GENETIC INTERACTIONS AND OSMOREGULATORY EFFECTS OF HUMAN DISEASE GENES BLOS4, VPS16B, VPS33B, AND WNK IN DROSOPHILA

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I. INTRODUCTION

The Blos4, Vps16B, Vps33B, and WNK genes are conserved in humans and Drosophila melanogaster and mutation in any one of them is implicated in a known human disease. The objective of studying these genes is to characterize their interactions and effects on osmoregulation to better understand the etiology of the implicated diseases.

BLOS4 takes its name from the protein complex in which it participates (biogenesis of lysosome-related organelle complex, or BLOC-1). It functions in membrane and vesicular trafficking, specifically the formation of organelles associated with the lysosome. Mutation in BLOS4 results in Hermansky-Pudlak Syndrome, an autosomal recessive disorder characterized by albinism, bleeding disorders, and buildup of waxy ceroid in cells due to lysosome abnormalities. The ceroid buildup can cause tissue damage, potentially leading to pulmonary fibrosis which can be fatal.

With-no-lysine Kinase 1 and 4, or WNK1 and WNK4, are vital for electrolyte and blood pressure homeostasis. They regulate the activity of cation chloride cotransporters (CCCs) which either absorb or secrete sodium (Na+) and potassium (K+) ions in the kidney. Mutations in WNK1 or WNK4 lead to Familial Hyperkalemic Hypertension (FHHt), also known as Pseudohypoaldosteronism Type II. This disease is autosomal dominant and therefore the most common of the three considered here and is characterized by elevated levels of sodium and potassium which in turn causes hypertension, hyperkalemia, and metabolic acidosis.
Finally, *VPS16B* and *VPS33B* are named for the group of protein complexes they help form (vacuolar protein sorting-associated protein complexes, or VPS-C). Similar to BLOC-1, these complexes’ primary function is membrane trafficking, more specifically the tethering of vesicles. In *Drosophila*, prior research has shown that *Vps16B* is essential for phagosome maturation, meaning that without Vps16B, flies are immunocompromised and highly susceptible to bacterial infection. Mutation in either gene in humans leads to ARC Syndrome (arthrogryposis-renal dysfunction-cholestat), which is a fatal autosomal recessive disease, with infants diagnosed with the syndrome rarely surviving beyond one year. Symptoms include congenital joint problems, facial dysmorphism, renal tubular dysfunction, acidosis, abnormal platelets, and similar to the observation of flies lacking *Vps16B*, ARC Syndrome patients are highly susceptible to infection.
The idea to study these particular genes came from earlier research suggesting that they genetically interact with *WNK*. *WNK* is vital to homeostasis and osmoregulation, activating CCCs through a cascade in which the activated *WNK* protein phosphorylates SPAK or OSR1 in humans (or FRAY, the homologous protein in *Drosophila*), which in turn phosphorylate CCCs, including the sodium chloride cotransporter (NCC) and sodium-potassium-chloride cotransporters (NKCC1 and NKCC2). The activation of *WNK* itself is determined by chloride levels: low chloride causes *WNK* to auto-phosphorylate and activate, while high chloride levels allow chloride ions to bind and inactivate *WNK*. This is especially critical in the human kidney, where activated NCC in the distal convoluted tubule absorbs sodium and chloride ions, thereby maintaining homeostasis.

**Figure B.** *WNK* is vital to homeostasis and osmoregulation.

As part of ongoing research on this pathway, a genetic screen (carried out in the Rodan lab by Jeff Schellinger and Gaelle Mercenne) identified a number of genes that potentially interact with
WNK. While expression of a gain-of-function (chloride-insensitive) mutant WNK in the nervous system results in lethality, the screen introduced a PiggyBac transposon (a mobile genetic element that inserts and disrupts at random sites along the Drosophila chromosome) and discovered that some flies were able to survive, suggesting that some gene had been disrupted by the PiggyBac to counteract the lethality of over-active WNK. DNA sequencing of the surviving flies identified two of those interacting genes to be Blos4 and Vps33B.

The primary hypothesis in these experiments is that there is physiologically significant genetic interaction between Blos4, Vps16B/Vps33B, and WNK. Since WNK is important for iono- and osmoregulation, we examined whether Vps16B and Vps33B are also important for osmoregulation in Drosophila.

II. METHODS

Gal4/UAS System
This research utilized the Gal4/UAS system, a common but powerful tool in genetics research. This system makes it possible to target a specific gene for over-expression or knockdown in specific cells or tissues. The yeast Gal4 gene codes for a transcription factor that binds and activates the Upstream Activation Sequence or UAS, which then recruits transcription machinery and drives expression of the target gene. This technique was used to drive expression of Blos4, Vps16B, or Vps33B interfering RNA in specific iono- and osmoregulatory epithelia of the fly to achieve tissue-specific knockdown.

![Diagram of Gal4/UAS System](image)

**Figure C.** The Gal4/UAS system makes it possible to target a specific gene for over-expression or knockdown in specific cells or tissues.
**Hindgut-Gal4**

A Gal4/UAS system tailored to specifically increase expression of a target gene in the *Drosophila* hindgut, *Hindgut-Gal4* (sometimes abbreviated HG>) has been proven effective for driving gene expression in the hindgut while excluding the midgut. Unlike humans with dual kidneys, *Drosophila* depend on the Malphigian tubules and hindgut for vital renal functions like ion transport and osmoregulation, with water and ions reabsorbed from the hindgut. This system was used in excretion assays for *Vps16B* and *Vps33B*.

**Figure D.** The *Hindgut-Gal4* system is expressed (green) in the *Drosophila* hindgut using green fluorescent protein.
Excretion Assay
The objective of this assay is to determine the effects of knocking down specific genes on salt tolerance. As in humans, dietary salt (NaCl) is essential for Drosophila but excess is harmful. Consuming extra salt can disrupt ion balance and homeostasis, which leads to increased excretion and even death. In this assay, flies are placed in humidity vials in which they have water only for 6-8 hours, then moved to vials for 16-18 hours containing either a normal diet or +0.3M NaCl. The flies are then placed in empty vials for 2 hours, after which the rates of excretion (visible in the empty vials) are measured and calculated per fly.

Percent Survival Assay
Genetic interactions can be identified and calculated using observed deviation from expected Mendelian outcomes. When a 1:1 ratio is expected between two offspring genotypes but a different ratio is observed it’s possible to calculate the statistical significance and make inferences about the genetic interactions responsible for the observed ratio. This technique was used to test suppression of WNK lethality for Blos4 and Vps33B and antibiotic viability tests for Vps16B.

Figure E. The survival assay uses deviation from expected Mendelian outcomes to identify statistically significant genetic interactions.
Chloride-Insensitive $\textit{WNK}^{\text{L421F}}$

Mutation of the chloride-binding site on WNK at residue 421, from leucine (L) to phenylalanine (F), makes it insensitive to chloride concentrations, which is normally a lethal mutation in \textit{Drosophila} when over-expressed in the nervous system. The over-expression of $\textit{WNK}^{\text{L421F}}$ in the nervous system makes it possible to determine whether another gene interacts with \textit{WNK} by disrupting that gene to see if it suppresses the normal \textit{WNK} lethality. This technique was used in survival assays for \textit{Blos4} and \textit{Vps33B}.

RNA Interference

Interference of ribonucleic acid or RNAi disrupts target mRNA molecules, making it possible to inhibit the expression of a specific gene. By neutralizing the mRNA molecules, translation of the target gene is effectively blocked, making inhibition possible even if the fly has a functional copy of the target gene. This technique was used in excretion assays for \textit{Vps16B} and \textit{Vps33B}.

Antibiotic Survival

As previously mentioned, \textit{Vps16B} flies are highly susceptible to infection and generally have low viability. Prior research has shown that \textit{Vps16B} mutants have underdeveloped phagosomes that make them immunocompromised. This technique involved introducing an cocktail of four antibiotics (ampicillin, erythromycin, kanamycin, and tetracycline) to the flies’ diet to investigate their effect on the viability of the flies.

III. RESULTS

1. Disruption of \textit{Vps16B} or \textit{Vps33B} increases excretion

Experimental results demonstrated that in both male and female flies (Fig. 1 and Fig. 2), disruption of either the \textit{Vps16B} gene or the \textit{Vps33B} gene in the hindgut results in a statistically significant increase in excretion. This confirmed the hypothesis that these genes have an osmoregulatory effect relevant to the \textit{WNK} cascade in the hindgut.
The results were more significant for high salt diets but also held true for females on a normal diet (Fig. 2). Flies with Hindgut-Gal4 and RNAi of the target gene (RNAi / HG>) excreted more than the control flies, which were flies that lacked either the RNAi (HG> / +) or the Hindgut-Gal4 driver (RNAi / +). Similarly, \( Vps33B^{5-20} \) mutants, meaning flies with a germline, loss-of-function mutation in \( Vps33B \), excreted at significantly higher rates than control flies (Fig. 3), which were wildtype (White Berlin, or wB) and \( Vps33B \)-rescue. This was true for males and females and was observed on normal and high salt diets for both.

2. Disrupting Blos4 or Vps33B suppresses the lethal effect of overactive WNK

Survival assays for Blos4 disrupted by RNAi and Vps33B mutants both demonstrated that disruption of either gene reliably suppresses the normal lethality of \( WNK^{L421F} \), confirming the hypothesis that they interact with WNK. In Blos4 RNAi, WNK lethality was suppressed in both
males (39%) and females (84%, Fig. 4). In the case of the germline \( Vps33B^{5-20} \) mutants, the effects were even more pronounced: 34% for males and over 100% for females (Fig. 5).

### Figure 4. RNA interference of Blos4 suppresses the lethality of WNK\(^{1.421}\) mutant

### Figure 5. Heterozygous germline loss-of-function mutation in \( Vps33B \) suppresses the lethality of WNK\(^{1.421}\) mutant

3. Antibiotics increase the viability of \( Vps16B \)

Introducing antibiotics to the fly food caused a statistically significant increase in the survival of flies with \( Vps16B \) knocked down in the hindgut, both for males (Fig. 6) and females (Fig. 7). This result is especially striking given that the concentration of antibiotic used (50 µg/mL per antibiotic) has the opposite effect on control flies. (Other research has concluded that antibiotics can deplete the flies’ gut microbiota, which likely accounts for this observation.)
IV. CONCLUSIONS

In summary, the five major conclusions of these experiments are:

- Hindgut knockdown of \textit{Vps16B} or \textit{Vps33B} using RNA interference in \textit{Drosophila} increases excretion and salt-sensitivity on both high-salt and regular diets.
- Germline loss-of-function mutation of \textit{Vps33B} also increases excretion and salt-sensitivity.
- Disruption of Blos4 suppresses the lethal effect of overactive, chloride-insensitive \textit{WNK}.
- Similarly, loss-of-function mutation in $Vps33B$ suppresses the lethal effect of chloride-insensitive $WNK$.
- Antibiotics increase the viability of flies with $Vps16B$ knocked down in the hindgut at a concentration that otherwise decreases viability in wild-type flies.

These results confirm the original hypothesis that there is physiologically significant genetic interaction between $Blos4$, $Vps16B$, $Vps33B$, and $WNK$, as well as establishing a valuable new laboratory method for increasing the viability of $Vps16B$ flies in future research.
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References:
