules. We also show that KBP interacts with several kinesins, as previously described (3), further confirming that KBP is indeed a kinesin binding protein and suggesting that KBP may regulate multiple aspects of intracellular transport along microtubules. Furthermore, we cannot exclude the possibility that KBP may be involved in signaling pathways mediated by primary cilium especially because one of the kinesins that we found in our yeast two-hybrid screen, KIF7, was shown to be a cilia-associated protein (18).

Figure 6. KBP has a cytoplasmic localization. (A) Confocal images of HeLa cells expressing HA-hKBP and stained with a mitochondrial marker (Mitotracker) showed that KBP has a cytoplasmic distribution and does not co-localize with mitochondria. (B) Co-expression of HA-hKBP and BicD2-N constructs in HeLa cells confirms KBP cytoplasmic localization. Scale bars 20 μm.

Taken together, these results provide further insights into the cellular mechanisms that are perturbed in GOSHS patients and that underlie the clinical phenotype.

KBP, SCG10 and microtubules

Microtubules are dynamic structures composed of α and β tubulin dimers. These structures are necessary for many processes in the cell, including transport and maintenance of neuritic processes (19–22). Microtubule dynamics are characterized by stages of catastrophe alternated with stages of polymerization; this process is called dynamic instability. Although dynamic instability occurs both at the plus and minus ends of purified microtubules in vitro, it is more pronounced at the plus ends (22).

Several proteins are known to regulate microtubule dynamics, including KBP (4). Our results show that KBP interacts with SCG10, a stathmin-like protein previously reported to be involved in microtubule dynamics (23,24). Moreover, during differentiation, SCG10 enhances neurite outgrowth, a phenomenon that is believed to depend on microtubule dynamics (24,25).

Our results strongly suggest that KBP’s role in microtubule organization/dynamics is not a result of its direct interaction/association with microtubules but is instead due to its interaction with SCG10. As KBP interacts with several kinesins, a possible function for KBP would be as an adaptor molecule for the transport of SCG10 to the growth cones via kinesin-related transport. However, our yeast two-hybrid screen showed that KBP’s interaction with the kinesins is via the kinesin motor domain. Knowing that the motor domain is
KBP implications in GOSHS and HSCR

GOSHS is a rare but severe genetic disorder characterized by central and enteric nervous system defects. Based on our results, we propose that the GOSHS phenotype is caused by a reduction in neuronal differentiation and perturbed microtubule dynamics due to a deregulation of SCG10 activity. The data we now present supports previous findings (4).

Since GOSHS patients frequently have Hirschsprung disease (HSCR), KBP interacting proteins could bring some new insights about HSCR development. Currently, the genetic basis of this condition can only be explained in ~20% of all the cases, with RET being the major gene involved (26). SCG10 has been previously identified as a down-regulated gene in a RET mouse model for HSCR (27). Furthermore, as the expression pattern of SCG10 is restricted to neuronal tissue, any perturbation of the KBP–SCG10–tubulin interaction will potentially result in neuronal development defects consistent with the GOSHS clinical phenotype. Finally, our observation that KBP is required for neuronal maturation of PC12 cells is consistent with our biochemical studies that showed an interaction between KBP and SCG10.

Conclusions

Our study shows that KBP is necessary for neuronal development and neuronal maturation. Furthermore, we show that KBP interacts with several microtubule-associated and microtubule-related proteins in vitro and in vivo, being likely involved in microtubule organization/stability. Our results provide new insights into perturbed cellular mechanisms that lead to GOSHS and suggest that KBP has an important role in modulating SCG10 function during neuronal development.

MATERIALS AND METHODS

Constructs

Design of vectors and oligonucleotides used are described in Supplementary Material, Table S1.

Cell culture and transfections

HEK293, NIH-3T3, HeLa and N1E/115 cells were cultured in DMEM high medium containing 4.5 g/l of glucose, 1-glutamine and pyruvate (Gibco), supplemented with 10% fetal calf serum (BioWhittaker) or with 10% newborn calf serum (Gibco) (for NIH-3T3) and 1% penicillin/streptomycin (Gibco), at 37°C and 5% CO2.

For transient transfections, 300 000 cells of HEK293, HeLa, 3T3 and N1E/115 were seeded in 6-well plates. Transfections were performed 24 h after seeding using jetPei (Promega).

Culture of primary cortical neurons

Primary cortical neurons were prepared from embryonic brains (E15–16) of C57Bl/6J mice. After mechanical dissociation, neurons were plated on 6-well plates previously coated with poly-D-lysine (2 μg/ml). Neurobasal medium (GIBCO) supplemented with B27-supplement (GIBCO), 0.5 mM glutamine (BioWhittaker), 1% penicillin/streptomycin (Gibco) and 2.5 μg/ml amphotericin B (Sigma-Aldrich) was used as culture medium. After 48 h, cells were treated with 10 μM cytosine arabinoside (Sigma-Aldrich) and incubated for another 48 h to inhibit non-neuronal cell growth.

Yeast two-hybrid screen

The yeast two-hybrid screen was performed using the Matchmaker 3 System (ClonTech) according to the manufacturer’s instructions. A detailed description can be found in Supplementary Material.

Cell lysates, co-immunoprecipitation assays and western blot analysis

Cell lysates were prepared as follows: cells were washed with ice-cold PBS and incubated with lysis buffer (200 mM NaCl, 20 mM Tris–HCl pH 7.8, 1% Triton X-100 and protease inhibitors) for 30 min on ice. Cell lysates were collected by scraping and cleared by centrifugation at 14000 rpm for 10 min in a pre-cooled (4°C) centrifuge. Supernatants were stored at −80°C before they were processed further for SDS–PAGE followed by western blot analysis. For immunoprecipitation, lysates were incubated with HA-coupled sepharose beads (Roche) ON at 4°C. Precipitates were washed with lysis buffer and protein was eluted in loading buffer. Antibodies used for western blot detection can be found in Supplementary Material.
Microscopy and image analysis
For immunofluorescence analysis, N1E/115, 3T3 and HeLa cells were cultured on poly-D-lysine coated cover slips. Two percent PFA was used as fixative agent for N1E/115 and HeLa cells and 3T3 cells were fixed in ice-cold methanol for 3 min at −20 °C. Cells were made permeable with 1% BSA and 0.1% Triton X-100 in PBS. Antibodies used for immunofluorescence can be found in Supplementary Material. Fluorescent images were made using a Leica TCS SP2 (AOBS) microscope.

In vitro polymerization of microtubules and high-speed fractionation
NIH-3T3 cells were lysed in tubulin buffer (Cytoskeleton) containing 0.1% Triton X-100. Lysates were centrifuged at 100 000 g for 1 h in an Optima™ MAX-E- Beckman Coulter ultracentrifuge. Supernatant fractions were incubated for 30 min at 37 °C with purified, taxol-stabilized microtubules, which were generated according to the manufacturer’s indications (Cytoskeleton™). Microtubules were pelleted down by centrifugation (100 000 g for 1 h) and both supernatant and pellet fractions were checked by western blotting.

PC12 culture, differentiation and transfection
PC12 cells were cultured in RPMI 1640 (Gibco) supplemented with 10% horse serum (Gibco), 5% fetal calf serum (Gibco) and 1% penicillin/streptomycin (Gibco), at 37 °C and 5% CO2.

For transient transfections, 50 000 PC12 cells were seeded in 12-well culture plates coated with laminin (Millipore). Twenty-four hours after, cells were transfected with pEGFP-N1 vector using lipofectamine, tissues were cryoprotected overnight in a 20% sucrose/5% gelatin solution. Afterwards, tissues were rapidly frozen in isopentane pre-cooled in liquid nitrogen to −60 °C. Frozen sections were cut at 14 μm, collected on Superfrost Plus microscope slides (BDH Laboratories), air dried and stored at −20 °C. Prior to labeling, sections were placed in warmed PBS (37 °C) for 15 min in order to remove the sucrose/gelatin, and then rinsed in PBS (2 × 5 min). Histochemical staining was done using a polyclonal rabbit anti-GFP antibody (Molecular Probes) as previously described (36). Sections were then stained with DAPI (5 min). In order to observe any co-localization of KBP RNA and FoxD3-GFP, bright field in situ images were converted into grayscale, inverted and then inserted into the red channel before being overlaid with FITC images using Adobe Photoshop software.

Fish and embryos
Zebrafish were kept and bred under standard conditions at 28.5 °C (30). Embryos were staged and fixed at specific hours post-fertilization (hpf) as described elsewhere (31). To better visualize in situ hybridization results, embryos were grown in 0.2 mM 1-phenyl-2-thiourea (Sigma) to inhibit pigment formation (30).

Antisense oligonucleotide (Morpholinos) injections
Splice blocking scg10a, scg10b and kbp morpholinos (SBMO) were designed to complement the sequences corresponding to the splice donor site at the predicted exon2/exon3 junction for scg10a, exon1/exon2 for scg10b and exon3/exon4 for kbp (Supplementary Material, Table S1).

The MOs (Supplementary Material, Table S1) were diluted in sterile filtered water over a concentrations range of 1–5 μg/μl. Approximately 1 nl of diluted MO was injected at the 1–2-cell stage using a gas-driven microinjection apparatus to determine the effects of knocking down single genes or combinations of them. The standard control MO from Gene Tools was injected as a negative control for the MO experiments. A p53 MO (Supplementary Material, Table S1) was injected in combination with other MOs to eliminate potential apoptotic side effects of MO injections (32).

Whole mount in situ hybridization and double-labeling immunohistochemistry in zebrafish
Digoxigenin-labeled riboprobes that complement scg10a, scg10b and kbp mRNAs were generated by linearization of pcCR TOPO II vectors that contained partial ORFs of the genes. SCG10a and SCG10b plasmids were linearized with EcoRV (New England Biolabs) and subsequently transcribed with SP6 polymerase (Promega). KBP plasmid was linearized with Sall (New England Biolabs) and transcribed with T7 polymerase (Promega). Embryos were collected and processed for whole-mount in situ hybridization as previously described (33). Digoxigenin-labeled probes were detected using a standard NBT/BCIP staining reaction.

For immunohistochemistry, FoxD3-GFP transgenic embryos (34,35) were fixed in 4% PFA. To obtain frozen sections, tissues were cryoprotected overnight in a 20% sucrose/PBS solution and placed for 1 h at 37 °C in a 20% sucrose/5% gelatin solution. Afterwards, tissues were rapidly frozen in iso-pentane pre-cooled in liquid nitrogen to −60 °C. Frozen sections were cut at 14 μm, collected on Superfrost Plus microscope slides (BDH Laboratories), air dried and stored at −20 °C. Prior to labeling, sections were placed in warmed PBS (37 °C) for 15 min in order to remove the sucrose/gelatin and then rinsed in PBS (2 × 5 min). Histochemical staining was done using a polyclonal rabbit anti-GFP antibody (Molecular Probes) as previously described (36). Sections were then stained with DAPI (5 min). In order to observe any co-localization of KBP RNA and FoxD3-GFP, bright field in situ images were converted into grayscale, inverted and then inserted into the red channel before being overlaid with FITC images using Adobe Photoshop software.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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