Ion Channel Contributions to Wing Development in Drosophila melanogaster

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ABSTRACT

During morphogenesis, cells communicate with each other to shape tissues and organs. Several lines of recent evidence indicate that ion channels play a key role in cellular signaling and tissue morphogenesis. However, little is known about the scope of specific ion-channel types that impinge upon developmental pathways. The Drosophila melanogaster wing is an excellent model in which to address this problem as wing vein patterning is acutely sensitive to changes in developmental pathways. We conducted a screen of 180 ion channels expressed in the wing using loss-of-function mutant and RNAi lines. Here we identify 44 candidates that significantly impacted development of the Drosophila melanogaster wing. Calcium, sodium, potassium, chloride, and ligand-gated cation channels were all identified in our screen, suggesting that a wide variety of ion channel types are important for development. Ion channels belonging to the pickpocket family, the ionotropic receptor family, and the bestrophin family were highly represented among the candidates of our screen. Seven new ion channels with human orthologs that have been implicated in human channelopathies were also identified. Many of the human orthologs of the channels identified in our screen are targets of common general anesthetics, anti-seizure and anti-hypertension drugs, as well as alcohol and nicotine. Our results confirm the importance of ion channels in morphogenesis and identify a number of ion channels that will provide the basis for future studies to understand the role of ion channels in development.

KEYWORDS

Ion channels
channelopathy
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wing
development

Ion channels are well known for their importance in excitable cells such as neurons and muscle cells, but there is also growing evidence that ion channels play a key role in regulating developmental signaling pathways, even in tissues that are non-excitable in adults. Evidence for the importance of ion channels in development can be found in the number of human syndromes associated with morphological defects caused by ion channel mutations. These defects commonly include craniofacial, limb, and digit dysmorphisms. For example, a gain-of-function missense mutation in CACNA1C, a gene encoding an L-type calcium channel, causes Timothy Syndrome (Splawski et al. 2004). Timothy Syndrome is associated with a high incidence of small upper jaw, thin upper lip, low-set ears, syndactyly (fusion of the digits of the hands or feet), and dental defects (Splawski et al. 2004). Similarly, Anderson-Tawil Syndrome, caused by mutations in the gene encoding the inwardly-rectifying potassium channel Kir2.1, leads to syndactyly and clinodactyly (curvature of the fingers or toes) as well as low-set ears, small lower jaw, cleft palate, and dental abnormalities (Plaster et al. 2001). Other channelopathies associated with a high incidence of morphological abnormalities include Temple-Baraitser Syndrome, caused by a gain-of-function mutation in the voltage-gated potassium channel Kir2.1, leads to syndactyly and clinodactyly (curvature of the fingers or toes) as well as low-set ears, small lower jaw, cleft palate, and dental abnormalities (Plaster et al. 2001). Other channelopathies associated with a high incidence of morphological abnormalities include Temple-Baraitser Syndrome, caused by a gain-of-function mutation in the voltage-gated potassium channel EAG1, Birk-Barel Syndrome, caused by a mutation in the two-pore potassium channel KCNK9, and Keppen-Lubinsky syndrome, caused by disruption of the inwardly-rectifying potassium channel GIRK2 (Barel et al. 2008; Chong et al. 2015; Masotti et al. 2015; Simons et al. 2015).

While the importance of ion channels in development is becoming increasingly apparent, the mechanisms by which ion channel mutations disrupt developmental signaling pathways are not fully understood. Ion
channels control the transmembrane potential ($V_{\text{mem}}$) of cells. Cells within an organism have varying resting potentials creating a “bioelectric” pattern across tissues. This pattern is important for proliferation and migration as well as correct left-right patterning, tissue and organ patterning, and organ size (Levin et al. 2017; Levin 2014). Changes in this transmembrane potential pattern result in significant defects in development across multiple organisms. In planarians, changing the $V_{\text{mem}}$ gradient can cause amputated trunks to regrow heads in place of tails, resulting in two-headed organisms (Durant et al. 2017). In Xenopus laevis, clusters of hyperpolarized cells are found at the locations of eyes during embryogenesis (Pai et al. 2012). Depolarization of these cells results in eye malformation while hyperpolarization of non-eye cells can induce the formation of ectopic eyes (Pai et al. 2012). The $V_{\text{mem}}$ pattern has been found to be important within mammalian systems as well, leading to the proposal of a “bioelectric prepattern” dictating the formation of the face (Adams et al. 2016).

In Drosophila melanogaster, ion channels have been found to play a key role in early development. In Drosophila ovarian follicles during oogenesis the $V_{\text{mem}}$ changes by developmental stage (Krüger and Bohrmann 2015; Woodruff et al. 1988). These changes in transmembrane potentials have been found to influence protein movement and distribution in the oocyte (Woodruff et al. 1988; Cole and Woodruff 2000). $V_{\text{mem}}$ patterns were found to correspond with distribution patterns of calcium channels, sodium channels, proton pumps, and gap junctions (Krüger and Bohrmann 2015). When the gap junction Innexin 2 is inhibited during oogenesis, defects in oocyte development occur, further supporting the importance of these ion channels in early Drosophila development (Bohrmann and Zimmermann 2008). Later on in Drosophila development, proper functioning of the inwardly rectifying potassium channel Irk2 has been found to be essential for wing growth and patterning, suggesting that ion channels continue to influence development in Drosophila beyond oogenesis (Dahal et al. 2012; Dahal et al. 2017).

While it is becoming increasingly evident that ion channels are important for development, it is still not fully known which ion channel types contribute to developmental signaling pathways. Drosophila melanogaster is an excellent model in which to address this question because the Drosophila wing is acutely sensitive to changes in developmental pathways. Disruptions of the BMP/Dpp, Notch, Hedgehog, or Wingless/WNT signaling pathways all cause changes in wing development which are easily observed such as abnormal changes in vein patterning and abnormal wing size or shape (Blair 2007). Disruption of the Drosophila ortholog of the Anderson-Tawil Syndrome associated potassium channel Kir2.1 (Irk2), has been previously found to cause severe wing defects, demonstrating that Drosophila wing development is sensitive to ion channel disruptions (Dahal et al. 2017; Dahal et al. 2012). Disruptions of other channels that play roles in development also cause Drosophila wing defects, making the Drosophila wing a useful system in which to identify ion channels that influence morphogenesis.

In this study, we used the Drosophila wing as a readout to screen for ion channels that impact development. We identified 180 ion channel related genes that are expressed in the Drosophila wing disc and then used loss-of-function Drosophila mutant lines or the UAS-GAL4/RNAi system to individually disrupt or knockdown ion channels. We then examined the wing phenotypes of the adult progeny of these lines. Using this approach, we identified 44 ion channel related genes which cause wing development abnormalities when disrupted or knocked down. In the interest of conducting a broad screen, we only looked at one loss-of-function or RNAi knockdown line per ion channel. While deeper interpretation of any of the candidates identified in this screen will require further confirmation of the phenotypes by CRISPR-knockouts, rescue experiments, and other characterizations in lines with differing genetic backgrounds, the results of our screen provide a starting point for further investigation of the role of ion channels in development.

**MATERIALS AND METHODS**

**Fly stocks**

The majority of the Drosophila melanogaster strains used were obtained from the Bloomington Drosophila Stock Center at Indiana University. We selected RNAi lines that were generated by the Transgenic RNAi project, completed in the lab of Norbert Perrimon at Harvard Medical School (Perkins et al. 2015). The irk1, irk2, and irk3 RNAi lines were obtained from the Vienna Drosophila Resource Center (VDRC, www.vdrc.at) (Dietzl et al. 2007). Flies were raised on standard cornmeal food at 25°. The $w^{1118}$ strain was used as the wildtype control and MS1096-GAL4 x $w^{1118}$ as the background control for MS1096-GAL4>UAS-RNAi crosses.

**Identification of ion channel library**

To build a library of ion channels for screening we used the Flybase RNA-seq database (flybase.org) to compile a list of ion channels expressed in Drosophila melanogaster (Gramates et al. 2017). To specifically identify ion channels expressed in the wing we overlaid this list with a library of genes expressed in the wing discs of third instar Drosophila larvae (Ibrahim et al. 2013). We chose to only screen ion channels that had loss-of-function mutant lines or RNAi lines readily available from the Bloomington Drosophila Stock Center or the Vienna Drosophila Resource Center, leaving us with a list of 180 ion channels to screen.

**Fly crosses and wing phenotype scoring**

For screening of UAS-RNAi strains, virgin MS1096-GAL4 females were crossed with males from each UAS-RNAi strain and their progeny were scored for wing phenotypes. MS1096-GAL4 fly wings were examined as controls for all RNAi knock down lines, and for candidates of interest identified using RNAi, the starting UAS-RNAi lines were also screened for wing phenotypes to control for possible background genotype impacts on wing morphology.

If homozygote mutants were viable, they were scored as homozygotes. If homozygotes were not viable, heterozygote mutants were screened directly for wing defects unless the balancer expressed Serate (Ser) or Curly (Cy) which would interfere with identification of wing defects. Mutant strains balanced with Ser or Cy marked chromosomes were crossed with $w^{1118}$ virgin females, and heterozygous progeny not expressing Ser or Cy were selected for scoring.

The wings of at least 20 males and 20 females were scored under a stereo microscope for each mutant strain and UAS-RNAi cross. We looked for abnormalities in vein patterning, vein thickness, trichome or bristle pattern, wing size, wing shape, or other notable changes when compared to controls. If any abnormality was observed, wings were mounted on a slide and further observed under a histology microscope (Nikon, eclipse 80i).

Candidates of interest were defined differently for those identified using loss-of-function mutant lines and those identified using MS1096 > RNAi knockdown. Heterozygous MS1096-GAL4 expressing flies have mild wing venation defects with variable penetration up to 100% for males and a lower penetrance for females (averaging 10.8%). For the MS1096 > RNAi knockdown lines we therefore defined candidates of interest as lines in which female progeny had wing defects.
with a percent penetrance two standard deviations above the mean penetrance of defects in heterozygous MS1096-GAL4 control female flies (at least 29%). We also examined the starting UAS-RNAi lines for wing phenotypes and only identified lines as candidates of interest if the penetrance of phenotypes was at least two standard deviations above both the starting UAS-RNAi line and the MS1096-GAL4 line.

Figure 1 Examples of observed vein and pigment defects
Disruption of 44 of the ion channel related genes screened using either loss-of-function mutations or RNAi wing-specific knockdown resulted in a wide variety of wing defects. Wild type wings have five longitudinal veins and two cross veins (A, left panel). Disruption of the 44 candidates of interest from the screen commonly resulted in abnormal wing pigment (A), posterior cross vein bifurcations (B), incomplete cross veins (C), ectopic veins (D), or longitudinal vein bifurcations (E). Some channel disruptions resulted in wings with multiple venation defects (F). The left column shows wildtype wings with matching wing sections enlarged for comparison with wing defects in right column. Arrows mark defects. The scale bar in the lower right corner represents 500 μm and applies to all panels in the figure.
Less than 1% of w^1118 (WT) have visible wing defects. For mutant lines we therefore defined candidates of interest as lines with a wing defect penetrance greater than 20%. This threshold was set intentionally high for mutant lines even though wildtype flies have very low penetrance of wing defects to reduce the likelihood of including false positives among the candidates of interest.

**Data Availability**
A full list of all RNAi lines screened can be found in Supplementary Table 1 and a full list of loss-of-function mutant lines screened can be found in Supplementary Table 2, with their observed phenotypes and percent penetrance. We provided the stock numbers from the Bloomington Drosophila Stock Center at Indiana University so that the same fly lines may be purchased and our studies can be replicated. Supplemental material available at Figshare: https://doi.org/10.25387/g3.7640345.

**RESULTS AND DISCUSSION**
To identify ion channel genes associated with morphological development, we compiled a library of ion-channel related genes expressed in the Drosophila melanogaster third instar wing disc (Ibrahim et al. 2013). We examined wings of flies that harbor loss-of-function alleles of these ion channels. When mutant alleles did not exist, we drove expression of siRNA against ion channels using MS1096-GAL4. MS1094-GALA drives expression in the dorsal compartment of the wing pouch throughout the third instar larval stage allowing us to specifically assess the impact of knocking down an ion channel in the wing disc during development (Capdevila and Guerrero 1994; Lunde et al. 1998).

A total of 128 loss-of-function mutant lines and 61 UAS-RNAi lines were scored. One fourth of the ion channel screens induce significant wing phenotypes upon loss-of-function or knockdown in the wing. These phenotypes range from mild to severe, with mild defects including abnormalities in bristle patterning or wing pigmentation, incomplete wing veins, bifurcations of the wing veins, and the presence of ectopic abnormalities. In total, 44 unique ion channels that contribute to wing development were identified. The majority of the identified genes (81.8%) have not been previously identified as impacting wing development, and 31 of these genes have human orthologs (Table 3). To further examine the candidates of interest, we divided them into six groups based on ion channel type (calcium, sodium, potassium, chloride, ligand-gated cation channel, and other) (Tables 1 and 2). The majority of the ion channels identified in our screen (29.5%) were ligand-gated cation channels, but channels from several categories were represented (Table 4).

We found a range in penetrance of defects among the candidates of interest in our screen, with some ion channel disruptions (such as Best2 knockdown) resulting in 100% penetrance of wing defects and other channel disruptions giving much lower penetrance of defects. This variability in penetrance could be because increased expression of other ion channels can compensate for reduced function of one ion channel. For example, when irk2 is deleted or knocked down with RNAi, irk1 and irk3 expression increases (Dahal et al. 2012). Each of the ion channels that affected wing morphology was a member of an ion channel family that similarly affects transmembrane potential. It could be that variability of penetrance reflects the differing abilities of ion channels to compensate for other members of the family. Alternatively, the variability in penetrance could be because ion channel disruptions likely impact development by changing the transmembrane potential pattern. Transmembrane potential is regulated by a large number of channels and ions and thus is likely subject to a fairly large amount of biological noise. It has been found that in the nervous system, transmembrane potential often varies due to sources of cellular and molecular noise (Faisal et al. 2008). Transmembrane potential is likely subject to the same noise in non-nervous system tissue, leading to the variability in penetrance that we found in the results of our screen.

**The ppk, IR, and Best families are highly represented among the identified ion channels**
Among the 44 ion channels that contribute to morphogenesis identified in our screen, several belonged to three gene families: the pickpocket family, the ionotropic receptor family, and the bestrophin family. Five of the identified ion channels (rpk, ppk, ppk17, ppk25, and ppk30) belong to the pickpocket family. Pickpocket family genes encode Degenerin/epithelial sodium (Na+) channels (DEG/ENaCs). Totalling 31 members, the pickpocket family is one of the largest families of ion channel genes in Drosophila melanogaster. These channels are non-voltage gated, amiloride-sensitive sodium channels, and some have been characterized as ligand or mechanosensory-gated (Zelle et al. 2013). Their functions are not well understood, but they have been implicated in chemosensory and mechanosensory roles, with some members playing roles in pheromone detection required for proper male courtship behavior (Ben-Shahar 2011; Adams et al. 1998; Lu et al. 2012; Starostina et al. 2012). While possible developmental functions of the pickpocket genes in Drosophila melanogaster have not been previously investigated, many of the pickpocket genes exhibit changing expression patterns throughout early development, supporting the hypothesis that they may play roles in morphogenesis (Zelle et al. 2013). Interestingly, in both Drosophila melanogaster and in mammals, DEG/ENaC channels have been recently implicated in neuronal roles, with some studies...
### Table 1 Summary of candidates identified in screen of RNAi knockdowns

| Stock ID#  | Gene name | Protein Function                  | % Penetrance Male | % Penetrance Female | % Penetrance Total | Phenotype                                              |
|-----------|-----------|-----------------------------------|-------------------|---------------------|--------------------|--------------------------------------------------------|
| Calcium Channels |          |                                   |                   |                     |                    |                                                        |
| BL27263   | Stim      | CRAC channel regulator            | 100               | 100                 | 100                | Wings small and malformed, thick veins, blisters       |
| BL31295   | nan       | Transient receptor potential channel | 84                | 32                  | 54                 | L5 incomplete, L5 bifurcation                          |
| BL31292   | wtw       | Transient receptor potential channel | 69                | 39                  | 53                 | PCV incomplete                                         |
| Sodium Channels |       |                                   |                   |                     |                    |                                                        |
| BL25847   | rpk       | DEG/epithial sodium channel        | 100               | 85                  | 92                 | L5 incomplete or bifurcation, L4 bifurcation           |
| BL27088   | ppp25     | DEG/epithial sodium channel        | 22                | 37                  | 30                 | PCV bifurcation, L5 bifurcation                        |
| BL25810   | ppp30     | DEG/epithial sodium channel        | 73                | 30                  | 55                 | PCV incomplete, L4 & L5 bifurcations, L5 incomplete,    |
| Potassium Channels |      |                                   |                   |                     |                    |                                                        |
| VDRC 28430| Irk1      | Inwardly rectifying K+ channel     | 100               | 91                  | 95                 | L5 & L4 bifurcations, loss of ACV, thick veins         |
| VDRC 4341 | Irk2      | Inwardly rectifying K+ channel     | 93                | 85                  | 89                 | L5 & L4 bifurcations, loss of ACV, thick veins         |
| VDRC 3886 | Irk3      | Inwardly rectifying K+ channel     | 100               | 30                  | 47                 | L5 & L4 bifurcations, loss of ACV, thick veins         |
| Chloride Channels |     |                                   |                   |                     |                    |                                                        |
| BL42654   | Best2     | Calcium activated chloride channel | 100               | 100                 | 100                | Wings small and severely malformed                     |
| BL39040   | Best3     | Chloride channel                  | 100               | 40                  | 66                 | Small narrow wings (male), missing ACV, L2 bifurcation, PCV incomplete |
| Ligand-gated Cation Channels |       |                                   |                   |                     |                    |                                                        |
| BL62391   | Ir7b      | Ionotropic receptor               | 86                | 97                  | 92                 | PCV incomplete or bifurcation, L5 bifurcation          |
| BL34678   | Ir76a     | Ionotropic receptor               | 97                | 46                  | 74                 | PCV incomplete, L5 bifurcation                         |
| BL53975   | Ir94h     | Ionotropic receptor               | 24                | 29                  | 27                 | Bristle defects                                        |
| Other     | Inx3      | Gap junction channel              | 98                | 38                  | 68                 | PCV incomplete, L4 bifurcation, L5 bifurcation, L5 incomplete |

At least 20 female and 20 male flies were scored for each line.
BL, Bloomington Drosophila Stock Center number, VDRC, Vienna Drosophila Resource Center number. PCV, posterior cross vein, ACV, anterior cross vein, L, longitudinal vein.
*Function predicted by sequence similarity.
Table 2 Summary of candidates identified in screen of loss-of-function mutant lines

| Stock ID# | Gene Name | Protein Function | % Penetrance Male | % Penetrance Female | % Penetrance Total | Phenotype |
|-----------|-----------|------------------|-------------------|---------------------|-------------------|-----------|
| BL14156   | sj        | Voltage-gated Ca\(^{2+}\) channel | 48                | 60                  | 54                | Bristle defects |
| BL13682   | SERCA     | Calcium-transporting ATPase | 67                | 76                  | 40                | Ectopic veins, ectopic bristles, pigment defect |
| BL38067   | bn2       | Calcium channel activity | 20                | 34                  | 28                | PCV bifurcation |
| BL19967   | m\(a\)-A; B; C | Calcium channel regulator | 15                | 50                  | 31                | PCV bifurcation |
| BL38075   | ppk       | DEG/epithelial sodium channel | 42                | 52                  | 47                | PCV bifurcation |
| BL58557   | ppk17     | DEG/epithelial sodium channel | 18                | 31                  | 25                | PCV bifurcation |
| BL37430   | NaCP60E   | Voltage gated sodium channel | 81                | 100                 | 90                | PCV bifurcation, pigment abnormality |
| BL74      | na        | Sodium leak channel complex component | 37                | 5                   | 22                | Bristle defects, ectopic vein |
| BL42469   | unc80     | Sodium leak channel complex component | 62                | 24                  | 43                | PCV bifurcation, ectopic vein, pigment defect |
| BL23397   | unc79     | Sodium leak channel complex regulator | 9                 | 36                  | 23                | PCV bifurcation |
| BL13221   | Teh1      | Sodium channel regulator | 70                | 91                  | 81                | Black spots below L5 |
| BL59167   | Task6     | Two-pore domain potassium channel | 100               | 100                 | 100               | PCV bifurcation |
| BL59589   | SLO2      | Calcium activated potassium channel | 6                 | 37                  | 23                | PCV bifurcation |
| BL22837   | Shaker    | Voltage-gated potassium channel | 21                | 85                  | 54                | PCV bifurcation |
| BL37284   | KCNQ      | Voltage-gated potassium channel | 20                | 51                  | 29                | PCV bifurcation |
| BL6879    | Best1     | Calcium activated chloride channel | 70                | 100                 | 85                | PCV bifurcation |
| BL1687    | Rdl       | GABA-gated chloride channel | 92                | 95                  | 94                | L2 incomplete, ectopic bristles, pigment defect |
| BL6353    | GluCl\(\alpha\) | Glutamate-gated chloride channel | 60                | 100                 | 80                | Bristle defects |
| BL44812   | Or47a     | Olfactory receptor | 32                | 51                  | 42                | PCV bifurcation |
| BL56583   | Ir\(67\)a | ionotropic receptor | 80                | 75                  | 77                | PCV bifurcation, L3 bifurcation, thick veins |
| BL31033   | Ir\(84\)a | Ionotropic receptor | 50                | 34                  | 34                | PCV bifurcation, thick veins |
| BL43017   | Ir\(92\)a | Ionotropic receptor | 20                | 23                  | 22                | Bristle defects, abnormal vein pigment |
| BL25551   | Ir\(94\)g | Ionotropic receptor | 30                | 8.6                 | 20                | Bristle defects, abnormal vein pigment |
| BL37066   | GluRIIB   | Non-NMDA ionotropic glutamate receptor | 31                | 56                  | 44                | PCV bifurcation |
| BL59216   | mACHR-A   | G-protein coupled acetylcholine receptor | 21                | 56                  | 38                | PCV bifurcation, L4 incomplete |
| BL24880   | nACHRa7   | Nicotinic acetylcholine receptor | 42                | 67                  | 52                | PCV bifurcation |
| BL20783   | nACHRa6   | Nicotinic acetylcholine receptor | 26                | 32                  | 29                | PCV bifurcation |
| BL41424   | nACHRa5   | Nicotinic acetylcholine receptor | 15                | 43                  | 26                | PCV bifurcation |
| BL59187   | CG18549   | Ion channel regulatory protein | 69                | 97                  | 83                | PCV bifurcation |

At least 20 female and 20 male flies were scored for each line.
BL, Bloomington Drosophila Stock Center number.
PCV, posterior cross vein, L, longitudinal vein.
*Function predicted by sequence similarity.
suggestions that they may directly modulate synaptic processes (Hill and Ben-Shahar 2018; Younger et al. 2013). Our results suggest that some members of the pickpocket families may play roles in developmental signaling, further expanding the diverse functions of this family.

Another gene family highly represented in our screen is the ionotropic Receptor family. Seven of the candidates of interest (Ir7b, Ir67a, Ir76a, Ir84a, Ir92a, Ir94g, and Ir94h) belong to the ionotropic Receptor family, including three (Ir76a, Ir84a, Ir92a) belonging to the Antennal Ionotrope Receptor subfamily and four (Ir7b, Ir67a, Ir94g, Ir94h) belonging to the Divergent Ionotrope Receptor subfamily. Ionotropic Receptor family members are similar in sequence to ionotropically gated receptors (iGluRs), but they lack glutamate-interacting residues and are thus thought to be non-responsive to glutamate (Benton et al. 2009). These channels are ligand-gated and primarily thought to play chemosensory roles in taste and odor reception (Rytz et al. 2013). The Antennal Ionotropic Receptors are mostly expressed in the antennae and are thought to play roles in odor reception while the Divergent Ionotropic Receptors are expressed in gustatory neurons and play roles in taste (Rimal and Lee 2018). These receptors are expressed at low levels during development and in the developing wing disc, and our results suggest that they play roles in morphogenesis of the wing in addition to their chemosensory roles.

Three members of the Bestrophin family, Best1, Best2, and Best3, were found to contribute to wing morphogenesis. Bestrophins are non-voltage gated chloride channels. Interestingly, disruption of Best2 resulted in the most severe wing defects of all of our candidates of interest. Wing-specific Best2 RNAi expression (using the MS1096-GAL4 driver) caused the wings to be completely shriveled and malformed (Figure 2D). There is evidence that Best2 may be a calcium activated chloride channel (CaC). Best1 may be both a CaC and a volume regulated anion channel (VRAC) (Chien et al. 2006; Chien and Hartzell 2007). Our results indicate that the Bestrophins play a key role in Drosophila wing development suggesting that the chloride current is important for correct morphogenesis. Indeed, five chloride channels were identified in our screen (Table 3).

### Multiple genes identified have human orthologs associated with morphological defects

We found several of the ion channels that impact Drosophila wing development have human orthologs with mutations that are associated with morphological defects. Three of the ion channels from our screen, Irk1, Irk2, and Irk3 are the Drosophila orthologs of Kir2.1, which is a channel associated with Andersen-Tawil syndrome (Tristani-Firouzi et al. 2002; Yoon et al. 2006b; Yoon et al. 2006a). We have previously described the effects of Irk/Kir2.1 disruption on fly and mouse development (Dahal et al. 2012; Dahal et al. 2017; Belus et al. 2018). Here we identify seven additional ion channels that have human orthologs that are associated with morphological defects as part of channelopathies in humans (Table 5). These include Task6 (KCNK9), Nan (TRPV4), unc80 (UNC80), narrow abdomen (NALCN), and the nicotinic acetylcholine receptors nAChRα5, nAChRα6, and nAChRα7 (CHRNA7). Interestingly, the genetic lesions that cause the channelopathies associated with these genes in humans are all loss-of-function mutations (Table 5). The Drosophila lines scored in our screen are also loss-of-function or knock-down lines, but it is important to note that human channelopathies usually occur as a result of heterozygous mutations while the majority of the Drosophila lines we looked at were homozygous, representing a more severe reduction in ion channel function.

Twik related acid-sensitive K+ channel 6 (Task6) encodes a two-pore-domain potassium channel and is the Drosophila ortholog of the human KCNK9 gene. Heterozygous KCNK9 loss-of-function mutations in humans cause Birk-Barel syndrome, a channelopathy associated with craniofacial defects including elongated face, downturned eyelids, protruding ears, and cleft palate (Barel et al. 2008).

Another channel identified in our screen, Nanchung (Nan), a transient receptor potential channel, is the Drosophila ortholog of TRPV4. Both loss-of-function and gain-of-function heterozygous TRPV4 mutations are associated with high number of skeletal dysplasia disorders.

### Table 3 Human orthologs of ion channel candidates identified in screen

| Drosophila melanogaster Gene | Human Ortholog |
|------------------------------|----------------|
| Calcium Channels             |                |
| stj                          | CACNA2D3       |
| SERCA                       | ATP2A1         |
| brv2                        | PKD1L2         |
| Stim                        | STIM1          |
| nan                         | TRPV6          |
| inaF-A;B;C                  | none           |
| wtrw                        | none           |
| Sodium Channels             |                |
| NaCP60E                     | SCN8A          |
| narrow abdomen              | NALCN          |
| unc80                       | UNC80          |
| unc79                       | UNC79          |
| rpk                         | ASIC2          |
| ppk                         | ASIC2          |
| ppk25                       | ASIC4          |
| ppk30                       | ASIC3          |
| ppk17                       | none           |
| Teh1                        | none           |
| Potassium Channels          |                |
| Task6                       | KCNK9          |
| SLO2                        | KCNT1          |
| Shaker                      | KCNA1          |
| KCNQ                        | KCNQ4          |
| Irk1                        | KCNJ2          |
| Irk2                        | KCNJ2          |
| Irk3                        | KCNJ2          |
| Chloride Channels           |                |
| Best1                       | BEST2          |
| Best2                       | BEST4          |
| Best3                       | BEST4          |
| Rdl                         | GLRA4          |
| GluCl                       | GLRA1          |
| Ligand-gated Cation Channels|                |
| GluRIB                      | GRIK1          |
| mAChR-A                     | CHRM1          |
| nAChRα7                     | CHRNA7         |
| nAChRα6                     | CHRNA7         |
| nAChRα5                     | CHRNA7         |
| Or47a                       | none           |
| Ir7b                        | none           |
| Ir67a                       | none           |
| Ir76a                       | none           |
| Ir84a                       | none           |
| Ir92a                       | none           |
| Ir94g                       | none           |
| Ir94h                       | none           |
| Other                       |                |
| CG18549                     | MFSK11         |
| lnx3                        | none           |

*Human orthologs were identified using the DRSC Integrative Ortholog Prediction Tool (Version 7.1) (Hu et al. 2011). Only human orthologs with a DIOPT score > 2 are shown.*
Table 4 Number of candidates identified for each ion channel type

| Ion Channel Type | Number of Candidates | Percentage of Total Candidates |
|------------------|----------------------|-------------------------------|
| Ligand-gated cation | 13 | 29.5% |
| Sodium | 10 | 22.7% |
| Calcium | 7 | 15.9% |
| Potassium | 7 | 15.9% |
| Chloride | 5 | 11.4% |
| Other | 2 | 4.5% |

that cause skeletal defects such as scoliosis and brachydactyly (shortening of the fingers) (Nilius & Voets 2013).

We found that wing morphogenesis was also affected by the reduced function of unc80 and narrow abdomen, the Drosophila orthologs of UNC80 and NALCN, respectively. Together with UNC79, these proteins form a cation channel complex (Lu et al. 2010). Loss-of-function homozygous mutations in NALCN cause infantile hypotonia with psychomotor retardation and characteristic facies-1 (IHPF1) and loss-of-function homozygous mutations in UNC80 cause infantile hypotonia with psychomotor retardation and characteristic facies-2 (IHPF2) (Bramswig et al. 2018; Stray-Pedersen et al. 2016; Al-Sayed et al. 2013). These are two closely related channelopathies associated with mild dysmorphic facial features (Bramswig et al. 2018; Stray-Pedersen et al. 2016; Al-Sayed et al. 2013). Some heterozygous mutations in NALCN, speculated to be dominate-negative mutations, cause congenital contractures of the limbs and face, hypotonia, and development delay (CLIFAHDD) (Chong et al. 2015). CLIFAHDD is a congenital disorder associated with severe craniofacial defects and limb deformities (Chong et al. 2015). In our screen, homozygous loss-of-function mutations in the Drosophila orthologs unc80 and narrow abdomen both caused wing defects, indicating that these two proteins may play conserved roles in morphogenesis.

Disrupted function of the nicotinic acetylcholine receptors nAChR5, nAChR6, and nAChR7 were also identified in our screen. These three nicotinic acetylcholine receptors are the Drosophila orthologs for the human α7 nicotinic acetylcholine receptor (encoded by CHRNA7). A 15q13.3 microdeletion syndrome, in which CHRNA7 and five other genes are deleted, causes facial and digital dysmorphisms (Sharp et al. 2008). Single-gene deletions of CHRNA7 also cause 15q13.3 microdeletion syndrome phenotypes, suggesting that deletion of CHRNA7 is the cause of the syndrome (Hoppman-Chaney et al. 2013). Our screen identified all three of the Drosophila orthologs of CHRNA7 indicating that this nicotinic acetylcholine receptor likely plays a conserved role in development.

**Ion channel compensation effects**

While we identified 44 ion channels in our screen, it is likely that our results underestimate the true scope of ion channels involved in wing development. Ion channels are often made up of multiple subunits or have multiple family members that are able to compensate for each other when a single channel is disrupted or deleted. In both developmental and non-developmental contexts (such as in cardiac cells) disruption of a single ion channel can cause upregulation of different ion channels to compensate, masking potential phenotypes (Dahal et al. 2012; Rosati and McKinnon 2004). This impact of compensation may be more significant for ion channels that come from large families with many members that could potentially compensate for the loss of one member. It is interesting to note that in the results from our screen, ion channels identified from large families such as the pickpocket family...
(with 31 members) gave more subtle phenotypes than those from smaller families such as the Bestrophin family (with only four members). This may be a result of ion channel compensation, with other ion channel family members being able to perform the function of the disrupted channels to prevent more severe defects from occurring.

Potential impacts
To confirm the results of the channels in our screen, more experiments will have to be done using rescues and disruptions in other background phenotypes. However, if conserved developmental roles are found for the channels identified in our screen, this would have important implications in human health as ion channels are one of the top targets of known drugs (Overington et al. 2006). We used the drug–gene interaction database (DGIdb, www.dgidb.org) to look for known drugs that act upon the human orthologs of the ion channels identified in our screen (Cotto et al. 2018). We found that many of the human orthologs of the ion channels that we identified interact with common general anesthetics such as halothane, sevoflurane, isoflurane, and desflurane. Other ion channels that impact wing morphogenesis in flies interact with anti-hypertension drugs such as amiloride, nilvadipine, verapamil, mibebradil. Another subset of ion channels that we found to impact morphogenesis interact with anti-seizure drugs such as topiramate, phencemide, ezogabine, zonisamide. If the ion channels identified in our screen have conserved roles in morphogenesis, the use of drugs like these during pregnancy needs to be examined closely. In addition, alcohol is known to act upon Kir channels, human orthologs of Irk1, Irk2, and Irk3, which were identified as modifiers of wing development (Dahal et al. 2012; Bates 2013). Furthermore, nicotine acts upon nico
tinic acetylcholine receptors, three of which were identified as modifiers of development in our screen (nAChRα5, nAChRα6, and nAChRα7). Our results may help to explain the known effects of maternal smoking on fetal development (Hackshaw et al. 2011).

Conclusion
Overall, our screen identified 44 ion channels that impact morphogenesis of the Drosophila melanogaster wing, underscoring the overall importance of ion channels in development. It will be interesting to investigate which specific morphogenic pathways are impacted by the disruption of these channels and the mechanisms by which these ion channels impinge upon these pathways.

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