Lgl2 Executes Its Function as a Tumor Suppressor by Regulating ErbB Signaling in the Zebrafish Epidermis

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Abstract

Changes in tissue homeostasis, acquisition of invasive cell characteristics, and tumor formation can often be linked to the loss of epithelial cell polarity. In carcinogenesis, the degree of neoplasia correlates with impaired cell polarity. In Drosophila, lethal giant larvae (lgl), discs large (dlg), and scribble, which are components of the epithelial apico-basal cell polarity machinery, act as tumor suppressors, and orthologs of this evolutionary conserved pathway are lost in human carcinoma with high frequency. However, a mechanistic link between neoplasia and vertebrate orthologs of these tumor-suppressor genes remains to be fully explored at the organismal level. Here, we show that the pen/lgl2 mutant phenotype shares two key cellular and molecular features of mammalian malignancy: cell autonomous epithelial neoplasia and epithelial-to-mesenchymal-transition (EMT) of basal epithelial cells including the differential expression of several regulators of EMT.

Further, we found that epithelial neoplasia and EMT in pen/lgl2 mutant epithelial cells is promoted by ErbB signalling, a pathway of high significance in human carcinomas. Intriguingly, EMT in the pen/lgl2 mutant is facilitated specifically by ErbB2 mediated E-cadherin mislocalization and not via canonical snail–dependent down-regulation of E-cadherin expression. Our data reveal that pen/lgl2 functions as a tumor suppressor gene in vertebrates, establishing zebrafish pen/lgl2 mutants as a valuable cancer model.

Introduction

Tumor suppression is a concept first formulated in Drosophila after emerging evidence that recessive mutations can lead to the formation of cellular overgrowth [1,2]. To date, more than 50 tumor suppressor genes have been identified in Drosophila [3]. Their deficiencies result in benign hyperplasias to malignant neoplasms. Amongst these tumor suppressors, mutations in lethal giant larvae (lgl), cause malignant neoplasia in imaginal discs and the brain when transplanted into wild type adult host flies [4,5]. In Drosophila neuroblasts, lgl function is essential for localization of the cell fate determinant Numb, mislocalization of which in lgl mutant larvae prevents the neuroblasts from dividing asymmetrically and therefore causes neuroblastoma [6–9]. Furthermore, it has been proposed that lgl prevents tumor formation by antagonizing the activation of Dpp signaling by semaphorin 5c in the brain [10]. Although epithelial overgrowth phenotypes have been reported in lgl mutant larvae in Drosophila [1], the mechanism by which lgl manifests its effects on epithelial growth remains to be understood. Nevertheless, it is known that along with lgl, two other tumor suppressor genes, discs large (dlg) and scribble (scrib), primarily act in the maintenance of apico-basal cell polarity in epithelial cells [11].

The establishment as well as the maintenance of apico-basal cell polarity and eventually the depolarization of a cell is a complex process, involving several factors. In recent years, a conserved mechanism for the establishment and maintenance of apico-basal cell polarization has emerged, which mainly involves two pathways. Accordingly, the formation of the apical domain is controlled by the Par (partitioning defective) pathway, which consists of the PDZ domain containing proteins Par3, Par6, and atypical protein kinase C (aPKC). In contrast, a pathway consisting of disc-large (dlg), scribble (scrib) and lethal giant larvae (lgl) regulates the formation and maintenance of the baso-lateral domain [12,13]. Intriguingly, only mutations in genes that act in the baso-lateral pathway (e.g. lgl, dlg, scrib) lead to a neoplastic growth phenotype in Drosophila [2,14–16].

The two vertebrate orthologs of the Drosophila lethal giant larvae gene have conserved functions in the maintenance of cell polarity and tissue homeostasis. Disruption of lgl1 function results in the loss of apical functional complex in neuroblasts and hyperplasia of the brain in mouse [17]. Furthermore, it has been shown for human melanoma cell lines, that a human homolog of lgl, hugl1 is significantly down-regulated. Artificial induction of hugl1 in these cell lines reduces their migratory potential with concomitant transcriptional up-regulation of the cell adhesion molecule E-cadherin (E-cad) and a down-regulation of matrix-metalloproteinases (matrix), both of which are known to be involved in suppression of epithelial-to-mesenchymal transition (EMT), a process which enables an epithelial cell to gain mesenchymal or migratory properties [18]. Recently, a significant correlation between the loss of hugl1 and a poor clinical prognosis for cancer patients has been shown [19].

The forward genetic approach in zebrafish has revealed a novel function for the second lgl ortholog, pen/lgl2, in maintenance of the...
epidermal integrity, which is a stratified epithelium. The pen/lgl2 deficiency primarily results in the loss of hemidesmosomes, cellular junctions that mediate cell-matrix adhesion [20]. It has been shown that Lgl2 localizes to the lateral domain of the epidermal cells and regulates hemidesmosome formation by mediating the targeting of ITGa6, a component of hemidesmosomes, to the membrane [21]. Furthermore, epithelial cells in the pen/lgl2 mutant exhibit altered epidermal cell morphology as well as cell polarity and enhanced epidermal growth [20,21]. Recently, it has been shown that in colorectal and breast carcinoma cell lines, a member of the ZFH family of repressors ZEB1 regulates the levels of Lgl2. The loss of ZEB1 function restores Lgl2 levels and the epithelial phenotypes in tumor cells, which suggests that Lgl2 acts as an effector of ZEB1 in tumor suppression [22].

From the analysis of several cancer models it is evident that autocrine self-stimulation with growth factors is one of the hallmarks of tumorigenicity [23,24]. However, whether activation of growth factor signaling is a consequence of the loss of basolateral pathway components remains unclear to date. Moreover, whether lgl1 and lgl2 deficient clonal cell populations can promote tumor formation in vertebrate tissues, a typical characteristic of tumor suppressor genes, remains unresolved. Here, we show that lgl2 deficient clones indeed promote tumor formation in the zebrafish epidermis. Moreover, pen/lgl2 mutant basal epidermal cells undergo EMT. Using biochemical analysis, chemical inhibitors and genetic interaction studies, we demonstrate that these phenotypes are a consequence of an over-activation of erbB signaling involving at least one erbB family member, erbB2. Our microarray and immuno-histological analysis reveal that activation of erbB signaling facilitates EMT by transcriptional up-regulation of key EMT regulators and a reduction in the membrane localization of E-cad, a known suppressor of EMT.

Results

lgl2 functions as a tumor suppressor gene in the zebrafish epidermis

We have previously shown that zebrafish pen/lgl2 larvae show overgrowth of epidermal cells (Figure 1A and 1B; and [20]). As pen/lgl2 mutant larvae die at 4–5 days post fertilization (dpf), it was not clear whether these hyper-proliferating cells would be able to form tumor like structures. To test this, we transplanted cells from pen/lgl2 homozygous mutant donor individuals, into wild type lgl2 deficient clones indeed promote tumor formation in the epidermis. Moreover, pen/lgl2 mutant basal epidermal cells undergo EMT. Using biochemical analysis, chemical inhibitors and genetic interaction studies, we demonstrate that these phenotypes are a consequence of an over-activation of erbB signaling involving at least one erbB family member, erbB2. Our microarray and immuno-histological analysis reveal that activation of erbB signaling facilitates EMT by transcriptional up-regulation of key EMT regulators and a reduction in the membrane localization of E-cad, a known suppressor of EMT.

Figure 1. pen/lgl2 deficient epidermal cells form tumors in a cell autonomous fashion. DIC images of 5-day-old wild-type (A) and pen/lgl2 mutant larvae (B). DIC image of wt host with pen/lgl2 mutant skin clones (C). Close-up of a tumor in DIC (D) and GFP channel (E). In comparison to 5 day wild-type larvae (A) the pen/lgl2 mutant larvae exhibit neoplasias, most prominently in the ventral jaw region (B). Seven days after the transplantation of pen/lgl2 mutant cells at blastula stage, recipients develop tumor like structures in the skin (C). These tumor like structures (D) contain GFP labelled cells (E) indicating that they are derived from mutant clones.

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hosts during blastula stage. In order to trace the donor cells in the wild type recipients, donor embryos were transgenic for ubiquitously expressed GFP. The recipient larvae with epidermal cells from mutants or wild type siblings were monitored for potential tumor development. On the 7th day after the transplantation, 17 of the 24 (71%) larvae that had received pen/lgl2 deficient cells (GFP marked) in the epidermis developed epidermal tumors (Figure 1C and 1D). In contrast, none of the larvae that had received cells from wild type siblings developed epidermal tumors (n = 77). Fluorescence microscopic analysis of these tumors revealed that they contain GFP positive cells, indicating that the lgl2-/- cells are inducing the formation of epidermal tumors (Figure 1E). Interestingly, lgl2-/- cells in other tissues, including brain, did not show any visible hyperplasia at this stage. We conclude that pen/lgl2 mutant cells are capable of inducing epidermal tumor formation, even if surrounded by wild type tissue. The levels of GFP expression varied in the tumor cells (Figure 1E). This may reflect a variegated expression of GFP. However, we cannot rule out the possibility that some wild type cells also contribute to the neoplastic tissue. Our results support the notion that lgl2 acts as a tumor suppressor gene specifically in the epidermis.

Loss of pen/lgl2 function results in EMT of basal epidermal cells

Along with uncontrolled cell proliferation, migratory behavior mediated by EMT is another hallmark of cancer cells [23]. In pen/lgl2 mutant larvae, epidermal cells not only hyper proliferate but also exhibit different morphological shape as evident from the changes in the keratin organization from basal polygonal in wild type to peri-nuclear and spindle shaped in pen/lgl2 mutants (Figure 2A and 2B, [20]). Such alterations in cell morphology are correlated with acquisition of EMT [26–28], we investigated the localization of E-cad in pen/lgl2 mutants. We found that the membrane localization of E-cad was severely reduced in the mutant epidermis, while the cytoplasmic fraction appeared to be increased compared to the wild type siblings (Figure 2C and 2D).

To observe the behavior of basal epidermal cells of pen/lgl2 mutants, we generated a transgenic line driving the expression of GFP in basal epidermal cells under the control of AN-p63 promoter [29]. Accordingly, tg(AN-p63::Gal4,UAS::GFP) zebrafish larvae show GFP expression exclusively in this cell type (Figure S1). To test whether pen/lgl2 mutant cells acquire migratory properties, we performed time lapse studies of tg(AN-p63::Gal4,UAS::GFP);lgl2-/- larvae. These revealed that, in contrast to their wild type siblings, basal epidermal cells in pen/lgl2 mutant larvae dramatically alter their shape, form numerous lamellipodia like structures and exhibit net displacement over time (Figure 2 and Videos S1, S2, S3, S4). From an apical perspective, the observed changes in cell morphology also lead to an increase in average cell area (Figure 2G). The mutant epidermal cells appear larger when compared to wild type cells as they exhibit a highly flattened morphology and develop lamellipodia like cell protrusions.

We conclude that in absence of pen/lgl2 function the basal epidermal cells lose their epithelial morphology and acquire the morphology of migratory (mesenchymal) cells indicating that these cells undergo the morphological changes associated with EMT.

Expression profile of pen/lgl2 mutant larvae reveals differential transcriptional regulation of known molecular regulators of EMT

To understand if pen/lgl2 mutants also show molecular signatures of EMT and if so, which of the known EMT related genes are active in pen/lgl2 mutant basal epidermal cells, we performed a genome wide expression profiling of pen/lgl2 mutant larvae showing neoplastic overgrowth at 108hpf using the Agilent...
microarray platform. Subsequent statistical analysis revealed 117 genes to be significantly differentially regulated in pen/lgl2 mutant larvae (FDR; \( P < 10^{-6} \)) (Figure 3A and Table S1). Amongst these differentially regulated genes, we found a very strong transcriptional induction of mmps such as mmp9 (11.1 fold) and mmp13 (3.1 fold), which are known regulators of EMT, mainly in the context of malignancy [30–32]. Further, we found a set of cytotkeratins, krt5, ckt1, ckt2, and collagens to be down-regulated within a range of 3.5 to 4.4 fold, which is consistent with the previous analysis of the role of cytokeratins in EMT [28]. Moreover, genes involved in cell cycle regulation (e.g. histone-b, jun-b, N-ras), cell survival (e.g. sgk1) and tight-junction formation (e.g. cldn-7, cldn-e, cldn-c, cldn-i) were also up-regulated from 3.6 to 5.6 fold (Figure 3A).

The canonical way to achieve EMT is to down-regulate E-cad at the transcriptional level. The transcriptional repressors, mainly those of the snail family, play an important role in this process [27,33–35]. Intriguingly, we observed robust up-regulation of E-cad expression at the mRNA level (Figure 3A). By performing quantitative RT-PCR in pen/lgl2 mutant larvae, we detected 8 times higher E-cad RNA levels in mutants compared to the wild type sibling controls (Figure 3B). Further, examination of E-cad by western blot analysis revealed increase in protein levels in pen/lgl2 mutant larvae compared to their wild type siblings (data not shown). Thus, although the membrane localization is drastically perturbed (Figure 2C and 2D), E-cad protein levels are higher in pen/lgl2 mutants. We further estimated the expression levels of snail family members. Consistent with up-regulation of E-cad levels, none of the snail family members shows increased expression in pen/lgl2 mutants. These data indicate that in pen/lgl2 mutant larvae EMT of basal epidermal cells is facilitated by mis-localizing E-cad rather than its snail mediated repression.

We confirmed the tissue specificity of differential expression of the genes mmp9, sgk1, cldn7, krt5 and E-cad by in-situ hybridization (ISH) (Figure 3C–3L). We verified the up-regulation of mmp9 as well as sgk1 and down-regulation of krt5 specifically in the basal epidermal cells. Interestingly, the up-regulation of the tight-junction gene cldn7 was observed mainly in the cells that form epidermal cell aggregates in the ventral jaw region and in the fin-fold (Figure 3H).

Our analyses indicate that basal epidermal cells in pen/lgl2 larvae exhibit both the morphological and transcriptional characteristics of cells undergoing EMT. We conclude that pen/lgl2 function is essential to suppress epidermal neoplasia and EMT. Thus, pen/lgl2 acts as a recessive tumor suppressor gene in vertebrates. Furthermore, canonical snail mediated repression of E-cad is not involved in EMT in pen/lgl2 mutant. Instead, EMT is facilitated by removal of E-cad from the plasma membrane.

![Figure 3. pen/lgl2 mutant epidermal cells differentially express EMT regulators.](https://www.plosgenetics.org/figs/doi/10.1371/journal.pgen.1000720.g003)

** = \( P < 0.005 \)
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Inhibitors of ErbB signaling suppress cell proliferation and EMT in basal epidermal cells of pen/lg2 mutant larvae

As human carcinomas are often a consequence of massive over-activation of growth factor signaling [36], we assayed pen/lg2 mutant larvae for the phosphorylation level of the mitogen activated protein kinase Erk, a common member of growth factor signaling cascades, by western blot [37]. We found elevated levels of phosphorylated Erk in the mutants compared to their wild type siblings (Figure 4A). To identify which growth factor signaling is activated, we treated larvae from heterozygous pen/lg2 carriers with inhibitors for three different receptor tyrosine kinases (RTKs), FGFR, IGFR and EGF/R, starting at 96 hpf just prior to the appearance of the pen/lg2 mutant phenotype. These treatments revealed that an inhibitor of ErbB (PD168393) reduced the levels of phosphorylated Erk (Figure 4B) and rescued the epidermal neoplasia phenotype as well (Figure 4D and 4E). Similar rescue in the epidermal phenotype was observed with another ErbB inhibitor AG1478 (data not shown). In contrast, Inhibition of FGFR (SU5402) neither affected the levels of phosphorylated Erk (Figure 4C) nor did it rescue the epidermal overgrowth phenotype (data not shown). Similarly, there was no rescue in the epidermal overgrowth phenotype when IGFR signaling was inhibited using AG1024 (data not shown). Genotyping of an entire clutch treated overgrowth phenotype when IGFR signaling was inhibited using AG1024 (data not shown). Genotyping of an entire clutch with KeRatin (PD168393) revealed the expected Mendelian proportion (27 out of 120) of pen/lg2 homozygous larvae. We further asked whether the EMT and over-proliferation phenotypes were suppressed after treatment with PD168393. Indeed, cytokeratin and E-cad antibody stainings of inhibitor treated pen/lg2 mutant larvae were indistinguishable from wild type siblings (Figure 4H and 4K). Consistently, epidermal cell proliferation and mmp9 transcript levels were strongly reduced (Figure 4L–4N, Figure 4O–4Q). However, hemidesmosomes did not form in PD168393 treated pen/lg2 mutant larvae as revealed by electron microscopic analysis (Figure S4).

Our data indicate that loss of functional pen/lg2 results in over activation of ErbB signaling which promotes over proliferation of basal epidermal cells as well as cellular EMT by transcriptional modulation of EMT regulators. We further conclude that over-activation of ErbB signaling is not the cause for the absence of hemidesmosomes in the pen/lg2 mutant. This observation further indicates that disruption of pen/lg2 primarily affects hemidesmosome formation, which is consistent with the previous analysis [20,21].

erbB2 promotes EMT but not the cell proliferation in the basal epidermal cells of pen/lg2 mutant larvae

In mammals four ErbB receptors are known, ErbB1 to ErbB4, which get activated upon the binding of ligands such as EGF, HBEGF, neuregulins, betacellulin [38]. Ligand binding leads to the formation of homo- or heterodimers amongst these receptors, resulting in signal transduction [38]. Our bioinformatic and phylogenetic analysis coupled with the previous analysis of some of the family members [39,40] revealed that with the exception of erbB2, all other members of this family exist in duplicates in the zebrafish (Figure 5A); erbB2 as well as pcs/erbB3b zebrafish mutants exhibit defects in glia development and regeneration [39–42]. We found erbB2 to have an additional epidermal phenotype in the finfold (Figure 5C) and it is also expressed in the epidermis (Figure 5E). To determine which of the ErbB receptors is activated in pen/lg2, we performed loss of function studies in a pen/lg2 mutant background. We knocked down erbB1a with a splice site antisense morpholino. Injections of erbB1a morpholino did not interfere with the pen/lg2 phenotype but reproduced the cardiovascular phenotype published earlier [43] (data not shown). However, we cannot exclude the involvement of erbB1a in promoting the EMT and growth phenotype in pen/lg2 mutant larvae as we found the effect of the morpholino to decrease beyond 40hpf, possibly due to dilution effects (Figure S2). Double mutants of pcs/erbB3b/pen/lg2 mutation did not show suppression of the pen/lg2 neoplastic phenotype and immuno-histological analysis using the pan 1–6 Cytokeratin antibody did not reveal any reduction in strength and initiation of EMT phenotype (data not shown, Figure 5I). Taken together, these results demonstrate that erbB2 function is not essential for neoplasia and EMT phenotype in pen/lg2 mutants.

In zebrafish, lg2 and erbB2 are both located on chromosome 12 (23.6mb distance). To study double mutants, choosomal recombinants were made (see Materials and Methods). We investigated progeny from lg2+/−, erbB2+/− double heterozygous fish (3 crosses; n = 296), which were sorted for the morphological epidermal lg2 phenotype at 108hpf as well as 132hpf and subsequently genotyped. We identified 40 larvae that were homoyzozygous for both lg2 and erbB2. In 37 of these, no epidermal phenotype was detected up to 132hpf (Figure 6C). In contrast, all lg2 larvae with erbB2+/− or erbB2+/+ genotype showed strong phenotypologies or lethality at the same stage (Figure 6B, Table S2). In a separate experiment, we analyzed Cytokeratin and E-cadherin localization, mmp9 expression and BrdU incorporation in lg2+/−, erbB2−/− double mutants at 108hpf when the lg2 phenotype becomes apparent. The morphology of the epidermal cells in the double mutant larvae, as revealed by Keratin staining, appeared completely normal at 108hpf Figure 6D–6F. Occasionally, epidermal cells in pen/lg2; erbB2 double mutant larvae exhibited milder changes in Keratin organization, indicative of altered cellular morphology at 132 hpf (data not shown). E-cadherin localization appeared normal in the epidermis of the double mutants (Figure 6G–6I) and mmp9 expression levels in the epidermis of double mutant larvae were comparable to those in wild type (Figure 6M–6O). Interestingly, however, the BrdU incorporation analysis revealed that (Figure 6J–6L) there is no significant decrease (t-test, p>0.05) in the epidermal cell proliferation in a predefined area in lg2−/−,erbB2−/− double mutants (29±1.0, n = 3) as compared to lg2−/−,erbB2+/− double mutants (32±9, n = 3) larvae.

Our data suggest that erbB2 but not erbB3b mediated signaling is responsible mainly for the EMT phenotype in pen/lg2 mutant basal epidermal. We did not observe rescue in the epidermal cell-proliferation phenotype in pen/lg2; erbB2 double mutants and epidermal cell morphology was altered as well at later stages indicating that erbB2 deficiency doesn’t completely rescue the phenotype. Since the inhibitors completely rescue both the proliferation as well as EMT phenotypes, we propose that other ErbB receptors might be involved in promoting epidermal cell-proliferation in pen/lg2 mutants.

Discussion

Impaired cell polarity is one of the hallmarks of carcinoma but was considered to be a secondary effect for a long time. Recent findings however suggest it can also be a cause rather than a consequence of tumor progression [18,19,44,45]. In many epithelia, the apico-basal polarity is established by the activity of the apical aPKC-Par3-Par6 pathway and baso-lateral Lgl-Scrib-Dlg pathway. Surprisingly, only loss of function in baso-lateral pathway components leads to epithelial or brain neoplasia phenotypes in Drosophila [45]. In vertebrates, two components of
Figure 4. Inhibition of ErbB signaling restores the epidermal morphology and cell cycle in pen/lgl2 mutant larvae. Western blot analysis of Erk1/2 phosphorylation in untreated wild-type and pen/lgl2 larvae (A), larvae treated with PD168393 (B) and larvae treated with SU5402 (C). DIC images of wild-type (D) and pen/lgl2 mutant (E) treated with the ErbB inhibitor PD168393 and genotyped. The pan 1–8 cytokeratin antibody staining (F–H), anti E-cad staining (I–K), anti BrdU antibody staining (L–N) and in situ hybridization staining for mmp9 (O–Q) of the epidermis of wild-type (F, I, L, O) pen/lgl2 mutant (G, J, M, P) and mutant larvae treated with PD168393 (H, K, N, Q). In comparison to wild-type larvae (A, left lane, 44kD) Erk shows higher level of phosphorylation in pen/lgl2 mutant larvae (A, right lane 44kD). The levels of Erk phosphorylation are equal in the mutants treated with PD168393 (B) but not with SU5402 (C). The α-Tubulin levels (55kD) are indicative of equal protein loading. The epidermal cell morphology, E-cad localization, cell proliferation and mmp9 expression in pen/lgl2 larvae, treated with PD168393 (H, K, N, Q) appears similar to wild-type larvae (F, I, L, O) than the untreated mutant larvae (G, J, M, P). Note that PD168393 treated (rescued) larvae were genotyped to confirm their genotypes.

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this pathway, lgl and dlg, have multiple orthologues [45]. Loss of lgl1 function results in brain hyperplasia in the mouse [17]. In zebrafish loss of lgl2 function leads to over-proliferation of epidermal cells [20]. Here, we demonstrate that transplantation of pen/lgl2 homozygous mutant cells during blastula stage into wild type embryos results in the formation of tumor-like structures in the epidermis after 7 days. This analysis clearly shows that lgl2 behaves as a tumor suppressor gene in vertebrates.

The basal epidermal cells in pen/lgl2 mutants do not form hemidesmosomes and display an altered cell morphology instead of the polygonal cell shape found in wild type larvae [20]. E-cad localization at the plasma membrane is reduced in the mutant epidermal cells indicating that epidermal cells undergo EMT. Indeed, GFP labeling of basal epidermal cells revealed that in pen/lgl2 mutant larvae these cells show continuous changes in their morphology, project lamellipodia and move as a sheet. This sheet like movement has been described as a metastable cell state in the context of EMT [28]. Consistent with the cellular analysis, the expression profiling of lgl2 mutant basal epidermal cells revealed differential expression of the known EMT markers and regulators such as matrix-metallopeptinases and keratins. Intriguingly, the levels of E-cad RNA are high in the pen/lgl2 mutant. This is in contrast to known developmental scenarios of EMT such as the delamination of neural crest cells or gastrulation where E-cad expression is downregulated at the transcriptional level by a transcriptional repressor, snail [34,35]. In addition, structural components of tight junctions, such as several claudins, are also up-regulated in pen/lgl2 mutants. Thus, the expression profiles of cell adhesion molecules in pen/lgl2 larvae resemble several human breast carcinomas including an inflammatory breast cancer (IBC), a highly aggressive subtype of human breast cancer, which has been characterized by E-cad and erbB2 over-expression [46–51]. The up-regulation of claudin genes is intriguing. However, it is not clear whether Claudins localize properly in the epidermal cells. Even if the Claudins do localize properly, the in-situ expression analysis suggests that claudins are up-regulated mostly in the cellular clumps that are formed in the median finfold or in the ventral jaw region. It is thus clear that claudins are not expressed during the process or EMT but rather when the mesenchymal cells re-acquire partial epithelial phenotypes while forming tumor-like aggregation. However, further analysis is required to test this notion.

How does deficiency in components of the baso-lateral pathway lead to epithelial cell proliferation and EMT? It has been suggested that the protein Scrib stabilizes the coupling between E-cad and the Catennins and thus behaves as a regulator of epithelial cell adhesion and migration [52]. Our data show that for Lgl2 the mechanism to suppress EMT and tumor formation is fundamentally different. Lgl2 may not manifest its function by stabilizing the coupling between E-cad and Catennins. Previously, we have shown that neither the loss of maternal nor zygotic lgl2 function primarily affect E-cad localization [20,21]. Consistent with mammalian data, knockout of E-cad in the mouse or zebrafish epidermis does not result in EMT [21,33–55]. This indicates that although the loss of E-cad facilitates EMT [56], it may not be sufficient to induce it in animal models.

We show that inhibition of erbB2 signaling, either genetically or by small chemical inhibitors, leads to suppression of EMT and a neoplastic phenotype in pen/lgl2 mutant larvae. Intriguingly, E-cad membrane localization is restored in these larvae, indicating that the loss of E-cad is a consequence of activation of ErbB signaling rather than a cause of it. Thus, our analyses presented here suggest that Lgl2 acts as a tumor suppressor by regulating the amplitude of ErbB signaling in the epidermis. Since we did not observe snail-mediated down-regulation of E-cad, we propose that an erbB2 dependent pathway in pen/lgl2 mutants leads to the destabilization of E-cad at adherens junctions. Indeed in pen/lgl2 mutant epidermal cells, known modifiers of E-cad function, such as wood9 and sgk1 are up-regulated (Figure 3). While Mmps are known to be involved in ecto-domain shedding of E-cad [57,58], Sgk1 functions in the phosphorylation of Ndr1, a protein involved in vesicular recycling of E-cad [59,60]. Additionally, recently published data from cell culture suggests an involvement of RTK signaling in the destabilization of adherens junctions via Numb [61]. Here, Numb functions as an adapter protein coupling...
Figure 6. *erbB2* promotes EMT in the epidermis of *pen/lgl2* mutant larvae. DIC images of the morphology of wild-type (A), *lgl2,erbB2*<sup>+/2</sup> (B) and *lgl2,erbB2*<sup>2+/2</sup> (C) larvae at 108hpf. Keratin (D–F), E-cadherin (G–I), BrdU (J–L) and mmp9 (M–O) staining in wild-type (D, G, J, M), *lgl2,erbB2*<sup>+/2</sup> (E, H, K, N) and *lgl2,erbB2*<sup>2+/2</sup> (F, I, O) larvae at 108hpf. The DIC images of the morphology reveals that cellular clumps, a typical characteristic of *pen/lgl2* mutant larvae are present in *lgl2,erbB2*<sup>+/2</sup> (B) but are absent in *lgl2,erbB2*<sup>2+/2</sup> (C). The keratin, E-cadherin and mmp9 staining in *lgl2,erbB2*<sup>2+/2</sup> (F, I, O) appear similar to wild-type (D, G, M) indicating *erbB2* promotes the EMT phenotype in *pen/lgl2* mutant larvae. Note that, in *lgl2,erbB2*<sup>2+/2</sup> larvae (L) BrdU incorporation does not decrease to wild-type levels (J).

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E-cadherin to the Par polarity-complex. This interaction was shown to be sensitive to elevated levels of RTK signaling, leading to mislocalization of E-cadherin [61]. Further analysis involving loss of function of mmp11 and gsk3 as well as studies of Numb localization in the pen/lg2 mutant larvae would be necessary to clarify their contributions to the EMT phenotype.

The primary function of Lgl2 in hemidesmosome formation [20,21] is not dependent on ErbB signaling as hemidesmosomes do not form in pen/lg2 mutants even after the inhibition of ErbB signaling (Figure S4). This suggests that the loss of hemidesmosomes might be the cause but not a consequence of the activation of ErbB signaling. This hypothesis is supported by the fact that components of hemidesmosomes (e.g. Ing6/Ingb4), physically interact with the ErbB2 receptor tyrosine kinase [62–64]. Thus, it is possible that mislocalization of hemidesmosomal components in pen/lg2 mutants lead to an activation of ErbB2.

ErbB signaling plays an important role in the development of human carcinomas, as it is able to induce proliferation and EMT and further is able to suppress apoptosis [36,65]. This is in particular true for ErbB2 which is over-expressed in more than 25% of all breast carcinoma [66]. A causal link between ErbB activation and loss of cell polarity, which is one of the hallmarks of carcinomas, has recently been established. It has been shown that activated ErbB2 associates with Par6-aPKC leading to disruption of the apico-basal polarity [67]. While ErbB2 regulates apico-basal cell polarity by interacting with apical polarity components like Par6-aPKC, our analyses presented here suggest that one of the basolateral pathway components, Lgl2, regulates the activation of ErbB2. Thus, there seems to be a reciprocal interaction between cell polarity regulators and ErbB2 signaling. Interestingly, although ErbB2 activation induces cell proliferation, the association of ErbB2 with Par6-aPKC was not essential for regulating cell proliferation indicating that ErbB2 affect the cell proliferation independent of disruption of apico-basal cell polarity [67]. Our data suggest that at the organismal level loss of ErbB2 does not affect the epidermal cell proliferation phenotype in lg2 mutants but only prevents EMT. Since inhibitors, which do not discriminate between various ErbB family members rescue both, proliferation as well as EMT phenotypes, it appears that activation of more than one ErbB family member might be involved in promoting epidermal neoplasia in pen/lg2 mutants. Thus, cell proliferation and EMT phenotypes are not coupled in pen/lg2 mutant larvae.

The ErbB signaling pathway includes multiple ligands and receptors in vertebrates. ErbB1 and ErbB4 represent discrete receptors in vertebrates. ErbB1 and ErbB4 represent discrete orthologues were obtained from Genebank (http://www.ncbi.nlm.nih.gov) and through Ensembl (http://www.ensembl.org/Danio rerio) databases. Alignments were performed using ClustalW. Phylogenetic analysis was run using neighbor joining, maximum parsimony and minimum evolution algorithms, (MEGA4, http://www.megasoftware.net/). All analysis methods showed similar tree morphology with comparable bootstrap values (1000 replicates). Human orthologues (Genebank ID): ERBB1: 1956, ERBB2: 2064, ERBB3: 2065, ERBB4: 2066. Zebrafish paralogs (Ensembl transcript ID): erbB1a: ENSDART00000027219, erbB1b: ENSDART00000031151, erbB2: ENSDART00000003932, erbB3a: ENSDART00000049893, erbB4a: ENSDART00000092114, erbB4b: ENSDART00000100398

Generating ΔNp63::Gal4, UAS::GFP transgenic fish

The ΔNp63 promoter was cloned by enzymatic restriction of BAC dkey-13d19 with HhaI (NEB) and subsequent blunt-end cloning of a resulting 4.96 kb fragment into a plasmid containing a Gal4,UAS::GFP expression cassette and mini-TOL2 sites (based on [68,69]). Transgenesis was achieved by simultaneous injection of plasmid DNA and transposase RNA at 1-cell stage using WPI PV930 pneumatic injection system followed by F1 screen for GFP positive larvae.

BrdU labelling

5-day-old wild type and pen/lg2 mutant larvae were incubated with 10 mM BrdU solution in 2% DMSO in embryonic medium (E3) for 2 hours. After treatment, larvae were washed several times in E3 fixed overnight in 4% PFA in PBS at 4°C. Staining was performed as described below.

Immunohistochemistry

For IHC procedures the following antibodies were used: Anti-BrdU antibody ab6326 (abcam); anti Cytokeratin antibody Ks pan
1–8 (Progen Biotechnologie) and anti-E-cad antibody (BD Transduction Laboratory). Embryos were either fixed in 4% PFA (E-cad, BdiU) or in Dent’s fixative (cytotherin). After downgrading the larvae to 0.1 M phosphate buffer (PB), they were washed with PBTr (PB+0.8% Triton X-100) five times and blocked in 10% normal goat serum. For BdiU staining, larvae were treated with 4 N HCl for 20 min., washed in PB and blocked in 1% BSA for 1–3 hours. Antibodies were diluted as: Ks pan 1–8 (1:10), anti-E-cad (1:250), anti-BrdU (1:50) and samples were incubated at room temperature for 4 hours or overnight at 6–8°C. Afterwards, larvae were washed five times in PBTr, incubated with Cy3 or Alexa 488 antibody or anti-rat antibodies, post fixed in 4% PFA and upgraded in 70% glycerol for fluorescence/light microscopy.

In situ hybridization
DIG-labeled RNA probes for cldn7, kat5, mmp9 and sgk1 were prepared from larval 5dpf total cDNA obtained from pen/lg2 or mutants. In situ hybridization was performed using Intavis in-situ robot (model: insituPro VSi).

Probe templates have been amplified with the following primer combinations by PCR reaction and subsequently cloned into pGEM-T Easy Vector (Promega). DIG-labeled probes were synthesized using Sp6 and T7 RNA polymerases (Roche).

E-cad: 5’-TTACTTCCTGTCTTTGCTGCT-3’; 5’-TCTATGATTGTCGTTTCCT-3’
cldn7: 5’-TGGCACATAAAGGACTGCAA-3’; 5’-CGATGAGAAATAGCTGTGACC-3’
erbB2: 5’-GTGATCGAGAAGCAAGATCAG-3’; 5’-CATCA-GTCTCCAGATCTCCA-3’
kat5: 5’-CAGGAGTGCTAGTGCCTTCC-3’; 5’-CGGGTT- GTTGAGGGTCTTGAT-3’
mmp9: 5’-GCTGCTCATGAGTGGGAC-3’; 5’-CGGAG- CTCTGCGATTACG-3’
sgk1: 5’-ATGGAAGGACGTCACCTTCC-3’; 5’-GCGTAA-AAGCCTTGTGCAATTG-3’

Transplantations
Transplantations were carried out at blastula stage. After transplantations, donor and corresponding recipients embryos were cultured together (3–5 in number) in a 24 well plate. The pen/lg2 mutant donors were identified at 4.5dpf by phenotype or by molecular genotyping at blastula stage (RLFPL). The host larvae that received mutant or wild type clones (GFP-positive) in the skin were sorted and further raised up to 7–8 dpf and analyzed by microscopy for tumor phenotypes.

Inhibitor treatment
For screening, all inhibitors (SU5402; AG1024; PD168393; AG1478 all Calbiochem) were used at 10–50 μM concentration in 1% DMSO in E3 Medium starting at 96hpf. For BdiU, IHC and ISH experiments, PD168393 and AG1478 were used at a concentration of 10 μM starting at 96hpf.

Western blot and detection
For western blots, mutants were identified by the development of the characteristic lg2 mutant phenotype at 108hpf. Three times 40 mutants and equivalent number of wild type larvae were collected at 108hpf and than treated with either DMSO as a control, or inhibitors for ErbB signaling (10 μM) or FGFR signaling (20 μM) for 12 h. Subsequently, larval tails were collected by cutting posterior to the swimming bladder on ice. Proteins were extracted in DXB (25 mM Hepes, pH 6.8, 50 mM KCl, 1 mM MgCl2, 1 mM DTT, 250 mM sucrose) containing Roche Complete protease inhibitors (Cat No. 11836153001) and Pierce Halt phosphatase inhibitors (Cat No. 78420). Protein concentration was determined using OD at 280 nm (NanoDrop). For PAGE, NuPage 4–12% gradient gels (Invitrogen) were used. After transfer, equal loading was re-checked by poinseau red staining. Antibodies used were: p-ERK (M9692; 1:600), Erk (M5670; 1:500), α-Tubulin (T9026; 1:10000) (all from Sigma).

Secondary antibodies used were HRP conjugated anti-mouse (p-Erk, Tubulin) and anti-rabbit (Erk). Detection was performed using chemiluminiscence (ECL*; GE Healthcare).

Microarray analysis
The microarray experiment was conducted using tissues (tail) posterior of the anal opening of wild type (Tuebingen) and pen larvae at 5 dpf. RNA for microarray analysis was extracted from four biological replicates using TRIZOL Reagent (Gibco BRL, Eggenstein, Germany). Complementary RNA was prepared from 1 μg total RNA from each replicate as described in the Agilent Low RNA Input Linear Amplification kit manual (Agilent Technologies, Palo Alto, CA, USA). Double-stranded cDNA was synthesized using the reagents from this kit, and Cy3- or Cy5-labeled cRNA was prepared by cDNA in vitro transcription in the presence of cyanine 5-CTP or cyanine 3-CTP dyes. Fluorescence labeled cRNA was then purified with the Qiagen RNEasy spin columns, according to the manufacturer’s protocol (Qiagen, Hilden, Germany). After purification, cRNA was fragmented and used to hybridize to the zebrafish G2519F 4X44 microarray platform containing 4 duplicated arrays of the 22,000 probe-set design (Agilent Technologies, Palo Alto, CA, USA). Four biological replicates were conducted, including two dye swap experiments to minimize the effect of any potential dye bias. Hybridization, washing, and scanning were performed according to the manufacturer’s protocol. The microarrays were scanned on a Genepix Axon 4000B scanner (Molecular Devices, Union City, CA, USA) at five micron resolution with five-line averaging. Raw expression values from each probe set were extracted using the Genepix Pro 6.0 feature extraction software, and features were flagged manually for poor quality. The data were then analyzed in the R statistical programming environment using the Bioconductor module Limma [70,71]. Duplicate probe sets on each array were considered as technical replicates for the analysis in addition to the 4 biological replicates on separate arrays. A standard linear model for differential expression, with the Limma module in Bioconductor, was used to identify genes up and down regulated in the mutant versus wild type experiment. The resulting p-values from the hypothesis tests were adjusted for multiple testing with the false discovery approach (FDR) to control for false positives [72]. In addition, the empirical Bayes approach automatically adjusts raw p-values for multiple testing and generates a B-statistic that may also be used for ranking differentially expressed genes [70]. All microarray data may be accessed through the ArrayExpress repository on the European Bioinformatics Institute database website (http://www.ebi.ac.uk/microarray-as/ae/).

Quantitative real-time PCR
As for the microarray experiment, larval tails posterior to the anal opening were used as tissue sample (3 technical replicates of each biological replicate; 3 biological replicates). Total RNA was isolated using TRIZOL reagent (Invitrogen) and cDNA was synthesized using AMV cDNA Synthesis Kit (Invitrogen) with oligo-dT15 primers (Promega). SYBR Green (BioRad) was used for quantitative real-time PCR. Time emission readings were recorded with DNA Engine Opticon 2 (MJ Research), and analyzed as described [73]. gapdh was used for normalization.
Genotyping of mutant larvae

Genotyping to identify mutants was done by a PCR based restriction fragment length polymorphism (RFLP) method for Igl2- and erbB2- or by DNA sequencing for erbB3b mutants. The RFLP analysis was done as follows: the mutations in erbB2 cause a loss whereas in Igl2 a gain of a restriction site. PCR product from genomic DNA samples obtained by the primer pair: 5'-ATGCAATACCTCTCTGGAGTACG-3'; 5'-TGTTGCTCTTA-GTGAGGCCAGG-3' (for Igl2) or 5'-TGAGATGCCTGTTAGCTGGC-3' and 5'-GGACTCAGGAAAAGG ACTTAG-3' (for erbB2) was digested with either SfcI (for Igl2 mutation) or BsrGI (for erbB2 mutation) resulting in a genotype specific DNA band pattern (Figure S3). The erbB3b genotyping was done by scoring for the premature stop at position 156 bp by DNA sequencing of a PCR product obtained from the genomic DNA samples with either SfcI or BsrGI leading to a genotype specific DNA band pattern in agarose gel electrophoresis. The erbB3b mutation was scored by sequencing of a PCR product spanning the site of lesion. The nature of the mutation is a cytosine to adenine transversion leading to a premature stop codon after 156 bp.

Found at: doi:10.1371/journal.pgen.1000720.s003 (0.66 MB TIF)

Figure S4 Inhibition of erbB signaling does not restore hemidesmosome formation in pen/lg2 mutants. pen/lg2 mutant basal cells are unable to form hemidesmosomes, even after inhibition of ErbB signaling. EM cross section through larval skin 5dpf reveals hemidesmosome formation at the basal membrane in wild types (A, arrows) whereas pen/lg2 mutants (B) and pen/lg2 mutants treated with ErbB inhibitor PD168393 (C) lack these structures.

Found at: doi:10.1371/journal.pgen.1000720.s004 (1.58 MB TIF)

Table S1 Expression profile of pen/lg2 compared to wild type. Using microarray technique the expression profile of wild-type versus mutant zebrafish larval tails, posterior to the anal opening was analysed. A significance threshold of adj. p-value of 10^-6 (FDR) resulted in 117 genes to be significantly differentially expressed. Within those, most prominent genes, involved in EMT and cell cycle, as well as cytoskeleton rearrangements, can be found. Additionally, genes involved in the formation of tight and adherens junctions are present. The down-regulated genes are indicated in red in this table whereas up-regulated genes are indicated in black.

Found at: doi:10.1371/journal.pgen.1000720.s005 (0.03 MB XLS)

Table S2 Comparison of phenotypes and genotypes in erbB2/lg2 double mutant incrosses. Percentual distribution of epidermal neoplasia in pen/lg2 single- and lg2/erbB2 double mutants. Note that the loss of erbB2 strongly reduces the formation of the characteristic overgrowth phenotype in the pen/lg2 mutant background, even at late time points.

Found at: doi:10.1371/journal.pgen.1000720.s006 (0.03 MB DOC)

Video S1 Phenotype of basal epidermal cells in the wild-type larva. A 90-minute timelapse movie of Tg(Anp63:Gal4,UAS::GFP) wild-type zebrafish larva at 5dpf. Settings: 20×1-min interval, 10 frames per second. The GFP labelled basal epidermal cells in the wild-type larvae remain static.

Found at: doi:10.1371/journal.pgen.1000720.s007 (1.81 MB MOV)

Video S2 Phenotype of basal epidermal cells in the pen/lg2 mutants larva. A 90-minute timelapse movie of Tg(Anp63:Gal4,UAS::GFP), lg2-/- zebrafish larva at 5dpf. Settings: 20×1-min interval, 10 frames per second. The GFP labelled basal epidermal cells exhibit migratory behavior in the mutant larva. Cells show shape changes and development of lamellipodia like cell protrusions, indicating their mesenchymal character.

Found at: doi:10.1371/journal.pgen.1000720.s008 (6.63 MB MOV)

Video S3 Phenotype of basal epidermal cells in wild-type larva. A 240-minute timelapse movie of Tg(Anp63:Gal4,UAS::GFP) wild-type zebrafish larva at 5 dpf. Settings: 25×1-min interval, 10 frames per second. The GFP labelled basal epidermal cells remain static in the wild-type larva.
Video S4 Phenotype of basal epidermal cells in pen/1gl2 mutant larva. A 240-minutes time lapse movie of tg(Np63+Gal4.UAS:GFP) 1gl2−/+ zebrafish larva at 5dpf. Settings: 25 × 1-min interval, 10 frames per second. The GFP labeled basal epidermal cells exhibit migratory properties in the mutant larva. Cells show cell shape changes and formation of lamellipodia like cell protrusions indicating their mesenchymal character. 

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Author Contributions

Conceived and designed the experiments: SR MS. Performed the experiments: SR MPL. Analyzed the data: SR MPL MS. Wrote the paper: SR CNV MS.

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