

Research Article

Driver Mutations in Major Lung Cancer Oncogenes Can Be Analyzed in *Drosophila* Models

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Abstract

Lung cancer remains the leading cause of cancer-associated mortality. Despite recent promising achievements, the overall prognosis remains very poor. In order to integrate the advantages of adapted, transgenic animal models with a high-throughput procedure on the one hand and compliance with the 3R principles on the other hand, we have established and evaluated appropriate *Drosophila* models. To achieve this goal, we ectopically expressed oncogenes representing the most important driver mutations exclusively in the airway system. These oncogenes were either the human oncogenes or the corresponding *Drosophila* orthologs. We concentrated on two complementary read-out systems, 1) early larval lethality and 2) quantification of concurrently expressed GFP as a proxy for tumor mass. We could show that ectopic expression of *Egfr^{CA}*, *Ras^{V12}*, *Raf*, *Rolled (MAPK)*, *PI3K92E*, *Alk*, *Akt* and *Arm* can induce early lethality. Thus, they can be used in a straight-forward high-throughput screening approach and can replace mouse models to a considerable extent. Moreover, we could also show that measurement of tumor mass by a concurrently expressed marker (GFP) can be used to detect positive treatment results. Our results show that our *Drosophila* system provides a superb *in vivo* invertebrate screening system amenable to high-throughput approaches and thus effectively complements the toolbox for the development of novel anti-lung cancer treatments, while complying with the 3R principles.

1 Introduction

Lung cancer is still the leading cause of cancer induced deaths, with annually more than a million victims worldwide (Stewart and Wild, 2014). Most lung cancer cases are non-small cell lung cancers (NSCLC) that are characterized by extremely poor prognoses (Stewart and Wild, 2014; Travis et al., 2015). The development of NSCLCs most often depends on oncogenic driver mutations found in a relatively small number of genes (Luo and Lam, 2013; Ding et al., 2008; Kandoth et al., 2013; Rotow and Bivona, 2017). This oncogenic network comprises receptors and signaling systems that are involved in the control of the proliferative activity of cells. Consequently, growth factor receptors such as the EGFR as well as downstream components of the signaling cascades involved such as *Ras*, *Raf* or *Erk* are major oncogenes that underlie a great number of lung cancer cases.

Based on this finding, drugs that specifically target the corresponding gene products or associated signaling pathways are currently used as first- and/or second-line therapies. Although these drugs are tailored to the underlying driver mutations, their effectiveness is currently limited due to development of resistance, inefficient delivery or toxicity (Kandoth et al., 2013; Rosell et al., 2012). Therefore, novel compounds or combinations of compounds that specifically target these driver mutations or downstream signaling systems are urgently needed as effective therapeutic options.

Animal models of these different lung cancer subtypes, especially tailored transgenic mouse models, are currently an integral part of corresponding research pipelines. Currently, these mouse models are produced in different ways. Here, both the transfer of oncogenes with, e.g., adenoviruses, but also the use of inducible expression systems that limit oncogene expression to the target

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cells are of particular interest. In these models, the induction of oncogene expression can be time-controlled. These models are complemented by xenografts, in which tumors or metastases are directly transferred into susceptible mouse strains. Common to all these systems is that the tumors have to develop to reach a state comparable to that of a lung cancer patient. This is generally associated with high stress for the affected animals. Due to the development of the tumors, these burdens remain active over a very long period of time (Kwon and Berns, 2013).

In order to comply with the 3R principles and to reduce the consumption and harm to these animals, alternative systems are highly appreciated (Olsson et al., 2012). This is especially relevant as most 3R efforts have focused on toxicological topics rather than on using alternative transgenic systems based on invertebrate models such as *Drosophila melanogaster* or *Caenorhabditis elegans* (Kretlow et al., 2010). A highly promising strategy uses tailored invertebrate models to establish large-scale whole-animal screens that enable selection of effective and non-toxic compounds. Only very few invertebrate systems are principally suited for this complex task, with *Drosophila* being the most promising one. Recently, different *Drosophila*-based models were introduced to study chronic lung diseases such as asthma and COPD (chronic obstructive pulmonary disease) (Kallsen et al., 2015; Roeder et al., 2009, 2012). The inherent logic that underlies the use of *Drosophila* as a model for human lung diseases is the high degree of commonality regarding architecture, physiology and development (Roeder et al., 2012; Behr, 2010; Ruehle, 1932; Whitten, 1957; Ghabrial et al., 2003; Andrew and Ewald, 2010).

Drosophila has been used as a model for a great variety of different human diseases including various types of cancer. In addition, *Drosophila* has been very successfully used to establish high-throughput screening formats that have led to the development of new therapeutic strategies for a number of different cancer types (Bang et al., 2019, 2016; Das and Cagan, 2010; Markstein et al., 2014). This type of screening using tailored *Drosophila* methods not only shortcuts research pipelines, it also fully complies with the 3R principles by leading to a reduction in vertebrate animal testing (Kretlow et al., 2010).

Recently, the first models for human lung cancer based on tailored *Drosophila* have been implemented and used to identify alternative treatment strategies. Levine and Cagan focused on the *Ras* oncogene and developed a screening system based on ectopic overexpression of constitutively active *Ras* concurrently with a knockdown of the tumor suppressor *Pten* in the airway system to screen for compounds that rescue this lethal phenotype (Levine and Cagan, 2016). We have described a similar approach for constitutively active EGFR, which is also an oncogene underlying lung cancer development (Bossen et al., 2019). In both experiments, large-scale screening approaches led to the identification of novel combination treatments that might help to fight lung cancer in the future using an experimental system that complies with the 3Rs.

Here, we explore the complex oncogene network known to underlie most cases of human lung cancer and exploit whole animal screening approaches. By using tailored *Drosophila* mod-

els representing the most important oncogenic driver mutations, we developed a series of personalized lung cancer models. Ectopic overexpression in the *Drosophila* airway system of most of these oncogenes induced substantial structural changes including epithelial meta- and hyperplasia. We established two complementary read-out systems based on either early lethality or tumor mass that proved to be suitable for evaluating the effectiveness of test substances in large-scale screening approaches. For both read-out indicators, we sketch technical concepts for automated measurements. These concepts are both based on *in-situ* contactless optical technology; hence they are non-consumptive and repeatable along larval development. With these *Drosophila* models, novel tools for the development of personalized lung cancer treatments are at hand that fully comply with the 3R principles.

2 Animals, materials and methods

Fly strains and husbandry

Fly stocks were raised on cornmeal (CM) agar medium. The Gal4/UAS system (Brand and Perrimon, 1993) was used to ectopically overexpress oncogenes specifically in the airway epithelium. Three different trachea-specific driver lines were utilized to trigger expression of the corresponding genes. The crosses and experiments were carried out at 25°C, 60–70% humidity and 12 h/12 h light/dark cycle. The performance of the Gal4/UAS system (btl-Gal4) was improved by using elevated temperatures (30°C). Temperature-sensitive experiments employing temperature-sensitive expression control systems (btl-Gal4; Gal80ts) were incubated at 18°C until hatching and development of the first larval stages and switched to 29°C for induction of target gene expression. Transgenic fly strains used for crosses: control line *w¹¹¹⁸* (BDSC_5905), tracheal driver lines for trachea *ppk4-Gal4* (Wagner et al., 2008, 2009) and *btl-Gal4* (Maria Leptin lab, Heidelberg, Germany), *btl-Gal4*; *tubP-Gal80ts*, *DSRF-Gal4* (Gervais and Casanova, 2011), *UAS-Egfr^{CA}* (BDSC_9533), *UAS-hEGFR-Pvr* (BDSC_58432), *UAS-hEGFR-dEgfr* (BDSC_58415), *UAS-Pvr* (BDSC_8428), *UAS-Apc-RNAi* (BDSC_34869), *UAS-InR* (BDSC_8250), *UAS-htl* (BDSC_5419), *UAS-btl* (BDSC_29045), *UAS-RI* (BDSC_36270), *UAS-Pi3K92E* (BDSC_8294), *UAS-Akt* (BDSC_8192, 50758), *UAS-Arm* (BDSC_4782), *UAS-Cdk4* (BDSC_6631), *UAS-p53* (BDSC_8422), *UAS-Thor* (BDSC_9147), *UAS-Myc* (BDSC_9675), *UAS-RPTK:Alk* (Ruth Palmer lab, Gothenburg, Sweden), *UAS-Ras* (BDSC_4847), *UAS-Ras(V12)* (Jose C. Pastor-Pareja lab, Beijing, China), *UAS-Raf* (BDSC_2033), *UAS-hRAF^{GOF}* (Markstein lab, Amherst, USA).

Drug treatment

Trametinib (Biomol, Hamburg, Germany), fluvastatin (Enzo, Lörrach, Germany), afatinib (Enzo, Lörrach, Germany), tepipenem pivoxil (Selleckchem, Biozol, Eching, Germany) and cladribine (Selleckchem, Biozol, Eching, Germany) were pipetted directly onto low-melt CM medium (5% (w/v) yeast extract (Becton Dickinson; Heidelberg, Germany), 5% (w/v) sucrose, 8.6% cornmeal, 0.5% (w/v) low-melt agarose, 0.1% (v/v) propionic

acid, 0.3% (v/v) methyl-4-hydroxybenzoate) to reach a final concentration of 100 μ M. DMSO (0.1%) and ethanol (0.9%) served as a negative control. Different concentrations were used for trametinib. The collected larvae (developed at 18°C) were deposited on low-melt CM medium and incubated at 29°C until use (late 3rd instar) for microscopy and GFP quantification.

Measurement of terminal cell branching

3rd instar larvae were deposited in a drop of glycerol on a slide. The larvae were exposed to 70°C for 20 s until death and arranged for microscopy. The pictures of the terminal cells were taken in the GFP channel (10x). To capture all branches of the terminal cell, a Z-stack projection was performed. The right terminal cell from the 2nd dorsal segment was chosen. GFP was quantified in the ImageJ plugin NeuronJ (Meijering et al., 2004) after identical contrast adjustment (1.550 pixels/ μ m).

Quantification of epithelial thickening and nuclei

The tracheae were dissected from 3rd instar larvae. The tracheae were placed on a drop of mounting mixture (50% ibidi mounting medium (ibidi, Germany) and 50% ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher Sci)), set on a slide and covered with a coverslip. The tracheal area at the seventh abdominal segment was selected for microscopy. The thickness of the epithelial layer of the dorsal trunk as well as of the primary and secondary branches was measured using the SZX16 fluorescence-stereomicroscope and the cellSens Imaging Software for Life Science Microscopy (Olympus, Germany). Quantification of nuclei was performed at the same tracheal segment (A7). The numbers of nuclei were counted on a 500 μ m section of the dorsal trunk, on a 200 μ m section of the secondary branch, and on a 400 μ m section of the secondary branch. For visualization of the nuclei, the SZX2-FUV filter and the X-Cite 120 Iris fluorescent lamp were used. Data were collected from three points per tracheal area from at least ten larvae.

GFP quantification

The GFP quantification was performed with 3rd instar larvae. Animals were incubated at 18°C for egg laying and development of the early larval stages and switched to 29°C for induction of target gene expression. For the use of 3rd instar larvae, the crossings were incubated at 29°C for three days before quantification. Treatment with drugs was done for two days before measurement. Three larvae per replicate were collected and homogenized in 200 μ L phosphate-buffered saline. The homogenate was immediately centrifuged and 100 μ L of the supernatant was used for measurement in a black 96-well plate. The fluorescence intensity was measured in a microplate reader (Synergy H1, BioTek Instruments) at 480 nm excitation and 510 nm emission wavelengths. Genotypes: *btl-Gal4*; *tubP-Gal80ts*, *UAS-GFP* > *UAS-Ras^{V12}* = *Ras^{V12}*; *btl-Gal4*; *tubP-Gal80ts*, *UAS-GFP* = control.

Measurement of hypoxia sensitivity

Twenty 3rd instar larvae were collected into a new tube per replicate. After all larvae were burrowed in the food, they were de-

posited in a desiccator for hypoxia treatment. Nitrogen was introduced until an O₂ level of 2-3% was achieved. The larvae were treated over a period of 25 min. The escaped larvae were counted every 5 min.

Statistics

Statistical analyses were performed with GraphPad Prism 7. Statistical significance was evaluated by Mann-Whitney test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3 Results

A complex oncogene network underlies the development of various forms of lung cancer (Ding et al., 2008; Sharma et al., 2007; Siegelin and Borczuk, 2014). We used appropriate *Drosophila* lines to determine whether ectopic activation of these oncogenes can induce cancer-like phenotypes in the airways and if these phenotypes can be quantified to develop valuable screening systems (Fig. 1A).

For this purpose, we induced ectopic expression of the corresponding genes exclusively in defined parts of the airway epithelium of *Drosophila* larvae using different airway-specific Gal4 driver lines (Roeder et al., 2009; Kallsen et al., 2015; Wagner et al., 2009) (Fig. 1B-D). We chose three different Gal4 lines that direct expression to different parts of the tracheal system, *btl-Gal4* (Fig. 1B), which induces expression in the entire airway system, *ppk4-Gal4* (Fig. 1C), which targets expression to the entire airway system except the so-called terminal cells, and the *DSRF-Gal4* driver (Fig. 1D), which specifically targets these terminal cells.

We first used the most specific driver line and induced expression of one of the three oncogenes *Egfr^{CA}*, *Ras^{V12}*, and *Sem* exclusively in terminal cells (Fig. 2). *Egfr^{CA}* expression in terminal cells showed, in comparison to controls (Fig. 2A,B), only minor effects on the number of terminal branches as well as on their total lengths (Fig. 2E,F). On the other hand, targeted expression of *Ras^{V12}* led to a strong increase in the number of branches as well as their total length (Fig. 2C,E,F). *Sem* overexpression in these terminal cells induced an intermediate phenotype with significantly higher numbers of branches and greater total length (Fig. 2D-F). The branching phenotype of the terminal cells with *Ras^{V12}* expression was accompanied with enlarged nuclei (Fig. 2G,H). These structural changes had a physiological equivalent, observed as changes of hypoxia sensitivity. In a classical substrate-leaving response, animals experiencing *Ras^{V12}* overexpression in terminal cells showed a higher hypoxia sensitivity compared to the controls (Fig. 2I). Although the observed phenotypical variations were substantial, this approach was not suited to be transformed into a high-throughput screening system as the induced changes were neither lethal nor were the changes in the overall fluorescence signal large enough to be accessible to quantification.

All other experiments were performed with driver lines that target expression into all airway cells (*btl-Gal4*) or to all cells except the terminal ones (*ppk4-Gal4*). We tested the entire set of oncogenes available to evaluate whether the induced pheno-

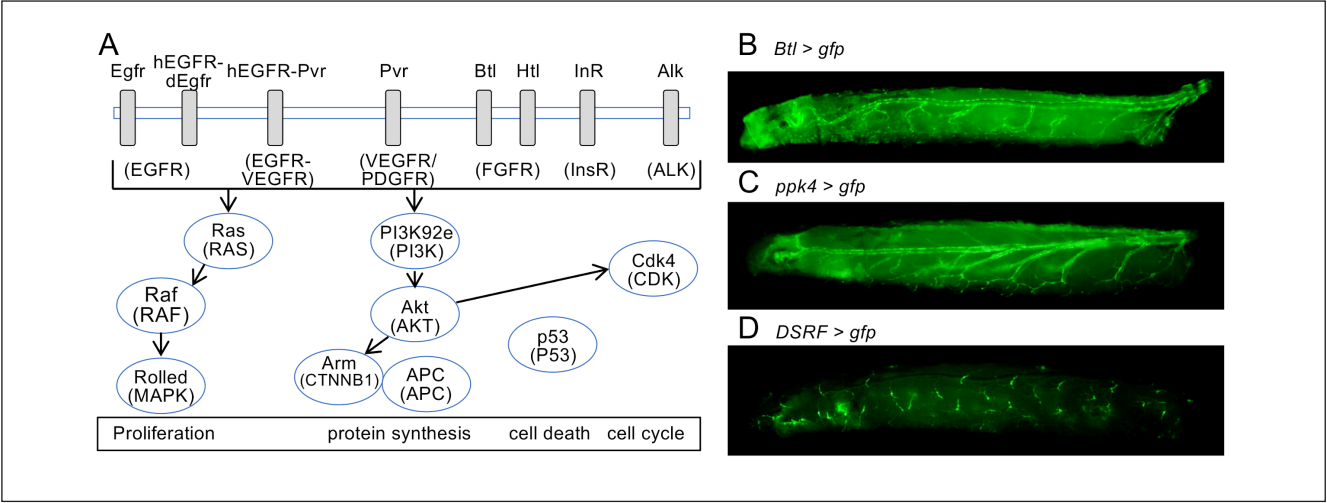


Fig. 1: Targeted expression of oncogenes in different parts of the tracheal system to study their relevance in the airway system (A) Schematic description of oncogenes used in this study (top of each icon) as well as the corresponding human orthologs (bottom of each icon; graph was modified according to Ding et al., 2008). (B-D) In this study, three different Gal4 driver lines were used that target expression to the entire airway system (B, *btl-Gal4*), to the airway system except the terminal cells (C, *ppk4-Gal4*), and to the terminal cells (D, *DSRF-Gal4*).

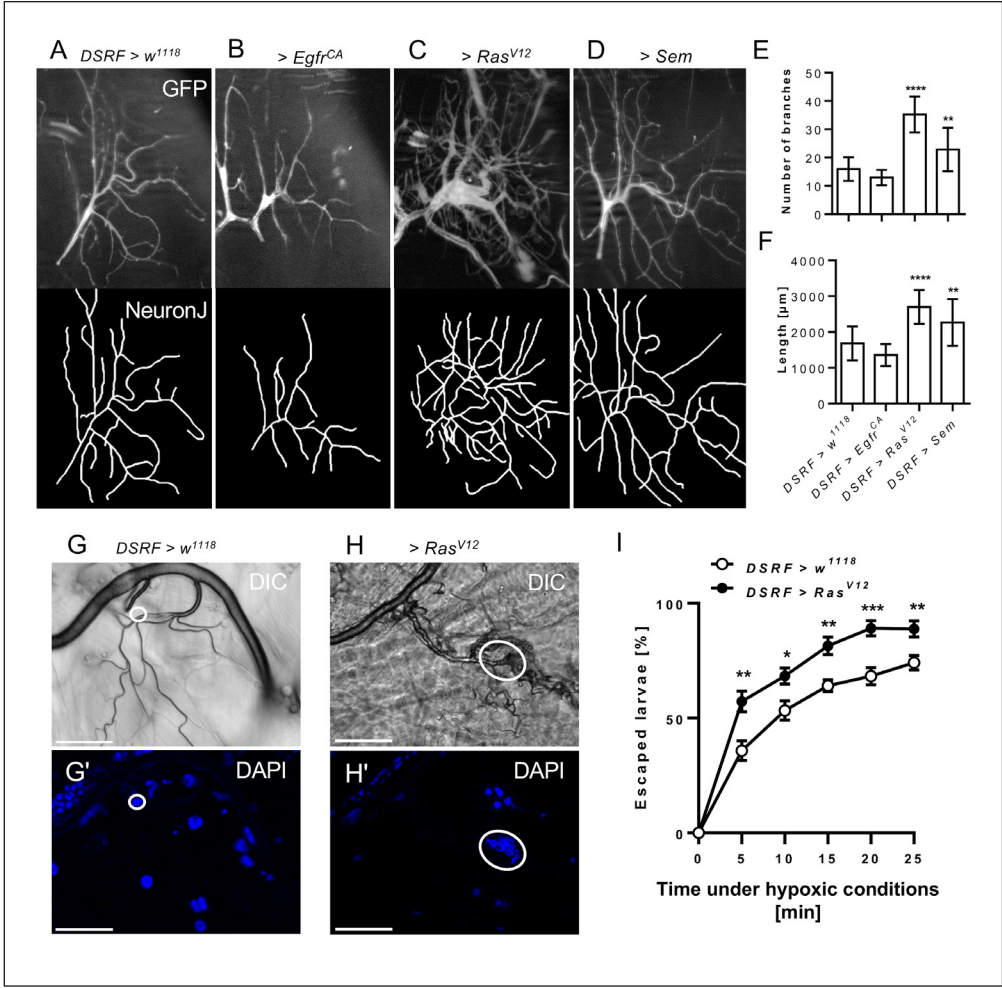


Fig. 2: Targeted expression of oncogenes to the terminal tracheal cells Overexpression of oncogenes was driven by the *DSRF-Gal4* line. (A-D) GFP expressing terminal cells with overexpression of *Egfr^{CA}* (B), *Ras^{V12}* (C) and *Sem* (D) (upper row). The branches of the terminal cells were traced and measured with NeuronJ (lower row). (E, F) Quantification of mean numbers (+ SD) of terminal branches and mean branch lengths (+ SD) (n = 14-46). (G, H) Enlarged nuclei of *Ras^{V12}* expressing animals (H) compared to the control (G). Nuclei were stained with DAPI (G', H'; blue). (I) Hypoxia sensitivity of *DSRF > Ras^{V12}* (n = 6) animals compared to the control (n = 12) was monitored over a period of 25 min. Scale bar = 50 μ m. Statistical significance was evaluated by Mann-Whitney test; * p < 0.05, ** p < 0.01, *** p < 0.001.

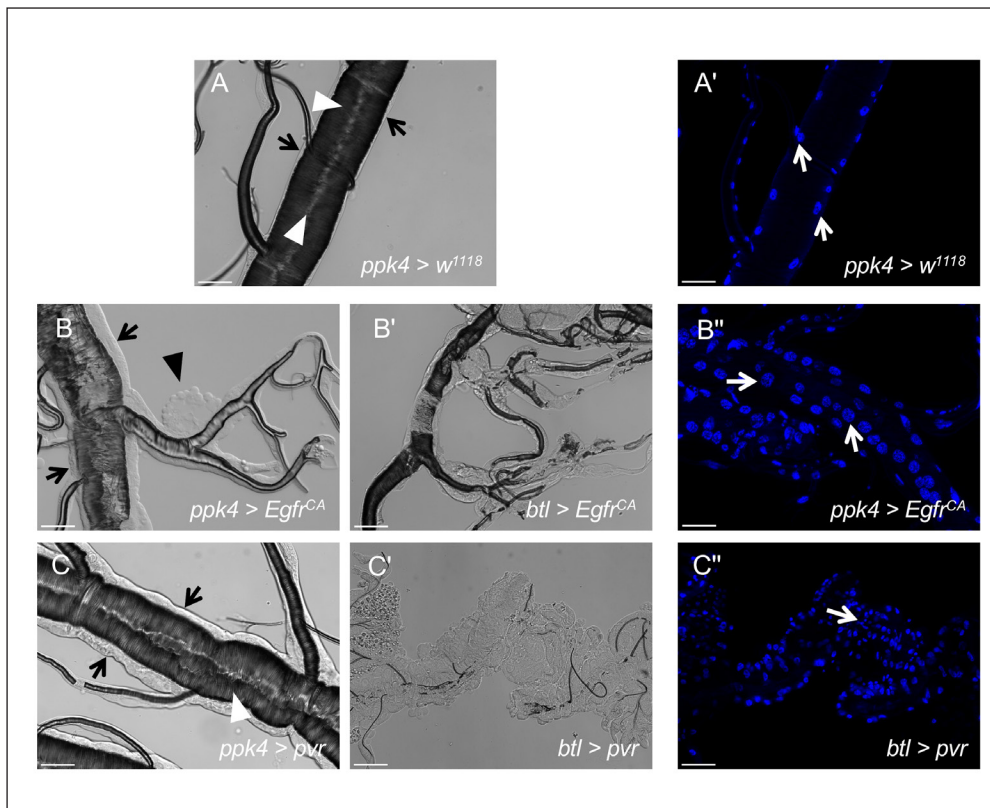


Fig. 3: Ectopic over-expression of oncogenes in the tracheal system induced massive structural changes

Transmitted light images and DAPI staining of the control (A) and trachea with *Egfr^{CA}* (B) and *Pvr* (C) expression. Expression with the moderate *ppk4-Gal4* driver is shown in B and C. Expression with the stronger *btl-Gal4* driver is shown in B' and C'. The epithelial layer is indicated by black arrows (A,B,C). Disordered cell masses are indicated by black arrowheads (B). The air-conducting lumen is indicated by white arrowheads (A,C). Number and size of nuclei are indicated by white arrows, compared to the control (A',B'',C''). Scale bar = 50 μ m.

types are suitable to develop high-throughput screening systems. These experimental interventions, which utilized both *Drosophila* orthologs of the corresponding human oncogenes as well as humanized flies carrying the human oncogenes, induced structural changes in the airways in comparison to matching controls (Fig. 3). Here, we show only the effects induced by ectopic over-expression of *egfr* and *pvr*, driven by the weaker *ppk4-Gal4* line and the stronger *btl-Gal4* line. Controls exhibited a regular appearance of the air-conducting lumen (Fig. 3A, white arrowheads), the epithelial layer (Fig. 3A, white arrows), and of the nuclei (Fig. 3A', white arrows). In flies ectopically expressing oncogenes, the most conspicuous change was a substantial thickening of the airway epithelium (Fig. 3B,C, black arrows). Over-expression of *egfr^{CA}* driven by *ppk4* (Fig. 3B) induced this thickening (black arrows) as well as larger and more nuclei (Fig. 3B'', white arrows), indicating an increase in cell number. Using *btl-Gal4* as a driver led to even stronger phenotypes with more disturbed airways (Fig. 3B'). Overexpression of *pvr* driven by *ppk4-Gal4* also led to increased epithelial thickening (Fig. 3C, black arrows) and structural changes within the air conducting lumen (white arrowhead). The number of nuclei was even higher (Fig. 3C'', white arrow) than observed in *ppk4-Gal4*, *UAS-egfr^{CA}* animals. Driving *pvr* expression with the stronger *btl-Gal4* driver led to an almost complete breakdown of tracheal structure (Fig. 3C'). To quantify these effects and relate them to the fly's equivalents of lung cancer, we focused on phenotypes directly associated with hyper- or metaplastic changes of the airway epithelium

and those that may be used as read-outs in high-throughput approaches. Accordingly, we decided to use epithelial thickening as a proxy of metaplastic transformation, and proliferation of airway epithelial cells as a proxy of hyperplasia.

The thickness of an unchallenged epithelial monolayer in the dorsal trunks of L3 larvae was about 2-3 μ m (Fig. 4A,C). However, we observed epithelial thicknesses of about 10 μ m in *Egfr*-overexpressing dorsal trunks (Fig. 4B,C). A similar epithelial thickness was also observed in animals specifically overexpressing *Htl*, *Ras*, and *Raf*. A second group of oncogenes, *Rolled*, *Myc*, *PI3K*, *Akt*, *Thor*, and *InR*, induced epithelial thickness in the range of 5 μ m, which in all cases differed significantly from the matched controls. In contrast, the increase in epithelial thickness for *Btl* and *Cdk4* was only marginal (Fig. 4C). In primary (Fig. 4D) and secondary branches (Fig. 4E) of the larval tracheal system, we observed very similar changes, but to different extents: the maximum epithelial thickness of primary branches (e.g., in *egfr*-overexpressing flies) was in the range of 5 μ m (Fig. 4D), whereas the maximum in secondary branches was about 3-4 μ m (Fig. 4E).

As mentioned, ectopic expression of a variety of oncogenes in the airway system induced by the *ppk4-Gal4* driver line led to substantial structural changes (Fig. 3, 4). In this system, we observed that ectopic expression of constitutive forms of *Drosophila EGFR* (*Egfr^{CA}*) was lethal in larval stages in almost all individuals. Shifting the expression control system towards stronger expression (driven by *btl-Gal4*) caused a greater number of onco-

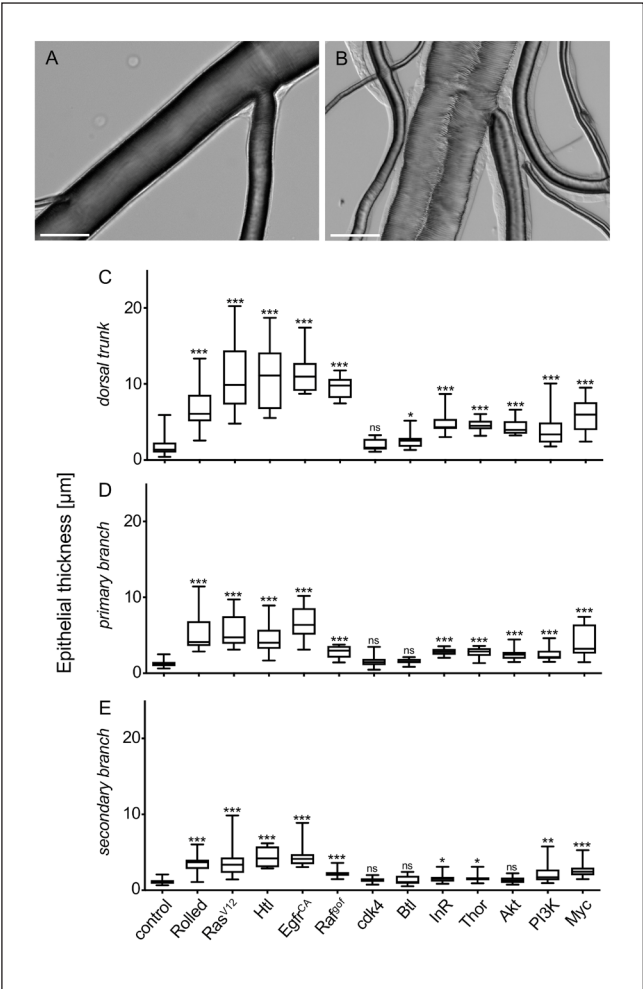


Fig. 4: Ectopic overexpression of oncogenes in the larval airway system induces thickening of the epithelial layer, a proxy for hyperplasia
Epithelial thicknesses differed substantially between tracheae of control flies (*w¹¹¹⁸*, A) and those ectopically overexpressing Raf (*ppk4 > Raf^{90f}*, B). Scale bar = 50 μ m. (C-E) Quantitative evaluation of the effects of ectopic overexpression of various oncogenes. Values obtained from larval dorsal trunks (C), larval primary branches (D), and larval secondary branches (E) are listed. N > 20 for each value. Mean values \pm SD are given. Statistical significance was evaluated by Mann-Whitney test; ns, not significant, * p < 0.05, ** p < 0.01, *** p < 0.001.

genes to exert lethal effects. Ectopically driven expression of *dEgfr^{CA}*, *hEGFR-dEgfr*, *pvr*, *Alk*, *Ras*, *Raf*, *Rolled*, *PI3K92E*, *Akt*, and *Arm* led to early lethality by the early third larval stage at the latest. In contrast, ectopic expression of *Btl*, *InR*, or *Cdk4* had no effect. These results are summarized in Table 1. This oncogene-induced lethality is ideally suited as a read-out system, because interventions that rescue this lethality could easily be identified by the occurrence of adult flies in the corresponding test systems. This approach has been used before for identifying novel combination

Tab. 1: Effects of oncogene overexpression on airway hyper- and metaplasia and larval death

<i>Drosophila</i> gene (human gene)	Tracheal phenotype, larval death (x)	
	moderate expression <i>ppk4-Gal4</i>	strong expression <i>btl-Gal4</i>
<i>dE</i>	+++ (x)	+++ (x)
<i>gfr^{CA}</i> (EGFR)		
<i>hEGFR-dEgfr</i>	-	++ (x)
<i>hEGFR-Pvr</i>	-	+
<i>Btl</i> (FGFR)	-	-
<i>Htt</i> (FGFR)	++	nd
<i>Pvr</i> (VEGFR/PDGFR)	++	+++ (x)
<i>InR</i> (InsR)	-	-
<i>Alk</i> (ALK)	+	+++ (x)
<i>Ras^{V12}</i> (RAS)	+	+++ (x)
<i>hRaf^{90f}</i> (RAF)	+	+++ (x)
<i>Rl</i> (MAPK)	+	+++ (x)
<i>PI3K92E</i> (PI3K)	+	+++ (x)
<i>Akt</i> (AKT)	-	+++ (x)
<i>Arm</i> (CTNNB1)	+	+++ (x)
<i>Apc</i> (APC)	+	+
<i>Cdk4</i> (CDK)	-	-
<i>Thor</i> (THOR)	+	nd
<i>Myc</i> (MYC)	+	nd
<i>P53</i> (TP53)	+	+

The phenotype induced by ectopic overexpression of the corresponding oncogene is given. Oncogenes of human origin are underlined. +, visible phenotype; ++, strong phenotype; +++, very strong phenotype; X, lethality; -, no effect; nd, not determined

treatments targeting Ras or EGFR in corresponding *Drosophila* lung cancer models (Levine and Cagan, 2016; Bossen et al., 2019).
Beside using lethality as a read-out for high-throughput approaches, alternative ways to quantify the tumor-associated phenotypes are highly appreciated, especially for those cases where early lethality cannot be used as a read-out. Thus, quantifying the GFP-fluorescence that is concurrently expressed together with the oncogene in the airway epithelium would be a suitable alternative (Fig. 5). To test this alternative, *Ras^{V12}* was ectopically expressed

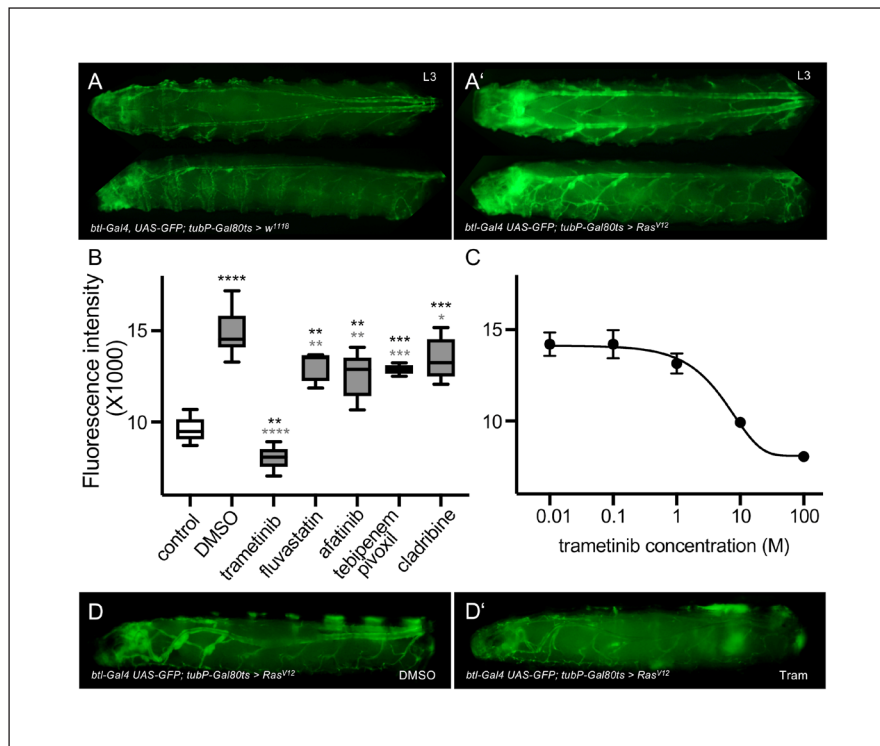


Fig. 5: Quantification of GFP expression in the airway system as a proxy of tumor mass

(A) GFP-expressing 3rd instar larvae after three days of induction are shown in dorsal and lateral view. (B) Measured fluorescence intensity of animals treated with different drugs (concentration: 100 μ M each). DMSO treated *Ras^{V12}* animals and animals without *Ras^{V12}* expression served as control (n = 4-15, whiskers show min and max). (C) Quantification of fluorescence intensity in *Ras^{V12}* overexpressing animals treated with different concentrations of trametinib shown in a dose-response curve (n = 6-9, means and SEM are shown). (D) *Ras^{V12}* expressing animals treated with trametinib (100 μ M) (D') have lower GFP expression compared to untreated animals (D). Statistical significance was tested by Mann-Whitney test; * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

together with GFP, enabling to quantify tumor mass. Comparable approaches based on quantification of fluorescence as proxies for tumor mass have been used only occasionally (Willoughby et al., 2013). Therefore, we used *Ras^{V12}* overexpression driven by the heat-inducible *btl-Gal4; tubP-Gal80ts* driver that also contained *UAS-GFP* and could observe a substantial increase in the GFP signal of 3rd instar larvae (Fig. 5A') compared with the corresponding controls (Fig. 5A). Quantification of the fluorescence signal using a microplate reader revealed significant differences between controls and animals showing a *Ras^{V12}* overexpression. Here, application of different compounds (100 μ M each) rescued the increased fluorescence (trametinib) or had no or only a very minor effect at this high concentration (fluvastatin, afatinib, tepipenem pivoxil, and cadriberine) (Fig. 5B). A more detailed analysis covering a large concentration range was performed with trametinib, the only compound that could rescue the phenotype (Fig. 5C). The experiment revealed a typical s-shaped curve with plateaus at very low and very high concentrations. The IC₅₀ value calculated from these data was 4.9 μ M. Fluorescence microscopy showed that the induced tracheal GFP signal was mostly reduced, implying that the tumor mass was reduced accordingly (Fig. 5D,D').

4 Discussion

In this study, we developed modular *Drosophila* models for a larger number of human lung cancer oncogenes and evaluated if these systems are amenable to high-throughput, whole-animal compound screening approaches. These research efforts should

not only provide very useful tools for lung cancer research, they may also reduce the number of vertebrates used in compound development pipelines. In two recent studies, tailored *Drosophila* systems were used to identify novel therapeutic strategies to fight specific lung cancer entities based on screening of FDA-approved compound libraries. For this, *Ras^{V12}* overexpression concurrent with *Pten* downregulation were used to identify a trametinib and fluvastatin as an effective strategy to fight *Ras^{V12}*-driven lung cancer (Levine and Cagan, 2016). Moreover, targeted overexpression of *Egfr^{CA}* was used to identify the EGFR agonist afatinib in combination with the STAT inhibitor bazedoxifene to effectively inhibit growth of EGFR-initiated tumors (Bossen et al., 2019). Both studies depended on a lethal phenotype that became apparent during early development and that was rescued by the successful pharmacological intervention.

The major aim of the current study was to evaluate if other driver mutations can principally also be transformed into high-throughput compatible screening systems. In order to achieve this goal, simple and reliable read-out systems that can be performed in microplate formats are mandatory. These specifications are only fulfilled by two different types of read-outs: the rescue of an otherwise lethal phenotype and the reduction of a quantifiable tumor mass by successful intervention.

Due to its simplicity, the rescue of a lethal phenotype has several advantages, especially as it has a digital design allowing to distinguish successful and failed interventions with low instrumental input. For automated recognition of living larvae besides dead ones, we focus on larval motility. Instead of applying video microscopy and analysis, we will evaluate a diffraction-based



optical technology (“Speckle” sensing) for this task. The technology was developed for the determination of beating frequencies in freely floating cardiomyocyte spheroids. To this end, the illumination will be altered to a multiplex beam pattern filling the well’s cross section. While cardiomyocyte signals are a sequence of almost identical peaks that may be easily analyzed, signals from larval motions will be irregular. Hence, the signal analysis will make use of noise analysis techniques in order to quantify the number of vital larvae per well.

On the other hand, quantifying tumor mass, in the current study via co-expressed GFP, allows to quantify effects beyond the simple yes or no experimental design, but it requires more sophisticated instrumental efforts. These strategies are in line with other successful high-throughput screening assays for specific cancer subtypes based on *in vivo* analysis of tailored *Drosophila* strains (Levinson and Cagan, 2016; Das and Cagan, 2010; Willoughby et al., 2013; Markstein et al., 2014). Broadly speaking, studies that utilize *in vivo* *Drosophila* high-throughput screening assays take advantage of the long history of using *Drosophila* to advance our general understanding of cancer biology (Gonzalez, 2013; Gateff, 1978; Harrison et al., 1995).

The recent findings that *Ras*^{V12} and *EGFR*^{CA} overexpression are well suited to develop high-throughput screening systems refuted the general assumption that diseases like lung cancer could not be modeled in *Drosophila* (Levine and Cagan, 2016; Bossen et al., 2019). The usefulness of this type of *Drosophila* cancer model was further supported by the development of personalized screening platforms based on transgenic *Drosophila* that can be used for different cancer entities (Bang et al., 2016, 2019).

In this study, we showed that most components of the oncogenic network underlying the vast majority of adenocarcinomas (Ding et al., 2008; Kandath et al., 2013; Paez et al., 2004; Travis et al., 2015) are able to induce lung cancer-like phenotypes in *Drosophila*. These phenotypes, primarily hyper- and metaplasia of airway epithelial cells, are not exact copies of the histological features of human lung cancer, but they are the closest equivalent in the fly and give rise to quantifiable and cancer-associated phenotypes.

The observation that the majority of the known oncogenes (Tab. 1) could induce structural changes related to hyper- and metaplasia in the fly’s airway epithelium implies that the model that has been proposed for mutations in *EGFR* or *Ras* could also be adapted to other oncogenes, raising the possibility of generating panels of personalized fly models for human lung cancers with different driver mutations. Beside the ectopic overexpression of constitutively active *Egfr* and *Ras*^{V12}, ectopic overexpression of *Raf*, *Rolled* (MAPK), *PI3K92E* (PI3K), *Akt* (AKT), *Alk* and *Armadillo* (CTNNB1) also induced lethal phenotypes, making them ideally suited to be developed into personalized high-throughput screening systems for the corresponding lung cancer subtypes (Tab. 1). The inherent features of the screening approach that is based on long-term treatment with the compounds of interest encompassing the entire development of the animals, ensures that only those candidate substances are assessed as positive that do not interfere with normal physiology or development.

The pharmacological data that can be obtained with this system are certainly in ranges that are also used in therapy. In the case of trametinib, for which we were able to determine an IC₅₀ of 4.9 μM, there is little clinical data. Trametinib is used as a combination therapy with dabrafenib in a variety of tumor diseases (Adachi et al., 2020). Taking into account the long residence time of trametinib, the concentration of trametinib is approximately 0.2 μmol/kg body weight, which could correspond to a concentration in body fluids of approximately 0.4 μmol/L. If we also take into account that the IC₅₀ value determined by us refers to the concentration in food, we are dealing with very similar concentration ranges. The same can be said about the comparison with the studies of Levine and Cagan (2016). Here, 0.5 μM trametinib was used, but in combination with a statin, which showed synergistic effects with a factor of 5–8. Again, the values were in very comparable concentration ranges.

To further expand the use of *Drosophila* as a screening tool for the identification of novel treatment strategies for those oncogenes that did not induce early larval lethality, alternative readouts would be highly appreciated. Therefore, alternative strategies that employ signals from markers concurrently expressed with the oncogenes, such as luciferase or GFP, are principally suitable, as they give a proxy for tumor mass (Markstein et al., 2014). Thus, we aimed to test this approach using concurrently expressed GFP. We could show that this strategy can be used to quantify increased cell mass and therewith higher GFP expression in the trachea only using microplate-based fluorescence analyses of whole animals, therewith further widening the group of oncogenes that can be used for personalized *Drosophila* screening approaches.

In order to transfer the tumor mass quantification, read out into an *in-situ* measurement ready for non-consumptive and automated operation requires fluorescence acquisition of intact larvae in the plate wells. One approach to this end was already described (Willoughby et al., 2013): The larvae are immersed in aqueous sucrose solution in order to take care for their proper position and orientation. Then fluorescence images are taken and transformed into one-bit (black/white) images using a constant threshold brightness. The number of white pixels is then used as the measure of tumor mass. In order to have a repeatable read out, a fluorescence quantification technique that works without liquid immersion on any possible position and orientation of the larvae in the well is preferable. Such a technique requires transparent feeding media and very high fluorescence collection efficiency as in an integrating sphere. No imaging nor image analysis is needed using this type of integrating technique.

Based on the finding of this study and of the two recent publications employing the first *Drosophila* lung cancer models (Bossen et al., 2019; Levine and Cagan, 2016), a panel of personalized *Drosophila* models amenable to high-throughput screening approaches can be installed that act as shortcuts for the development of novel therapeutic strategies. In order to be able to use personalized models of lung tumors, these must essentially be available, as patients cannot wait for them to be constructed. However, this is perfectly feasible with *Drosophila* systems. For this, the corresponding oncogene-overexpressing flies (without

the corresponding drivers) should already be available in a number of important combinations. The keeping of 100 or more oncogene combinations can be done easily. These would then only have to be crossed with the respective inducible drivers and the corresponding experiments could be completed within 2 weeks.

Expanding the application of the 3R principle beyond the field of toxicology holds the potential to substantially reduce the requirements for vertebrates as animal test systems (Blakemore et al., 2012; Graham and Prescott, 2015). Although these *Drosophila* systems offer a greater number of impressive advantages, limitations of these systems should not be overlooked. Only the tumor itself can be analyzed in the most accurate way. The tumor environment, however, remains the same as it is found in *Drosophila*. For this reason, it remains necessary to carry out confirmative experiments in, e.g., mouse models for those very few compounds that have been identified and characterized in depth in order to independently verify the results. Nevertheless, it should be noted that the use of suitable *Drosophila* models can lead to a massive reduction of the required mouse experiments.

In summary, here we have described a large number of personalized *Drosophila* lung cancer models that provide us with novel tools to identify suitable treatment strategies for lung adenocarcinomas. This valuable screening approach opens a new direction for cancer research, namely the identification of new anticancer drugs using high-throughput whole-animal screens.

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Conflict of interest

The authors declare no conflict of interest.

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