LIN28B increases neural crest cell migration and leads to transformation of trunk sympathoadrenal precursors

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RUNNING TITLE

LIN28B boosts cell invasive motility.

SOURCE DISCLOSURE

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DECLARATION OF INTEREST

The authors declare no potential conflicts of interests.

ABSTRACT

The RNA-binding protein LIN28B regulates developmental timing and determines stem cell identity by suppressing the *let-7* family of microRNAs. Postembryonic reactivation of *LIN28B* impairs cell commitment to differentiation, prompting their transformation. In this study, we assessed the extent to which ectopic *lin28b* expression modulates the physiological behavior of neural crest cells (NCC) and governs their transformation in the trunk region of developing embryos. We provide evidence that the overexpression of *lin28b* inhibits sympathoadrenal cell differentiation and accelerates NCC migration in two vertebrate models, *Xenopus leavis* and *Danio rerio*. Our results highlight the relevance of *ITGA5* and *ITGA6* in the LIN28B-dependent regulation of the invasive motility of tumor cells. The results also establish that *LIN28B* overexpression supports neuroblastoma onset and the metastatic potential of malignant cells through *let-7a* dependent and independent mechanisms.

INTRODUCTION

Neural crest cells (NCC) undergo substantial modifications in their morphology and function during embryogenesis. Their highly migratory and invasive nature allows them to reach distant body districts, whereas their pluripotency assures the specification of several cell lineages, including peripheral neurons and the adrenal medulla [1, 2]. The initial migratory wave of NCC is activated by the epithelial-to-mesenchymal transition (EMT). The morphological changes triggered by EMT sustain cell migration from the neural tube, invasion of the surrounding tissues, and spatial progression toward the peripheral sites where cells differentiate and unfold their intrinsic features [3]. Several NCC characteristics, such as motility, polarity, invasiveness, and plasticity, are largely shared by a number of highly metastatic tumors, including colon [4], breast [5], oesophagus [6] and neuroblastoma [7] tumors. Neuroblastoma is an embryonal malignancy responsible for

approximately 15% of cancer-related deaths during childhood [8]. It originates from the transformed sympathoadrenal cell precursors deriving from trunk NCC [9] and is characterized by vast clinical, genetic, and biological heterogeneity [10]. About half of patients with neuroblastoma are stratified as high-risk (HR), having an overall survival rate of less than 40% despite intensive multimodal therapy [11]. The main feature of HR patients with neuroblastoma is the prevalence of metastases at disease onset. The metastases to the liver, the bone marrow, the bone or lymph nodes are found in approximately 50% of HR patients. Like other pediatric cancers, neuroblastoma shows a low frequency of somatic mutations although alterations in several genes, including *LIN28B*, have been associated with this malignancy [12].

The RNA binding proteins LIN28A/LIN28B were initially identified as important regulators of developmental timing [13]. By inhibiting *let-7* microRNA biogenesis and through direct binding of the target RNAs, LIN28 regulates numerous cellular activities that are essential for embryogenesis [14], but it shows pro-tumorigenic features if maintained beyond the physiologically defined timeframe [15, 16]. In neuroblastoma, the pro-tumorigenic function of *LIN28B* has been attributed to either gene amplification or overexpression [7]. However, the lack of experimental models in which modulated levels of *LIN28B* can be studied during early developmental phases limits the possibility for a comprehensive investigation of the mechanisms that sustain NCC transformation.

In this study, we assessed the role of the ectopically expressed zebrafish *lin28b* gene in regulating trunk NCC migration and differentiation toward the sympathoadrenal lineage. In two vertebrate models, zebrafish (*Danio rerio*) and Xenopus (*Xenopus leavis*), we examined how the overexpression of *lin28b* affected the migration of trunk NCC during early embryonic development. We then analyzed whether *lin28b* determined the differentiation of NCC toward noradrenergic lineage. *In vivo*, a stable overexpression of the human *LIN28B* gene driven by the *dβh* promoter was adopted to evaluate the probability of neuroblastoma onset. In the tumor cells, we focused on evaluating the effects of the prolonged overexpression of *LIN28B* on cell motility and dissemination *in vitro* and in the *in vivo* xenograft model. Finally, we established the relevance of integrin-dependent signaling in the regulation of neuroblastoma cell migration upon *LIN28B* overexpression.

RESULTS

Lin28b overexpression impairs the differentiation of sympathoadrenal precursor

cells.

To estimate the effects of *lin28b* overexpression during embryonic development, we injected capped *lin28b* mRNA into 1-2-cell stage zebrafish embryos. We then assessed the ectopic expression of the corresponding transcript and protein at different developmental stages (Figure S1A) compared to the control (*ctrl*) embryos, injected with either the mCherry or the GFP mRNAs (Figure S1B). Wild-type uninjected embryos were used as a blank control for fluorescence screening, and as a reference sample for the evaluation of Lin28b protein levels in the immunoblot assay (Figure S1B). Of note, the injected fish showed no overt macroscopic phenotypes or developmental failures (Figure S1C). We then verified the functionality of Lin28b by observing a significant reduction of the *let-7a* expression in *lin28b* embryos (Figure S1D). To test if Lin28b affected the development of sympathoadrenal neurons, we analyzed the expression of tyrosine hydroxylase (*th*) and dopamine β-hydroxylase (*dβh*) as hallmarks of NCC differentiation toward the sympathoadrenal lineage. The ectopic expression of *lin28b* at early stages of development led to a marked reduction of both *dβh* and *th* mRNAs in the superior cervical ganglia (SCG) as compared to GFP-injected controls (Figure 1A). Moreover, TH protein levels also significantly decreased upon *lin28b* overexpression (Figure 1B). In addition, *lin28b*-injected embryos expressed lower levels of the SCG marker *zash1a* (Figure 1C), a transcription factor required for early sympathoadrenal cell specification [17]. These findings imply the involvement of *lin28b* overexpression in the loss of pro-differentiating signaling in sympathoadrenal cells already at early stages of embryonic development. Then, to verify the evolutionary conservation of Lin28b function in the tetrapods, we performed transient gain-of-function experiments on the Xenopus embryos. In this model, it is possible to specifically target the central nervous system and NCC without affecting the development of other tissues [18]. We therefore injected *lin28b* mRNA into one dorsal blastomere at the four-cell stage (Figure 1D, left panel) along with GFP mRNA in order to select and further analyze only embryos overexpressing *lin28b* in the developing central nervous system. Comparable to the effects observed previously in the zebrafish, the injected *lin28b* mRNA caused a significant reduction of the sympathoadrenal marker *reptin* in the Xenopus embryos (Figure 1D, right panel). To assure that the observed reduction of sympathoadrenal cells in *lin28b* embryos was not the result of damaged cell proliferation or induced apoptosis, we stained the SCG with either EdU or activated Caspase-3/TUNEL, respectively. We found no significant differences in the number of proliferating TH^+ cells in the SCG of injected embryos (Figure 1E). Similarly, Caspase-3 and TUNEL stainings showed no relevant activation of apoptosis in the SCG of *lin28b* larvae (Figures 1F and

S2). These results confirm that *lin28b* overexpression determines the failure of sympathoadrenal progenitor cell differentiation toward their functional counterparts without affecting their proliferation or viability.

Impaired cell differentiation promotes neuroblastomagenesis in *LIN28B*-overexpressing zebrafish.

To assess whether the *lin28b*-dependent block of peripheral sympathetic neural cell differentiation has pro-tumorigenic effects in fish, we generated the stable transgenic zebrafish line *Tg(dβh: hLIN28B*), referred to here as *hLIN28B*. For this purpose, *hLIN28B* was overexpressed in the peripheral sympathetic nervous system under the control of the *dβh* promoter. Tumor masses arose in the anterior abdomen of six-month-old transgenic animals (Figure 2A) with a 2.4% penetrance. The tumors were found in the interrenal gland (IRG), a common site of neuroblastoma onset [19], and consisted of small, undifferentiated, round tumor cells with distinct single nucleoli (Figure 2B). To molecularly characterize and assure the tumor type, we analyzed the expression of several neuroblastoma-specific markers. The tumor cells were strongly immunoreactive for TH (Figure 2C), indicating their peripheral sympathetic neuronal origin [20]. They were also positive for the HuC/D and the Synaptophysin markers (Figure 2C), demonstrating that they arose from sympathetic neuroblast precursors, like human neuroblastomas, and not from other NCC-derivatives, such as chromaffin cells. Together, these findings evince that the overexpression of *hLIN28B* in the peripheral sympathetic neuronal system of transgenic fish promotes neuroblastomagenesis with a penetrance below 5%, approximately as described for human patients with neuroblastoma.

Overexpressed *lin28b* increases the migratory capacity of trunk NCC

The different number of sympathoadrenal cells composing the SCG between the *lin28b* and control fish reflects the possible involvement of mechanisms regulating either NCC migration or their specification during the early phases of functional commitment. To assess this hypothesis, we used the *Tg(sox10:GFP)* zebrafish transgenic line [21]. In these fish, GFP expression is regulated by the NCC promoter *sox10*, allowing the real-time tracking of the NCC inside developing embryos. Transient overexpression of *lin28b* influenced the migration of trunk NCC, as confirmed by live cell imaging, without influencing the total number of GFP^+ cells (Figure S3A), their proliferation and viability (Figures S3B and S3C). Interestingly, although NCC from both the control and *lin28b*

embryos migrated ventrally along somites in narrow stripes as expected (Figure 3A; Supplemental Video S1A), the NCC overexpressing *lin28b* were first to reach their destination toward the dorsal aorta (Figure 3A; Supplemental Video S1B). To quantify this phenomenon, we measured the migration distances of NCC labeled with *crestin* riboprobe (Figure 3B) [22]. The average NCC migration distance was significantly longer in *lin28b* embryos compared to the controls at both monitored time points (Figure 3C). Importantly, the same behavior was also evident in the Xenopus embryos, where *lin28b*-overexpressing *sox10*$^{+}$ NCC cells [23] reached longer distances in the *lin28b* versus the control side of the embryo within the same timeframe (figure 3D). Together, these data prove that Lin28b regulates the migration of trunk NCC in different vertebrate models without affecting their proliferation and survival rates. Following, to test how effective Lin28b was in determining the sympathoadrenal lineage specification, we assessed the capability of NCC to form cell lineages other than the sympathoadrenal lineage. First, we analyzed the development of melanocytes originating from trunk NCC [24]. The amount of melanocytes in the yolk sac and the yolk extension was unaffected upon *lin28b* overexpression (Figure 3E). Moreover, a comparable number of sensory neurons composing the trunk NCC-derived dorsal root ganglia (DRG) was found in both control and *lin28b* larvae (Figure 3F). Furthermore, the DRG developed in the middle of the single somite boundary, as expected [25]. We then looked at the enteric neurons deriving from vagal NCC [26]. Upon staining with the anti-HuC/D antibody, no significant differences in the number of enteric neurons were found between the control and *lin28b* larvae (Figure 3G). The finding that *lin28b* embryos lacked trunk NCC-derived sympathoadrenal precursor cells but maintained vagal NCC-derived neurons indicates that Lin28b may play different roles in the designation of specific sub-populations of trunk NCC.

Overexpression of *LIN28B* increases the migratory capacity of SH-SY5Y cells

To functionally test the observed *in vivo* correlations between *lin28b* overexpression and the increased NCC migratory phenotype, we overexpressed *LIN28B* in the neuroblastoma cell line SH-SY5Y under a short-term (48 hours) and a long-term (7 days and 14 days) period. *LIN28* is known to be a master regulator of pluripotency in embryonic stem cells [23, 27]. Gene expression analysis indicated that 48 hours of *LIN28B* overexpression significantly increased the levels of the stemness-associated genes *Oct4*, *Sox2*, and *Nestin* (Figure 4A) while preserving colony formation and cell proliferation potentials upon long-term expression of *LIN28B* in SH-SY5Y cells (SH-SY5Y^{LIN28B}) and the controls (SH-SY5Y^{CTRL}) (Figures S4A and S4B). Importantly, while *LIN28B* and *let-7a* expression

patterns showed reciprocal expressions after short-term *LIN28B* induction, the *let-7a* levels reverted after prolonged overexpression (Figure 4B). These findings imply that intervals of *LIN28B* overexpression define molecular mechanisms that can be both *let-7a*-dependent and *let-7a*-independent. Long-term *LIN28B* overexpression provoked consistent changes in cell morphology (Figures 4C and S4C), which was accompanied by a notable reorganization of cytoskeleton microfilaments (Figures 4D and S4D). Indeed, SH-SY5Y^{CTRL} cells formed small and stellate-shaped cell clumps, whereas SH-SY5Y^{LIN28B} cells were bigger and cuboidal in shape. Given the role of cytoskeleton reorganization in cell motility [28] and the observed higher migratory rates of NCC upon *lin28b* overexpression *in vivo*, we analyzed the correlation between these phenomena *in vitro*. Ectopic *LIN28B* significantly increased the migratory properties of SH-SY5Y^{LIN28B} cells, as revealed by scratch and transwell assays (Figure 4E, F). A detailed analysis of cell movement using time-lapse imaging showed a sharp increase in total migrated distances (Figures 4G and S4E, Supplemental Movies S2A and S2B) and in the mean cell velocities of SH-SY5Y^{LIN28B} cells upon 7 days of *LIN28B* induction (Figure 4H). Notably, motility continued to progress upon *LIN28B* induction for 14 days (Figures S4F-H, Supplemental Movies S3A and S3B). These results provide further evidence that *LIN28B* overexpression is a determinant factor for defining the migration of neural crest-derived cells both *in vitro* and *in vivo*.

LIN28B promotes the invasive motility of neuroblastoma cells

The core feature of highly aggressive tumors is their metastatic potential determined by enhanced cell migration and invasion abilities. Both features are also mandatory for the proper function of NCC during embryogenesis. To investigate whether LIN28B may sustain cell invasiveness, we analyzed the capacity of SH-SY5Y^{LIN28B} cells to infiltrate the matrigel-coated Boyden chambers (Figure 5A, upper panel). This assay confirmed an increased capacity of SH-SY5Y^{LIN28B} cells to invade the basement membrane following a chemo-attractant gradient when compared to the control SH-SY5Y^{CTRL} counterpart (Figure 5A, graph bars). To confirm these findings *in vivo*, we injected either SH-SY5Y^{LIN28B} or SH-SY5Y^{CTRL} cells into the duct of Cuvier of *Tg(fli1:GFP)* zebrafish embryos with blood vessels marked in green. While SH-SY5Y^{LIN28B} cells showed a rapid spread inside the vessels and clear distribution throughout the trunk of the embryos one-day post injection, SH-SY5Y^{CTRL} cells did not disseminate in the same way (Figure 5B). This pro-invasive phenotype was sustained by EMT induction, as SH-SY5Y^{LIN28B} cells expressed

significantly higher levels of the mesenchymal markers N-cadherin, Snail, Twist1, and Slug compared to the control cells upon long-term *LIN28B* induction (Figures 5C and S5A). Consistently, the levels of *N-cadherin, Snail, Twist1*, and *Slug* genes were also significantly upregulated after *LIN28B* ectopic expression (Figure 5D). These findings explained the previously observed changes in SH-SY5Y^{LIN28B} cell morphology (Figure 4D) that aligned well with the mesenchymal-like phenotype. In line with these outcomes, the overexpression of *lin28b* in the zebrafish embryos led to a global activation of EMT (Figures 5E, 5F, and 5G) as well. Taken together, the results imply that LIN28B is sufficient to trigger EMT and to increase the invasive capacities of neural crest-derived neuroblastoma cells.

LIN28B activates integrin-signaling pathways

The migration of trunk NCC during body segmentation and somitogenesis is mediated by their interaction with the extracellular matrix (ECM) components. Integrins are a family of transmembrane receptors required for cell adhesion and their migration [29-31]. To investigate whether the acquired migratory and invasive potentials of *LIN28B*-overexpressing neuroblastoma cells could be attributed to integrin signaling, we evaluated the gene expression patterns of a number of integrins. Out of the nine transcripts analyzed, the most apparent upregulation was found for two integrins, α5-integrin (*ITGA5*) and α6-integrin (*ITGA6*). Both of these showed a substantial induction upon prolonged *LIN28B* overexpression (Figure 6A). Accordingly, the expression of the corresponding proteins, ITGA5 (CD49e) and ITGA6 (CD49f), was triggered in SH-SY5Y^{LIN28B} cells (Figures 6B and S5B-D) and confirmed by flow cytometry analyses (Figures 6C and 6D). Then, to assess the importance of the two transmembrane proteins in the regulation of SH-SY5Y^{LIN28B} cell motility, we examined their migration upon the addition of antagonizing primary antibodies. Pre-treatment with either the blocking antibodies anti-ITGA5 (ITGA5ab) or anti-ITGA6 (ITGA6ab) successfully abolished the migration of *LIN28B*-overexpressing cells (Figures 6E and 6F, Supplementary Videos S5-S8). Correspondingly, cell speed was dramatically reduced only in ITGA5ab- and ITGA6ab-treated SH-SY5Y^{LIN28B} cells, suggesting that the previously described pro-migratory phenotypes were largely hooked up by the two cell-surface integrins in *LIN28B*-overexpressing cells. Because the integrin clustering is essential for the early phases of cell adhesion to the substrate, we next assessed if the observed phenotype was associated with altered focal adhesions' (FAs) formation. While the SH-SY5Y^{CTRL} cells exhibited a diffuse cytosolic

distribution of FA adaptor proteins Paxillin, focal adhesion kinase (FAK), and Vinculin [32], the SH-SY5Y^{LIN28B} cells possessed these proteins organized on the plasma membrane as punctate, peripheral adhesions (Figures 7A and S6A). At day 7, these modifications were sustained mainly by the activation of two integrin/FAK-related signaling routes, including PI3K p85/AKT^{Ser473} and Src pathways (Figures 7B and S6B). Both signaling cascades are important players in regulating the integrin-based cell motility [33]. However, at day 14 only ERK activation was detected (Figure S6C), implying for a dynamic temporal regulatory switch between multiple signaling proteins in *LIN28B*-overexpressing cells. Taken together, our observations attribute to LIN28B a novel role in orchestrating the integrin-mediated cell spreading and migration.

DISCUSSION

The highly conserved RNA-binding protein LIN28B is expressed in developing tissues and is required for proper embryogenesis [13]. One of the most recognized roles of LIN28B involves the inhibition of the tumor suppressing *let*-7 family of microRNAs [14], contributing to the maintenance of cell pluripotency. The altered expression of *LIN28B* has been reported in various types of human cancer [4, 35, 36] while its role as an oncogene has been confirmed in different mouse models [7, 37]. In neuroblastoma, genomic amplification or overexpression of *LIN28B* are associated with the HR group of patients for whom the widespread metastatic disease is regularly present [7]. Nevertheless, *LIN28B*-driven mechanisms of tumorigenesis are still poorly characterized for neuroblastoma, especially because the onset of this tumor occurs during the early embryonic developmental stages.

In this study, we assessed the *in vivo* effects of fish *lin28b* overexpression on the NCC compartment. We relied on the zebrafish and Xenopus vertebrate models to demonstrate that the ectopic expression of *lin28b* led to a block in the differentiation of NCC-derived sympathetic progenitor cells without affecting their proliferation or vitality. We showed that Lin28b is required for the commitment of trunk NCC toward the sympathoadrenal cell lineage but is dispensable for the delineation of other NCC derivatives, such as pigmented cells and enteric neurons. This raises the possibility that Lin28b-derived signaling may act in specific NCC sub-populations in which the overexpression of *lin28b* beyond its physiological timeframe is sufficient for the malignant transformation of trunk sympathoadrenal cell precursors and for neuroblastoma's onset. In fact, the transgenic zebrafish model with *dβh*-driven overexpression of *hLIN28B* caused tumors in six-month-old fish. These tumors showed high immunohistochemical similarities with human

neuroblastomas and with previously reported neuroblastomas developed in zebrafish [38]. More importantly, the penetrance of LIN28B-positive tumors in fish aligned to the one previously described for human neuroblastomas. We then studied the molecular consequences of enforced short-term and long-term overexpression of the *LIN28B* gene *in vitro* in NCC-derived neuroblastoma cells. We could distinguish the short-term and long-term effects of *LIN28B* overexpression as respectively *let-7a*-dependent and *let-7a*-independent, sustaining previous findings [39]. Moreover, the LIN28B-driven acquisition of migratory and invasive properties was accompanied by substantial morphological changes and the increase of several mesenchymal markers, sustaining *LIN28B* as a promoter of EMT. During normal embryonic development, NCC undergo EMT and the subsequent migration is largely dependent on the cell-ECM crosstalk. Integrins are composed of diverse heterodimeric transmembrane receptors transducing the signals bidirectionally and regulating cell adhesion, migration, and invasion [40]. In our study, we identified a significant increase of two integrins, ITGA5 and ITGA6, upon long-term *LIN28B* overexpression. The importance of ITGA6 in inducing metastasis has been reported in several cancer types diagnosed in adults [41, 42], whereas in neuroblastoma, its involvement in cell dissemination was not previously described. Conversely, ITGA5 induction has been recently associated with a metastatic neuroblastoma phenotype [43]. Consistent with previous findings, our results highlight the importance of integrin-mediated pro-metastatic behavior in *LIN28B*-overexpressing neuroblastoma cells. They also sustain the role of LIN28B in facilitating a dynamic remodeling of cell adhesion proteins [44]. The recruitment of FA clusters on the plasma membrane of *LIN28B*-overexpressing cells implies their value for sustaining the migratory properties of tumor cells. In this scenario, the activation of downstream pathways at first involved the PI3K/AKT and Src kinases, followed by a delayed ERK engagement. All of them were described along with a shift toward more motile phenotypes and a favored FA formation [45]. These results imply that *LIN28B* overexpression promotes mesenchymal-like phenotypes whose motility is allowed by the Integrin/FAK axis and dynamic regulation of several intracellular pathways. Moreover, the striking expression of ITGA5/ITGA6 on the SH-SY5Y^{LIN28B} cell membrane may be critical for shaping the interaction between the ECM and tumor cells and, hence, for their motility and invasion capacities. Both characteristics are required for tumor cells' dissemination and metastasis formation. Our findings specify additional roles of LIN28B in the regulation of the interaction between neuroblastoma cells and their surrounding niche. Previous reports affirm the abundance of fibronectin and laminin, high-affinity binding targets of ITGA5 and ITGA6, within the trunk region of the zebrafish embryos [46, 47].

Their neutralization provokes the inhibition of NCC migration, pointing out the relevance of both ECM molecules for the proper function of NCC [47]. Hence, *hLIN28B*-overexpressing zebrafish could allow not only novel understandings of the cell-ECM interactions but may also serve as an *in vivo* model for evaluating the effects of selective integrin inhibitors in the treatment of metastatic neuroblastoma.

In conclusion, we confirmed the oncogenic potential of *LIN28B* and its association with neuroblastoma's onset in the zebrafish model. We showed a positive correlation between *LIN28B* overexpression and the induction of EMT, a process that led to the cell morphology transformation and increment of invasive traits of tumor cells. We additionally highlighted the complexity of the LIN28B-dependent pathways not necessarily involving *let-7a* miRNA. Further, we described the association between *LIN28B* overexpression and the pro-metastatic phenotype of neuroblastoma cells. This feature was triggered by activating *ITGA5* and *ITGA6* expression and by the accumulation of corresponding proteins on the cell membrane. Together, our results suggest that LIN28B sustains integrin-mediated cell spreading and migration by facilitating the formation of focal contacts. The two integrins, ITGA5 and ITGA6, could therefore be new molecular markers in pro-metastatic neuroblastoma. Further investigation on whether they might be potential targets in a group of HR patients remains to be performed.

MATERIALS AND METHODS

Animals. Wild-type (AB/TU) zebrafish [48] and the transgenic line *Tg(sox10:GFP)* [21] were staged and maintained as described previously [49]. All experiments using animal models were approved by the local Ethical Committees and by the Italian Ministry of Health (zebrafish authorization 86/2016-PR; Xenopus authorization 99/2012-A). Xenopus embryos were obtained and staged as described previously [17].

Cell lines. SH-SY5Y cells were purchased from the DSMZ (Germany) and grown in RPMI-1640 (Sigma-Aldrich) with the addition of 2 mM penicillin/streptomycin (Gibco), 2mM L-glutamine (Gibco), and 10% fetal bovine serum (FBS; Gibco). Cell line genotyping was conducted prior to the analyses.

SH-SY5Y^{CTRL} and doxycycline-inducible SH-SY5Y^{LIN28B} cell lines were obtained by a lentiviral infection; pLenti CMV CTRL Blast plasmid (Addgene #17492) and modified pLenti CMV/TO GFP Puro plasmid (GFP was replaced by *LIN28B* ORF, Addgene, #17481) were used. Lentiviral particles were produced by co-transfecting either the CTRL or *LIN28B*

plasmids with the packaging vector psPAX2 (Addgene #12260) and the envelope plasmid pMD2.G (Addgene #12259) into HEK-293T cells (ICLC) in the Opti-MEM culture medium (Gibco) with 1 mg/ml polyethylenimine (Sigma-Aldrich). The cells stably expressing the TetR control gene (SH-SY5Y^{CTRL}) were grown in 7 µg/ml of Blasticidin-S HCl (Gibco) enriched selecting medium. A population of SH-SY5Y^{LIN28B} cells was generated upon growth in 2 µg/ml of puromycin (Gibco) enriched selective medium.

Capped mRNA overexpression. The SP6 Message machine kit (Ambion) was used to transcribe synthetic capped RNA. Zebrafish embryos were injected with 50-100 pg of *lin28b* or *GFP* mRNA at the one-two-cell stage. Injected embryos were raised from 16 hours post fertilization (hpf) to 6 days post fertilization (dpf) and fixed with 4% paraformaldehyde for further analysis. Xenopus embryos were injected with *lin28b* or *GFP* mRNA in one blastomere at the four-cells stage and then fixed with 4% paraformaldehyde for further analysis.

Constructs and probes. The pCS2^{+}-mCherry construct was a kind gift of Dr. Enrico Moro (University of Padua). The pCS2^{+}-GFP was generated from the pME-GFP construct from the Multisite Gateway-based construction kit (Tol2Kit) [50]. Zebrafish *lin28b* cDNAs were obtained by PCR and cloned into the pCS2^{+} vector. The pcDNA3-FLAG-*LIN28B* construct used for the doxycycline-inducible SH-SY5Y cell line was a kind gift from Narry Kim (Addgene plasmid #51373). The following antisense RNA probes were also generous gifts: *dbh, th* [51], and *zash-1a* [52]. The *crestin* probe was generated by amplifying the zebrafish ORF from the wild-type whole embryo cDNA, and the PCR product was cloned into the pCR-II-TOPO vector (Invitrogen). Following sequence verification, antisense riboprobes were generated by *in vitro* transcription with a DIG RNA labeling kit Sp6/T7 (Roche). Antisense riboprobes were also generated following sequence verification through *in vitro* transcription with a DIG RNA labeling kit Sp6/T7 (Roche). The *dre-let-7a* probe was purchased from EXIQON. The Xenopus pCS2^{+}-*lin28b* plasmid was generated by RT-PCR and fully sequenced. The plasmids used for the preparation of *reptin* and *sox10* antisense RNA probes are described elsewhere [53, 54].

Transgenesis. The 5.2-kb promoter region of the *dβh* gene was PCR amplified from the zebrafish BAC clone CH211-270H11 (https://bacpacresources.org/) using the primers dβh-fw (#599): GCG TAC TCC CCC TTT TTA GG and dβh-rev (#600): TGT TGC TTT GTC GTC TTT TGA. The PCR product was cloned into the gateway p5-MCS vector (a kind gift of the Chien lab) using the KpnI/XhoI restriction sites. *LIN28B* was isolated from pcDNA3-Flag-*LIN28B* (Addgene #51373) by BamHI/NotI digestion and cloned into the gateway

middle entry vector pENTR1A using the same restriction enzymes. The full transgenesis construct was gateway assembled [50] to generate the pDEST5.2*dβh-LIN28B*-CG2. The embryos were injected with this DNA construct at the one-cell stage and grown to adulthood. Fin clips from the offspring were used for the genotyping of stable integration and the germline transmission of the transgene. The *Tg(dβh: hLIN28B)* was designated as the '*hLIN28B*' transgenic line in this article.

***In situ* hybridization.** Whole-mount RNA *in situ* hybridization (WISH) of zebrafish and Xenopus embryos was performed, as previously described [55, 17]. Briefly, fixed embryos were permeabilized with 10 µg/ml of Proteinase K and, after five hours of pre-incubation with Hybridization Mix (50% Formamide, 5X SSC, 0.01% Tween-20, 50 µg/ml of heparin, 500 µg/ml of t-RNA), they were incubated with 100 ng/µl of the antisense probe at 65° C. The second day, embryos were extensively washed and incubated in a Blocking Solution (2% sheep serum, 2 mg/ml of BSA in PBS/0.1% Tween-20) for two hours, followed by the addition of the Anti-Digoxigenin antibody (Anti-Digoxigenin-AP Fab fragment, Roche), diluted 1:3000. The embryos were washed several times in PBTw, followed by the incubation with the Staining Solution containing NBT/BCIP (Roche). Stained embryos were sectioned and mounted in 80% glycerol, and images were acquired with a Carl Zeiss Axio microscope.

Immunofluorescence. Immunofluorescence of whole zebrafish embryos was performed essentially as described [56]. Fixed embryos were permeabilized in cold acetone for seven minutes at -20° C, followed by three washes in PBTx (1% Triton X-100 in 1X PBS) for five minutes at RT in agitation. The embryos were blocked in 3% goat serum diluted in PBTx for one hour in agitation, and primary antibodies were then added overnight at 4° C. The embryos were washed three times in PBTx for 10 minutes and incubated with secondary antibodies in 5% goat serum/PBTx in the dark for three hours. Lastly, the embryos were washed two times in PBTx for 10 minutes and transferred in low melting 1.5% agarose (Sigma-Aldrich) and mounted in slides, and images were acquired with a Carl Zeiss Axio microscope and a Leica TCS-SP5-II confocal microscope. Primary and secondary antibodies are listed in Supplemental Table S1.

SH-SY5Y cells were fixed using 4% PFA in PBS for 15 minutes before permeabilization with 0.1% Triton X-100 diluted in PBS. To reduce the background fluorescence, the coverslips were blocked in 3% BSA prepared in PBS for one hour at room temperature. Cells were stained with phalloidin-TRITC, ITGA5, and ITGA6 antibodies. The cells in Figures S5C and S5C were incubated with 3% BSA without cell permeabilization. The

proteins were visualized with a Zeiss LSM 800 confocal microscope. For Paxillin, FAK, and Vinculin staining, the coverslips were fixed and permeabilized as previously described. The coverslips were subsequently treated with primary antibodies for one hour and after PBS washing with a secondary antibody in a dark humid chamber for 45 minutes. The coverslips were subsequently mounted on glass slides and observed by confocal microscopy (Zeiss LSM 800 confocal microscope). The number of FAs per cell was assessed using ImageJ software.

Western blot. Zebrafish-derived proteins were extracted from pools of 30 embryos through mechanical desegregation in Lysis Buffer (Tissue Extraction Reagent I, Life Technologies) supplemented with 1% Protease/Phosphatase Inhibitor Cocktail (Sigma Aldrich). The proteins from neuroblastoma cells were extracted with commercially available lysis buffer (Biosource International) from the samples pre-treated with doxycycline, as described previously [57].

Samples were centrifuged at 14,000 g for five minutes at 4° C, and the protein concentration was determined using the bicinchoninic assay (BCA) method (Pierce BCA Protein Assay Kit, Thermo Scientific). A total amount of 50 µg (zebrafish) and 20 µg (cell lines) of proteins were loaded in the Criterion™ TGX Stain-Free™ Precast Gels 4-20% (Bio-Rad) and then transferred on the nitrocellulose membrane (Midi-size LF PVDF Membrane, TransBlot®Turbo™, Bio-Rad, activated in 100% Methanol) through a semi-dry transfer with the Trans-Blot® TurboTM Transfer System (Bio-Rad). Membranes were blocked with Tropix® I-BLOCK (Thermo Fisher Scientific) for two hours, followed by incubation with primary antibodies at 4° C. The membranes were washed and incubated with secondary antibodies. Uvitec Cambridge was used for chemiluminescence detection, covering the membrane with ECL (ECL SelectTM Western Blotting Detection Reagent, GE Healthcare). The primary and secondary antibodies are listed in Supplemental Table S1.

EdU proliferation and TUNEL cell death assays. A proliferation assay was performed using a commercial kit, EdU-Click 488, as recommended by the manufacturer (Baseclick). Briefly, three independent pools of 15 living embryos were collected in microcentrifuge tubes, and 1 mM EdU was added, followed by incubation on ice for a variable time depending on their developmental stage. The embryos were washed with cold E3 medium on ice and then incubated for 30 minutes at 28.5° C. After that, embryos were fixed with 4% paraformaldehyde for two hours and permeabilized in 10 µg/ml Proteinase K. To detect proliferating cells, embryos were incubated in the Reaction Cocktail containing 10 mM of Dye Azide for three hours. In following, the embryos were used for the coupled

immunostainings to detect the SCG or the NCC. Cell death assay was performed using a commercial kit, Click-iT Plus TUNEL Assay, following manufacturer instruction (Life Technologies). Fixed embryos were permeabilized with 10 µg/ml Proteinase K and subsequently incubated with an Equilibration Buffer (TdT reaction buffer) for one hour. Next, the embryos were incubated with the TdT enzyme for 90 minutes at 37° C and then with the Reaction Cocktail for 30 minutes at 37° C. The embryos were fixed again in 4% paraformaldehyde for 30 minutes, and the immunofluorescence with the anti-GFP antibody was then performed (see Supplemental Table S1). The embryos were then mounted in 3% methylcellulose, and images were acquired through a Zeiss LSM 800 confocal microscope.

Fluorescent cell labeling, embryo preparation, and tumor cell implantation. After seven days of doxycyclin administration, SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells were labeled with the Vybrant® DiI Cell-Labeling Solution (Invitrogen) according to the manufacturer's instructions. The dechorionized (2 dpf) zebrafish embryos were anaesthetized with 0.003% tricaine (Sigma-Aldrich) and positioned on a 10 cm Petri dish coated with 3% agarose. Approximately 200 cells were injected within the duct of Cuvier of the anesthetized *Tg(fli1:GFP)* embryos using borosilicate glass capillary needles (OD/ID: 1.0/0.75 mm, WPI), a Pneumatic Picopump, and a micro-manipulator (WPI). After implantation, zebrafish embryos were maintained at 33° C. Embryos showing less than 30-40 cells after four hours post-injection were discarded from the analysis. At least 50 embryos per group were analyzed from three independent experiments. The embryos were live photographed using a Nikon C2 H600L confocal microscope (20X water dipping objective).

Time-lapse. *Tg(sox10:GFP)* embryos were injected with *LIN28B* mRNA and allowed to develop for 19-20 hpf. Anesthetized embryos were embedded in 1.5% agar, mounted in a heated chamber, and imaged with a Zeiss Axio Observer microscope for live cell imaging for eight hours. Quantification of displacement was measured in a 40 µm Z-stack, tracked using Fiji software. The quantification of the percentage of the migrated distance relative to Figure 3B was the measurement of the straight-line distance from the first position (neural tube) to the last position labeled with *crestin* riboprobe, with a higher number indicative of more distance between the first and last point. Statistical analysis was conducted in Excel followed by a two-tailed Student's t-test. Neuroblastoma cells were analyzed in a 37° C heated chamber with 5% CO_2. Cell motility was captured over a 12-hour period at 10 minute intervals, and tracks were recorded using the Fiji "manual tracking" plug-in. For

functional integrin blocking, the attached cells were pre-incubated for 30 minutes before migration analysis either with 10 µg/ml of mouse anti-human-ITGA6 or mouse anti-human-ITGA5 antibodies in 24 well plates (see Supplemental Table S1). The appropriate IgG antibodies (Mouse IgG2b, Millipore; Mouse IgG1, Santa Cruz Biotechnology) were used as negative controls. Cell motility was captured over a 12-hour period at 10-minute intervals.

The Wilcoxon test was used to assess the p-values quantifying the significance of the differences. All statistical analyses were performed using the R statistical software 'stats' package.

Flow cytometric analyses. Zebrafish single-NCC were obtained from the pool of 40 embryos. *Tg(sox10:GFP)* and wild-type zebrafish embryos were dissected to remove the head and washed, and the tails were mechanically desegregated by constant pipetting in Hanks' buffer (EMD Millipore Corporation). Single-NCC were obtained using Trypsin-EDTA (EMD Millipore Corporation) and Collagenase/Dispase (Sigma-Aldrich). After resuspending the NCC in Hanks' buffer, analyses were performed through Cytomics FC 500 (Beckman Coulter). Wild-type uninjected embryos were used as blanks.

SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells (0.3-0.5 x 10^6 cells) were incubated with PeCy5 anti-human CD49f (ITGA6, Becton Dickinson) and analyzed using a CytoFLEX Flow cytometer (Beckman Coulter). The percentage of CD49e (ITGA5) positive cells was validated upon coupled primary (1 µg) and secondary antibody labeling (see Supplemental Table S1). The percentage of CD49e- and CD49f-positive cells in both SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells were considered by setting an appropriate gate on the living cell population (Propidium iodide negative, 10 mg/ml, Roche). Unlabeled cells for each line were first acquired to ensure labeling specificity.

Colony formation assay. Two thousand cells were seeded in a 24-well plate using a MethoCult semi-solid medium (Stemcell Technologies) and were grown for two weeks. Colonies (foci) were visualized after incubation with MTT. The total colony number was counted under a light microscope in ten random fields and determined by Fiji software.

Transwell migration assay. SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells were seeded onto inserts without Matrigel (BD Biosciences) in a serum-free medium. Inserts were placed into wells containing a medium supplemented with 10% FBS. After 24 hours, the cells on the upper surface of the filters were removed, and the inserts were stained with Hoechst (Sigma-Aldrich) and cristalviolet. The numbers of migrated cells were counted under a light

and a fluorescent microscope in ten random fields. Using Fiji software, the number of migrated cells per image was determined.

Matrigel invasion assay. SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells (5×10^4) were seeded onto matrigel-coated transwell chambers (BD Biosciences) with an 8-µm pore size filter using an FBS gradient (0-10%) from the top to the bottom well. After 24 hours of incubation, the filter was removed and washed, and the cells that invaded the lower side of the filter were stained with calcein (Sigma-Aldrich). The absorbance was measured using the VICTOR™ Multilabel Plate Reader (PerkinElmer, Waltham, MA) at 495 nm.

Scratch test. SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells (3×10^4) were plated within each of the two-cell culture reservoirs separated by a 500-µm-thick silicone wall (IBIDI, Milano, Italy). The day after, the silicone insert was removed and the cells were allowed to grow for another 48-72 hours. Images were taken every 24 hours by a Nikon Eclipse TS100 microscope (Nikon Eclipse TS 100, Southern Micro Instruments, Marietta, GA) equipped with a Nikon Coolpix camera. Wound healing was analyzed by Fiji software for each time point.

RNA analysis. Total RNA was extracted from the pool of 30 embryos or cell pellets using TRIzol Reagent (Invitrogen), and 2 µg of RNA were used for cDNA synthesis using Super Script II (Invitrogen), according to the manufacturer's recommendations. The cDNA was subjected to PCR reaction using the AmpliTaq DNA polymerase (Thermo Fisher Scientific).

The expression of *lin28b*, cdh1, snail1a, twist1a, and vimentin transcripts was analyzed by real-time quantitative PCR (qPCR) using the SYBR Green PCR Master Mix (Applied Biosystems) in an Applied Biosystems 7900HT Fast Real Time PCR System. Primer dissociation curves were checked in each run to ensure primer specificity in human and zebrafish mRNA. The expression of *gapdh* was used as a normalizer in each sample, and triplicate PCR reactions were carried out. Primers were designed using the Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/input.htm) and are listed in Supplemental Table S2.

MicroRNA (miRNA) measurement by qPCR. Total RNA was isolated from SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} as well as from the pool of 30 embryos using the Qiazol Lysis Reagent (Qiagen), and 10 ng of total RNA solution were reverse transcribed using the miScript II RT Kit (Qiagen). Specific miRNA levels were quantified by qPCR using the TAQMAN microRNA Assay (Life Technologies) for *hsa-miR-let-7a* together with the universal RT

primer, according to the manufacturer's protocol. The relative quantities of the miRNAs were calculated using the Cq value after normalization to control the miRNA (*RUN6B*).

Quantification and statistical analysis. All data were expressed as the mean ± SEM of experimental triplicates. Statistical significance was determined using the Student's t-test; $p < 0.05$ was considered significant and was marked with an asterisk (*) on the graphs while $p < 0.01$ was marked with two asterisks (**).

The statistical significance of the cells' migratory differences related to time-lapse experiments was calculated using the Wilcoxon test (from the R software 'stats' package), and boxplots (using the R software 'stats' package) reported the quartile distribution of related variables.

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DECLARATION OF INTEREST

The authors declare no potential conflicts of interests.

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FIGURE LEGENDS

Figure 1. *Lin28b* overexpression causes sympathoadrenal cell loss. (**A**) *In situ* hybridization for the progenitor markers *dβh* and *th* in the superior cervical ganglia (dashed squares) of the control (*ctrl*) and *lin28b* embryos at 80 hpf. Scale bar: 100 µm. The fraction of embryos displaying the corresponding phenotype is provided in each panel. (**B**) The Western blot analysis of the indicated proteins in the control (*ctrl*) and *lin28b* embryos at 72 hpf. The molecular weights are indicated in Kilodaltons (KD). Protein quantification is shown in the right panel. The error bars represent SEM. $^{**}p < 0.01$. (**C**) Labeling of *zash1a* mRNA in 72 hpf control (ctrl) and *lin28b* zebrafish embryos. Dashed circles highlight the developing superior cervical ganglia. The fraction of embryos displaying the corresponding phenotype is provided in each panel. (**D**) The schematic experimental overview is presented on the left side. On the right side, a representative example of a Xenopus embryo (out of 76 embryos analyzed) at stage 35 is shown, indicating the expression of the sympathoadrenal marker *reptin* in the developing adrenal medulla (dashed squares). The lateral view of the *lin28b* injected side (right image) and the uninjected side (ctrl side, left image) of the same embryo is presented. (**E**) Proliferation of sympathoadrenal progenitors identified by tyrosine hydroxylase (TH) immunolabeling (red) coupled with EdU incorporation (green) in the control (*ctrl*) and *lin28b* embryos at 80 hpf. The quantification is shown in the right panel ($n = 30$ embryos for each group). *n.s.*: $p > 0.05$. Scale bar: 100 µm. (**F**) The control (*ctrl*) and *lin28b* embryos were stained for activated Caspase-3 (green) and TH (red) to detect apoptosis in the superior cervical ganglia at 80 hpf. Scale bar: 100 µm.

Figure 2. *LIN28B*-induced neuroblastoma in zebrafish. (**A**) Macroscopic views of the control (*ctrl*) and *hLIN28B* zebrafish at six months of age, showing a tumor mass found in the anterior abdomen (white dashed line). (**B**) Haematoxylin-Eosin (H&E) staining of the sagittal sections of the tumors in the IRG of six-month-old fish. Scale bar: 100 µm. (**C**) Immunohistochemical analyses of the sagittal sections of the *Tg(dβh:GFP)* control (*ctrl*) animals and the *hLIN28B* transgenic fish using tyrosine hydroxylase (TH), HuC/D, and Synaptophysin antibodies. Nuclei were counterstained with hematoxilin (purple). Scale bar: 100 µm.

Figure 3. Lin28b increased the migration speed of trunk NCC. (**A**) Sequential images of a time lapse movie in *Tg(sox10:GFP)* embryos showing the motility of NCC in *lin28b* embryos (white arrows) and controls *(ctrl)*. The reported frames were recorded at 19 hpf

(T0) after 109 minutes (T109) and 292 minutes (T292). Scale bar: 200 μm. (**B**) The representative scheme depicting the analysis of zebrafish trunk NCC migration. The migrated distance of NCC (purple) toward the dorsal aorta (dotted line) in the same region of the trunk (from somite 8 to somite 12) of embryos injected with the GFP mRNA (*ctrl*) or with *lin28b* mRNA (*lin28b*) has been analyzed. (**C**) A total of 50 injected embryos per group were stained with the *crestin* riboprobe at 19 hpf (upper panel) and 24 hpf (lower panel), displaying the migration of trunk NCC toward the ventral region of the notochord (dotted black lines). The quantification of the percentage of the migrated distance is shown in the graph. *$p < 0.05$. Scale bar: 100 μm. (**D**) The trunk *sox10*+ NCC of the uninjected (ctrl; left image) and *lin28b* injected (right image) sides of the Xenopus analyzed at stage 30 (out of 89 embryos analyzed). A migrated distance of NCC (black square brackets) toward the dorsal aorta is depicted. (**E**) Imaging of the control (*ctrl*) and *lin28b* embryonic pigment patterns at 48 hpf showed melanocytes populating the yolk sac and the equal yolk extension (dashed squares) between the two groups (the average number of melanocytes in controls = 29 ± 4 and *lin28b* embryos = 28 ± 5, *p-value* = 0.31). (**F**) The HuC/D antibody staining (green signal) revealed the presence of segmentally arranged dorsal root ganglia in the trunk of both the controls (*ctrl*) and *lin28b* embryos at 4 dpf (the average number of HuC/D$^+$ cells in controls = 20 ± 2 and *lin28b* embryos = 22 ± 3, *p-value* = 0.2). For individual cell counts composing the zebrafish dorsal root ganglia (DRG), the five most caudal DRG labeled with the HuC/D antibody were counted for each condition (*n* = 30 embryos per group). (**G**) The number of HuC/D-positive enteric neurons (green-positive nuclei) remained comparable between the *lin28b* embryos compared to the control (*ctrl*) siblings at 6 dpf (the average number of HuC/D$^+$ cells in controls = 36 ± 4 and *lin28b* embryos = 33 ± 5, *p-value* = 0.4. *n* = 30 embryos per group). Scale bar: 100 μm.

Figure 4. The Overexpression of *LIN28B* in SH-SY5Y cells potentiates their migratory phenotype. (**A**) The qPCR analysis showed a significantly increased expression of the stemness markers *Nestin*, *Sox2*, and *Oct4* upon 7 days of *LIN28B* overexpression. The results show the average of three biological replicate experiments. *GAPDH* was used as the internal control. The error bars represent SEM. **$p < 0.01$, *$p < 0.05$. (**B**) The *let-7a* level was evaluated at several time points upon LIN28B induction (48 hours [h], 7 days [d], and 14 days) through qPCR analyses. The results display the mean values of the relative expression ± error bars representing SEM. **$p < 0.01$; *n.s.*: $p > 0.05$. (**C**) The morphology of the SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells treated with doxycycline for 7 days (d). Scale bar: 100 μm. (**D**) Phalloidin (F-Actin) staining (green) of the SH-

SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells upon 0 hours (h) and 7 days (d) of doxycycline treatment. Scale bar: 50 μm. (**E**) The SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells were examined for their migratory capacity in a wound healing assay upon 7 days of doxycycline administration. Representative photographs are reported in the left panel. The percentage of wound closure at every time point was calculated relative to the wound width at 0 hours (h, right graph bars). *n.s.*: $p > 0.05$; $^{**}p < 0.01$. Scale bar: 1000 μm. (**F**) The transwell migration assay on SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells upon 7 days of doxycycline administration. Migrated cells were stained with DAPI (blue) and cristalviolet (insets) and counted in following for 10 separated fields. $^{**}p < 0.01$. Scale bar: 100 μm. (**G**) The cell motility tracks of SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells (n = 15 cells tracked) treated with doxycycline for 7 days. The red dots indicate the final cell position on their path. The distances are presented in μm. (**H**) The dot plot depicting the average cell speed (μm/min) of the SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells upon 7 days of doxycycline administration. $p = 1.4 \times 10^{-3}$.

Figure 5. LIN28B activates EMT in neuroblastoma cells and increases their invasive potential *in vivo.* (A) The assessment of transwell migration was determined 24 hours after seeding the SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells pre-treated with doxycycline for 7 days. The quantification of migration was determined by measuring the mean fluorescence intensity, and the results are presented on the histogram. $^{**}p < 0.01$. (**B**) Fluorescent microscopy images of the trunk region of the *Tg(fli1:GFP)* zebrafish embryos with marked vessels (green) injected at 48 hpf with SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells (red signal) 7 days upon treatment with doxycycline. Visualizations of cells at 4 hours post-implantation (hpi) and at 1 day post-implantation (dpi) are depicted. White arrowheads indicate cells disseminated throughout the trunk vasculature. n = number of animals analyzed. Scale bar: 100 μm. (**C**) The Western blot analysis for the indicated EMT markers on SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} upon 7 days of doxycycline. β-Tubulin was used to show equal protein loading. The molecular weights are indicated in Kilodaltons (KD). (**D**) The overexpression of *LIN28B* for 7 days increased the expression levels of the mesenchymal markers *Twist1*, *Snail*, *N-cadherin*, and *Slug*. *GAPDH* was used as an internal control. $^{**}p < 0.01$. (**E**) qPCR analysis for the indicated EMT markers in the ctrl and *lin28b*-overexpressing zebrafish embryos at 48 hpf. The results are normalized to the *gapdh* internal control. The error bars represent SEM. **, $p < 0.01$. (**F-G**) The western blot analysis of 72 hpf (**F**) and 96 hpf (**G**) zebrafish embryos extracts for the indicated EMT markers and Lin28b protein. β-Actin was used as a loading control. The molecular weights

are indicated in Kilodaltons (KD). The quantification of the same is shown in the right graph bars. *$p < 0.05$; **$p < 0.01$.

Figure 6. Ectopic expression of *LIN28B* triggers integrin expression and increases cell motility potential. (**A**) qPCR analysis for *ITGAs (ITGA1-6, 11) and ITGBs (ITGB1, 4)* upon 7 days of *LIN28B* overexpression. *GAPDH* was used as an internal control gene. *n.s.*: *$p < 0.05$; **$p < 0.01$. (**B**) Immunofluorescence on SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells upon 7 days of doxycycline administration for ITGA5 (green, upper panel) and ITGA6 (lower panel) protein deposition (white arrows). The nuclei were counterstained with DAPI (blue). Scale bar: 50 μm. Insets correspond to higher magnification of the ITGA5/6-positive cells. (**C-D**) The flow cytometry profile of SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells over 7 days of doxycycline administration, showing (**C**) ITGA5-positive (CD49e, $p = 0.05$, left panel) and (**D**) ITGA6-positive (CD49f, right panel, $p = 0.04$) cells. (**E**) The cell motility tracks of SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells treated with doxycycline for 7 days and incubated with a non-neutralizing IgG control antibody or with the blocking antibody (ab) against ITGA5 (ITGA5ab; 12 cells tracked) prior to the time-lapse imaging. The red dots indicate the final position of the cell migration path. The corresponding average cell speeds of SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} are depicted in the box plots. IgG plot: $p = 3.1 \times 10^{-6}$; ITGA5 plot: $p = 1 \times 10^{-1}$. (**F**) The cell motility tracks of SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells treated with doxycycline for 7 days and incubated with a non-neutralizing IgG control antibody or the blocking antibody (ab) against ITGA6 (ITGA6ab; 12 cells tracked) prior to the time-lapse imaging. The red dots indicate the final position of the cell migration path. The corresponding average cell speeds of SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} are depicted as the box plots. IgG plot: $p = 6 \times 10^{-4}$; ITGA6 plot: $p = 8.9 \times 10^{-1}$.

Figure 7. Ectopic expression of *LIN28B* triggers focal adhesion re-organization and activation of integrin/FAK-related signaling pathway.

(**A**) The immunostaining of the focal adhesion (FA) associated adaptor proteins Paxillin, FAK, and Vinculin (green) in combination with Phalloidin (F-Actin, red) is presented. Local redistribution of the proteins in the FAs is indicated by white arrowheads and evidenced as insets. The nuclei were counterstained with DAPI (blue). FAs of 5 cells within 3 random fields were counted, and data were presented as mean ± SEM from 3 independent experiments. **$p < 0.01$. (**B**) The Western blot analysis for the indicated markers on SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} upon 7 days of doxycycline administration. An antibody against β-Tubulin was used as a loading control. The molecular weights are indicated in Kilodaltons (KD).

Supplemental Figure 1. Generation of a *lin28b*-overexpressing zebrafish model. (**A**) Wild-type zebrafish embryos were injected with *lin28b* or GFP mRNA as a control (*ctrl)*. The levels of the *lin28b* transcript were assessed by RT-PCR analysis at several time points (18-80 hpf). *β-Actin* was used as an internal control. The transcript length is indicated as the number of base pairs (bp). An immunoblot (IB) assay was adopted for the analysis of the Lin28b protein levels upon injection at the same time points. β-Actin was used as a loading control. The molecular weight of the proteins was indicated in Kilodaltons (KD). hpf: hours post fertilization. (**B**) Left panel: images of 48 hpf control (*ctrl*) embryos injected with mCherry mRNA or GFP mRNA. The Brightfield images show no macroscopic alterations after the injection of the control mRNAs. Central panel: representative wild-type uninjected embryo at 48 hpf, showing the absence of endogenous mCherry and GFP fluorescent signals. Right panel: IB assay, followed by a long exposure to detect the wild-type Lin28b protein levels during the indicated developmental stages. The molecular weights are indicated in Kilodaltons (KD). (**C**) The brightfield images of the control (*ctrl*) and *lin28b* embryos at the indicated developmental stages, showing the absence of visible malformations in the *lin28b*-overexpressing fish and the corresponding *ctrl*. (**D**) The analysis of *miR-let-7a* mRNA levels at 72 hpf by whole-mount *in situ* hybridization (WISH). *Lin28b* embryos showed a marked loss of staining for *let-7a* when compared to the *ctrl*. The fraction of embryos displaying the corresponding phenotype is provided in each panel. Scale bar: 100 μm. The qPCR analysis (right panel) showed a significant down-regulation of the *let-7a* transcript in 72 hpf *lin28b* embryos compared to *ctrl*. The error bars represent SEM. $^{**}p < 0.01$.

Supplemental Figure 2. *Lin28b* overexpression provokes no apoptosis of sympathoadrenal precursors. Immunofluorescence staining of the superior cervical ganglia (SCG) for the TH sympathoadrenal marker (TH^+, green) of the 80 hpf control (*ctrl*) and *lin28b* embryos stained with the TUNEL assay for cell death evaluation (red signal). Scale bar: 100 μm.

Supplemental Figure 3. *Lin28b* does not influence the proliferation or viability of NCC. (**A**) Cytofluorimetric analysis revealed no significant differences in the percentage (%) of trunk NCC between the *lin28b* -overexpressing and the control (ctrl) *Tg(sox10:GFP)* embryos. Uninjected embryos (BLANK) at 19 hpf, 24 hpf, and 28 hpf were used for the flow cytometry parameters set-up. hpf: hours post fertilization; SS: side scatter; *n.s.*: $p > 0.05$. (**B**) Lateral views of the *Tg(sox10:GFP)* control and *lin28b* embryos stained with EdU (green) at 24 hpf. The related quantification of proliferating NCC in three independent

experiments is shown in the right panel (data represent means ± SEM; *n* = 30 embryos for each group). *n.s.*: $p > 0.05$. Scale bar: 100 µm. (**C**) Lateral views of the trunk NCC (sox10+, green) of the control (*ctrl*) and *lin28b* embryos analyzed with the TUNEL assay (red signal) at 24 hpf. The quantification of TUNEL-positive NCC of three independent experiments is shown in the right panel (data are shown as means ± SEM; *n* = 30 embryos for each group). *n.s.*: $p > 0.05$. Scale bar: 100 µm.

Supplemental Figure 4. Long-term *LIN28B* overexpression enhances the migration capability of neuroblastoma cells. (**A**) Representative images of clonogenic assays are shown for each cell line. Cells were analyzed for the foci formation during the course of 7 and 14 days (d) of doxycycline administration. The number of colonies was calculated by Fiji software (right graphs) after coloration with MTT, and the results are shown as the mean ± SEM of three independent experiments. *n.s.*: $p > 0.05$. (**B**) The Western blot analysis in SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells upon 7 and 14 days (d) of doxycycline administration using the PCNA and GAPDH antibodies as a loading control. The molecular weights are indicated in Kilodaltons (KD). (**C**) SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells were treated with doxycycline for 14 days (d), and changes in the cell morphology were observed and photographed by light microscopy. Scale bar: 100 µm. (**D**) Phalloidin staining (F-Actin) in SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells upon 14 days (d) of doxycycline treatment revealed a shift in the cell morphology and a reorganization of the stress fibers. Scale bar: 50 µm. (**E**) The single-cell motility tracks over time (h: hours) of SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} after 7 days (d) of doxycyclin induction. Asterisks (white for SH-SY5Y^{CTRL} and yellow for SH-SY5Y^{LIN28B}) indicate the same cell positioning over time. (**F**) The cell motility tracks of SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells (*n* = 17 cells tracked) treated with doxycycline for 14 days and captured by time-lapse imaging over a 12-hour period at 10 minute intervals using the Image J "manual tracking" plug in. The red dots indicate the final position of the cells during their migration path. (**G**) The dot plot depicting the average cell speed (µm/min) of SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells upon 14 days of doxycycline administration. The data are representative of three experiments. The error bars indicate SEM. $p = 1.5 \times 10^{-5}$. (**H**) The single-cell motility tracks over time of SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} after 14 days (d) of doxycyclin induction. The asterisks (white for SH-SY5Y^{CTRL} and yellow for SH-SY5Y^{LIN28B}) indicate the same cell positioning over time.

Supplemental Figure 5. Prolonged *LIN28B* overexpression triggers EMT. (**A**) The Western blot analysis showing the mesenchymal markers Twist1, Vimentin, Snail, and N-

cadherin and the epithelial E-cadherin after 14 days of *LIN28B* overexpression. GAPDH was used to show equal protein loading. The molecular weights are indicated in Kilodaltons (KD). (**B**) Immunofluorescence of the SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells over 14 days of doxycycline administration showed the induction of ITGA5 protein (green, upper panel) and ITGA6 (green, lower panel) in the SH-SY5Y^{LIN28B} cells (white arrows). Nuclei were counterstained with DAPI (blue). Scale bar: 50 µm. (**C-D**) Immunofluorescence analysis of the SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} cells over (**C**) 7 days (d) and (**D**) 14 days (d) of doxycycline administration, labeled with the ITGA5 antibody (green, upper panel) and ITGA6 (green, lower panel) without the membrane permeabilization. The staining with only secondary antibodies was used as a negative control (negative ctrl). The nuclei were labeled with Hoechst (blue). White arrows point out the integrin protein accumulation. Scale bar: 50 µm.

Supplemental Figure 6. Long-term *LIN28B* overexpression stimulates focal adhesion creation. (**A**) Confocal immunofluorescence images of SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} stained with antibodies against Paxillin, FAK, and Vinculin (green) after 14 days of doxycyclin administration. F-Actin filaments were labeled with phalloidin (F-Actin; red), and the nuclei were counterstained with DAPI (blue). Scale bar: 20 µm. Statistical analysis of the number of focal adhesions (FAs) per cell is presented. FAs of five cells of three random fields were counted. Columns represent the means from three independent experiments, and the bars represent the SEM. $^{**}p < 0.01$. (**B**) The Western blot analysis for the indicated markers on SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} upon 7 days of doxycycline induction. β-Tubulin was used as a loading control. The molecular weights are indicated in Kilodaltons (KD). (**C**) The Western blot analysis for the indicated markers on SH-SY5Y^{CTRL} and SH-SY5Y^{LIN28B} upon 14 days of doxycycline induction. β-Tubulin was used as a loading control. The molecular weights are indicated in Kilodaltons (KD).

Supplemental Video S1. Related to Figure 3. LIN28B increased the migration of trunk neural crest cells (NCC). *Tg(sox10:GFP)* embryos were injected with (**A**) mCherry (ctrl) or (**B**) *lin28b* mRNA and time lapse on trunk NCC was performed on 19 hpf embryos for eight hours.

Supplemental Video S2. Related to Figure 4. Overexpression of *LIN28B* in SH-SY5Y cells affects their pro-migratory potential. Time-lapse video of (**A**) SH-SY5Y^{CTRL} and (**B**) SH-SY5Y^{LIN28B} cells treated with doxycycline for 7 days and seeded into a plastic chamber. Frames were captured over a 12-hour period at 10 min intervals.

Supplemental Video S3. Related to Supplemental Figure 4. Overexpression of *LIN28B* in SH-SY5Y cells affects their pro-migratory potential. Time-lapse video of (**A**) SH-SY5Y^{CTRL} and (**B**) SH-SY5Y^{LIN28B} cells treated with doxycycline for 14 days and seeded into a plastic chamber. Frames were captured over a 12-hours period at 10 min intervals.

Supplemental Video S4. Related to Figure 6. Overexpression of *LIN28B* in SH-SY5Y cells affects their pro-migratory potential. Time-lapse video of (**A**) SH-SY5Y^{CTRL} and (**B**) SH-SY5Y^{LIN28B} cells pre-treated with doxycycline for 7 days and followed by treatment with 10 µg/ml of IgG negative control antibody for 30 minutes. Frames were captured over a 12-hour period at 10 min intervals.

Supplemental Video S5. Related to Figure 6. Pro-migratory potential of *LIN28B* overexpressing SH-SY5Y cells in impaired upon ITGA6 block. Time-lapse video of (**A**) SH-SY5Y^{CTRL} and (**B**) SH-SY5Y^{LIN28B} cells pre-treated with doxycycline for 7 days followed by 30 minutes treatment with 10 µg/ml of ITGA6 blocking antibody. Frames were captured over a 12-hour period at 10 min intervals.

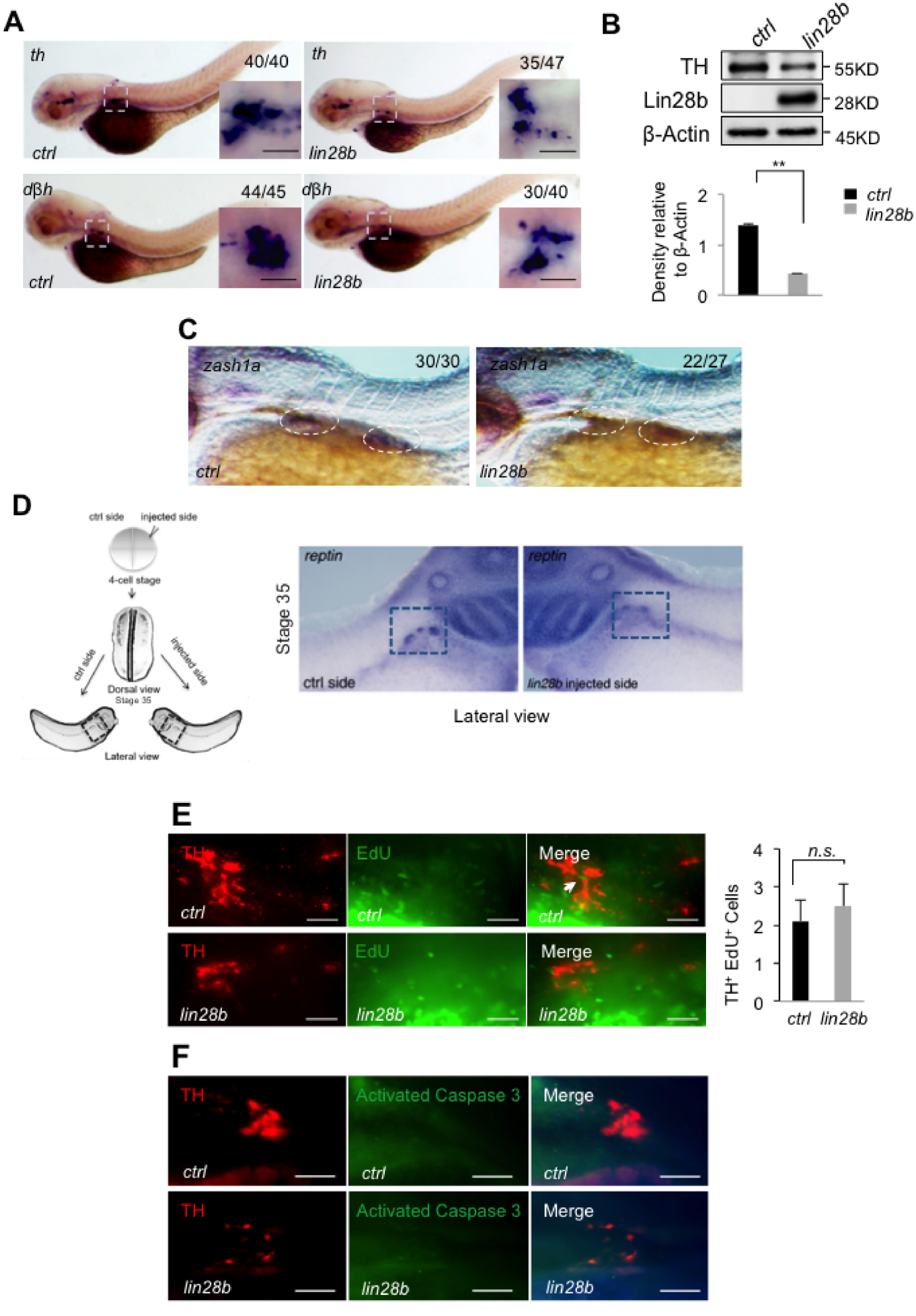
Supplemental Video S6. Related to Figure 6. Overexpression of *LIN28B* in SH-SY5Y cells affects their malignant phenotype. Time-lapse video of (**A**) SH-SY5Y^{CTRL} (**B**) and SH-SY5Y^{LIN28B} cells pre-treated with doxycycline for 7 days followed by 30 minutes treatment with10 µg/ml of IgG negative control antibody. Frames were captured over a 12-hour period at 10 min intervals.

Supplemental Video S7. Related to Figure 6. Pro-migratory potential of *LIN28B* overexpressing SH-SY5Y cells in impaired upon ITGA5 block. Time-lapse video of (**A**) SH-SY5Y^{CTRL} (**B**) and SH-SY5Y^{LIN28B} cells pre-treated with doxycycline for 7 days followed by 30 minutes treatment with 10 µg/ml of ITGA5 blocking antibody. Frames were captured over a 12-hour period at 10 min intervals.

Supplemental Table S1. List of primary and secondary antibodies used for immunofluorescence and western blotting studies.

Supplemental Table S2. List of primers used for RT-PCR and qPCR studies.

Figure 1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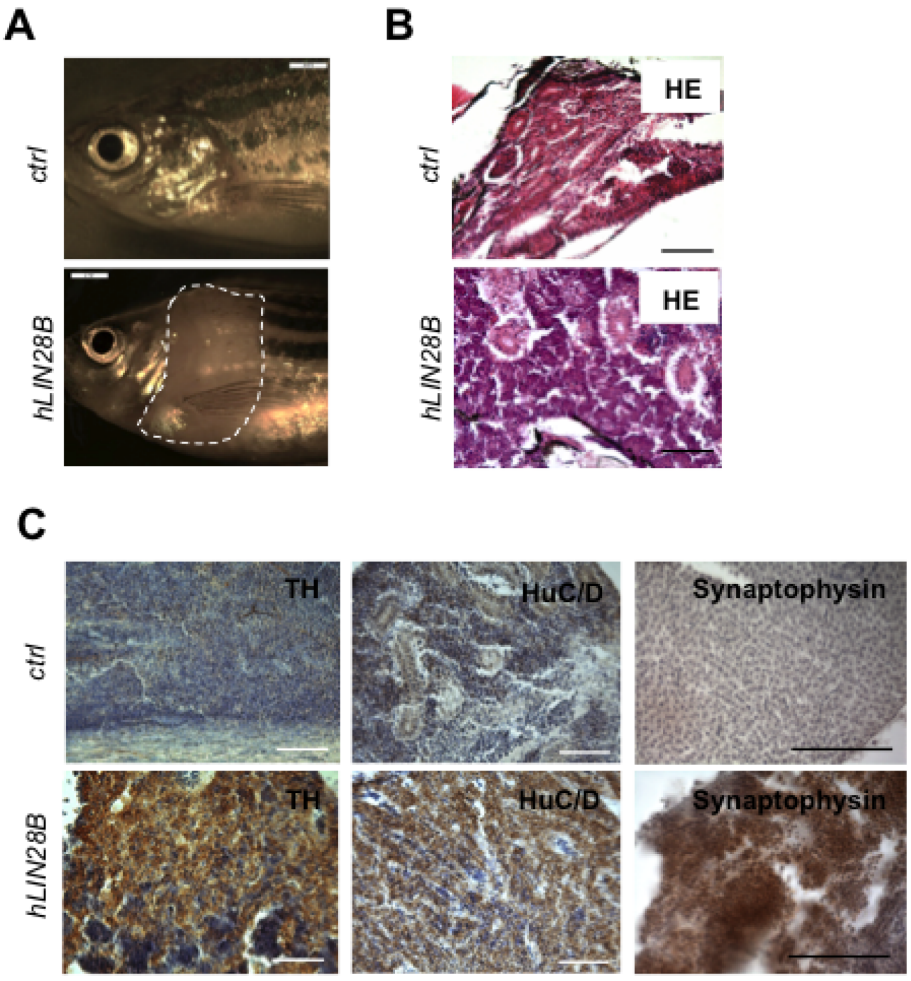
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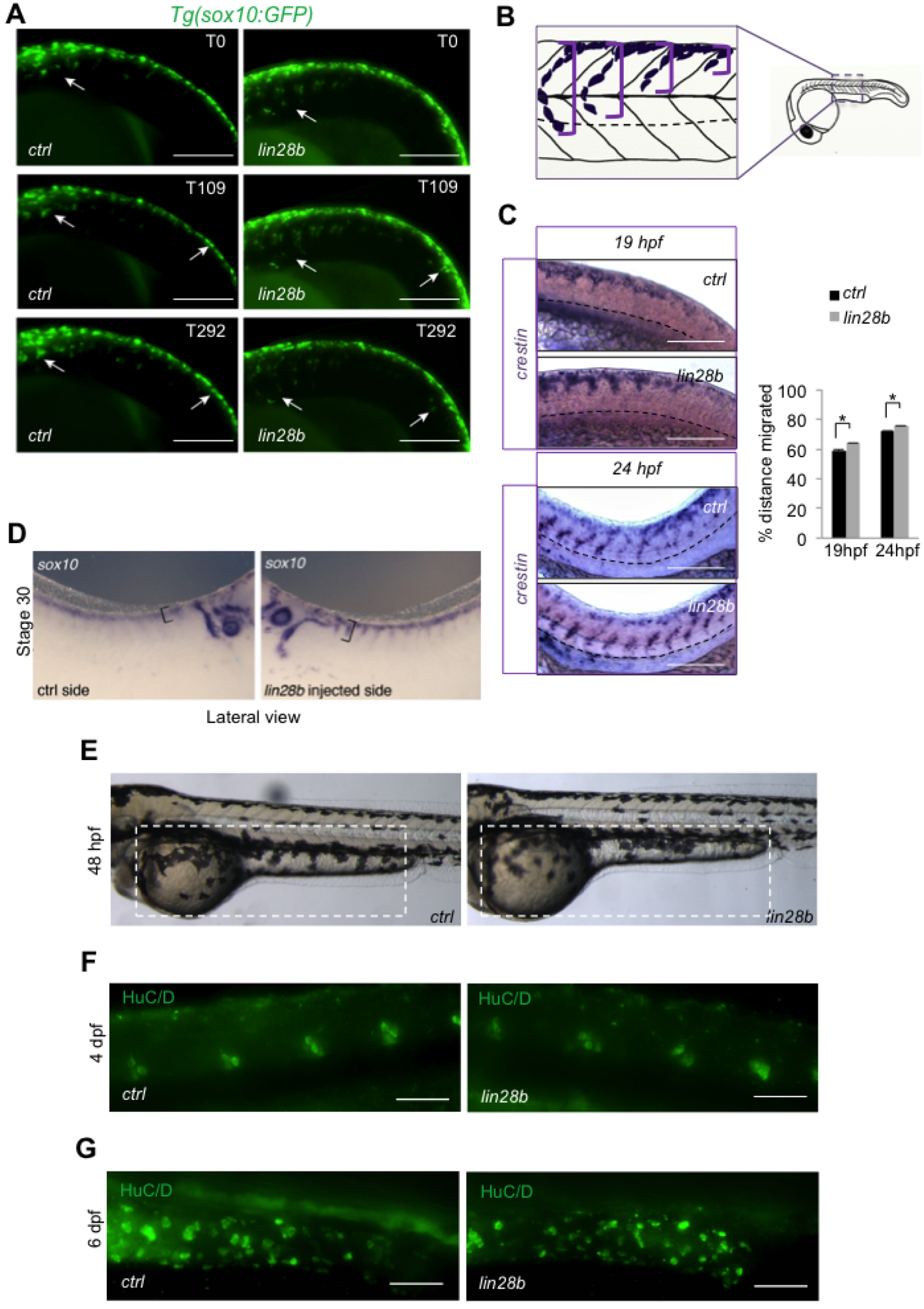
Figure 3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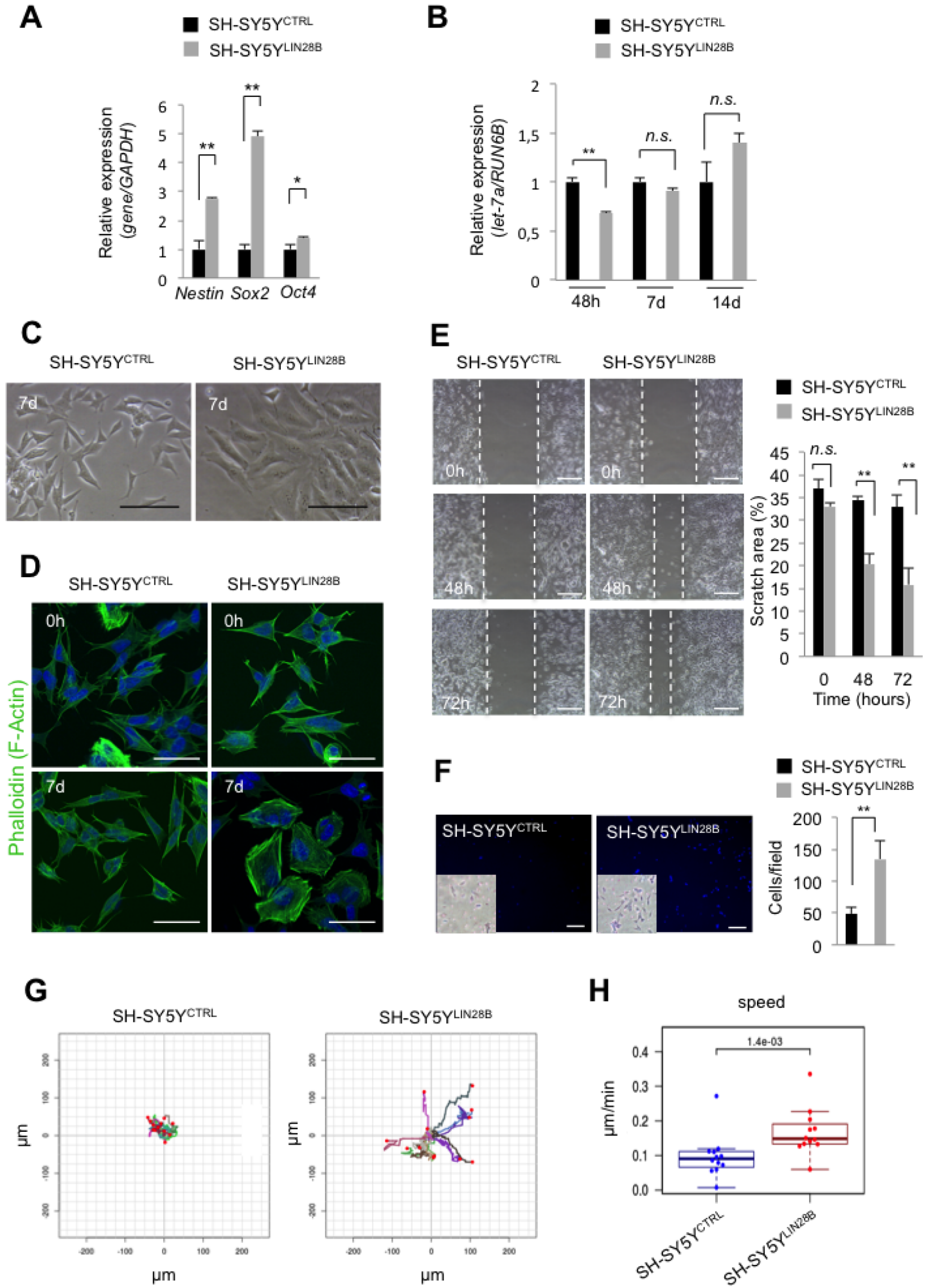
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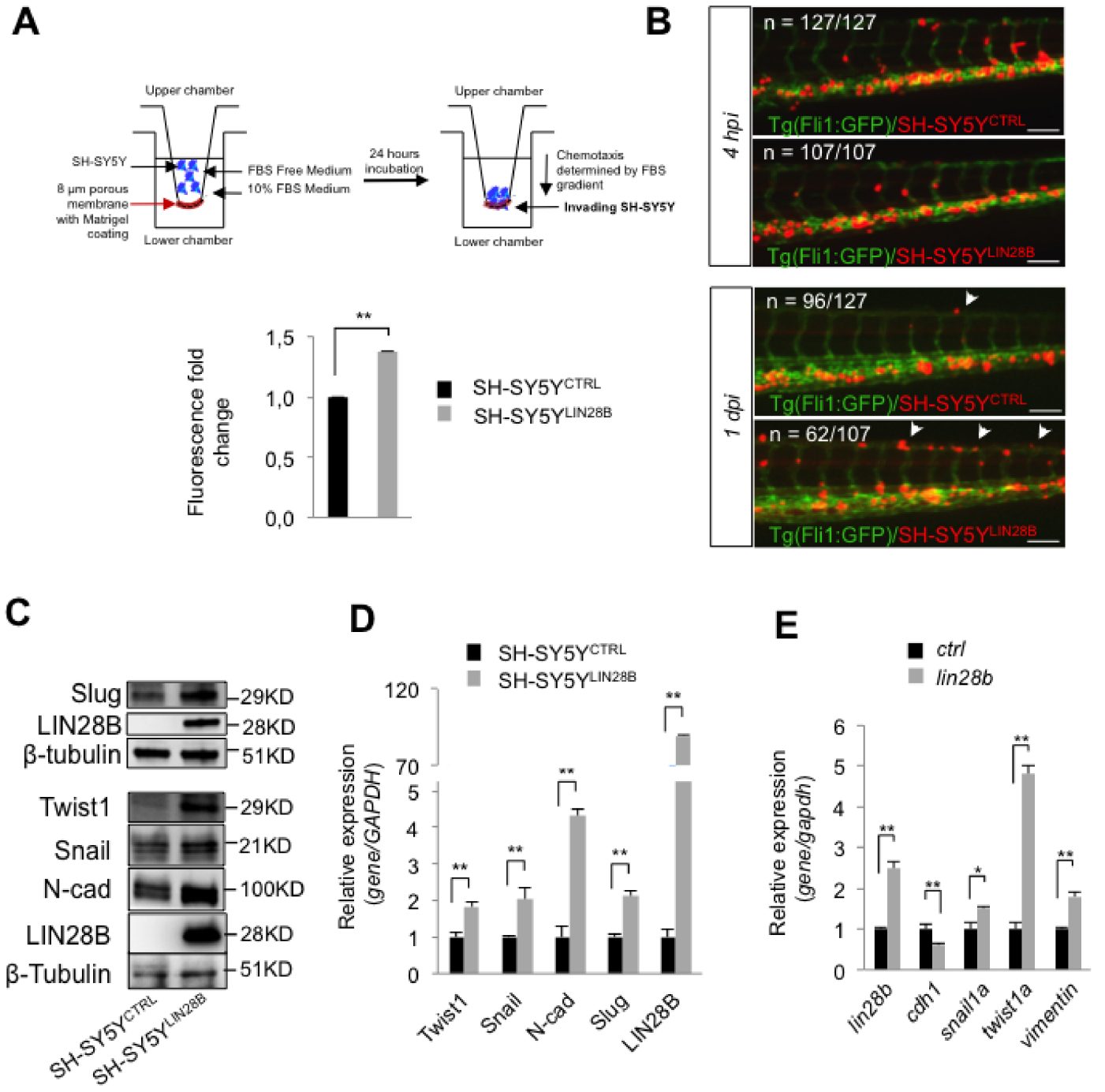
Figure 5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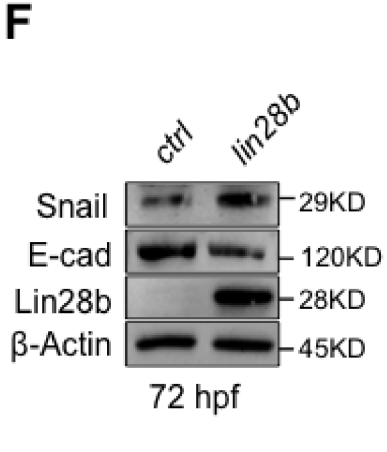
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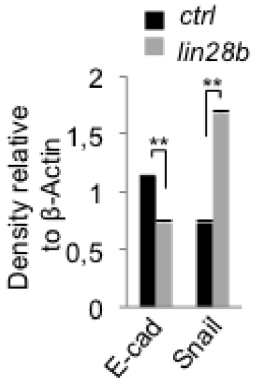
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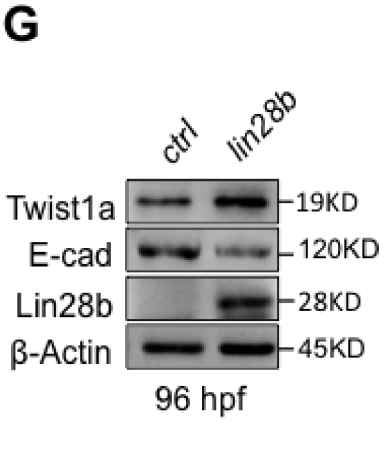
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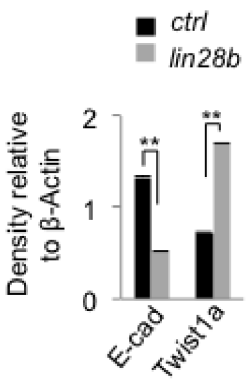

ctrl
lin28b
Density relative to β-Actin
2
1
0
**
E-cad
Twist1a

Figure 6

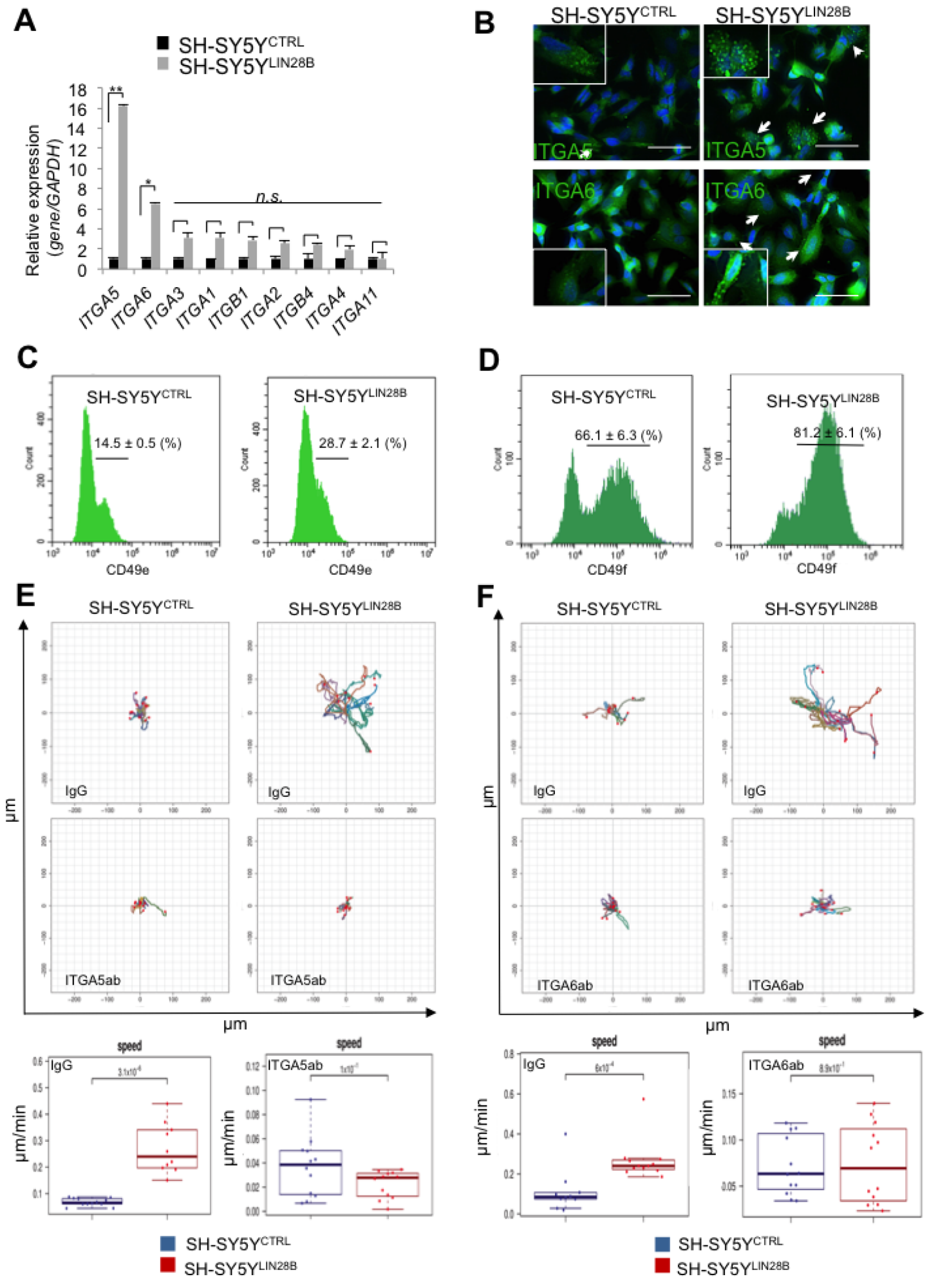
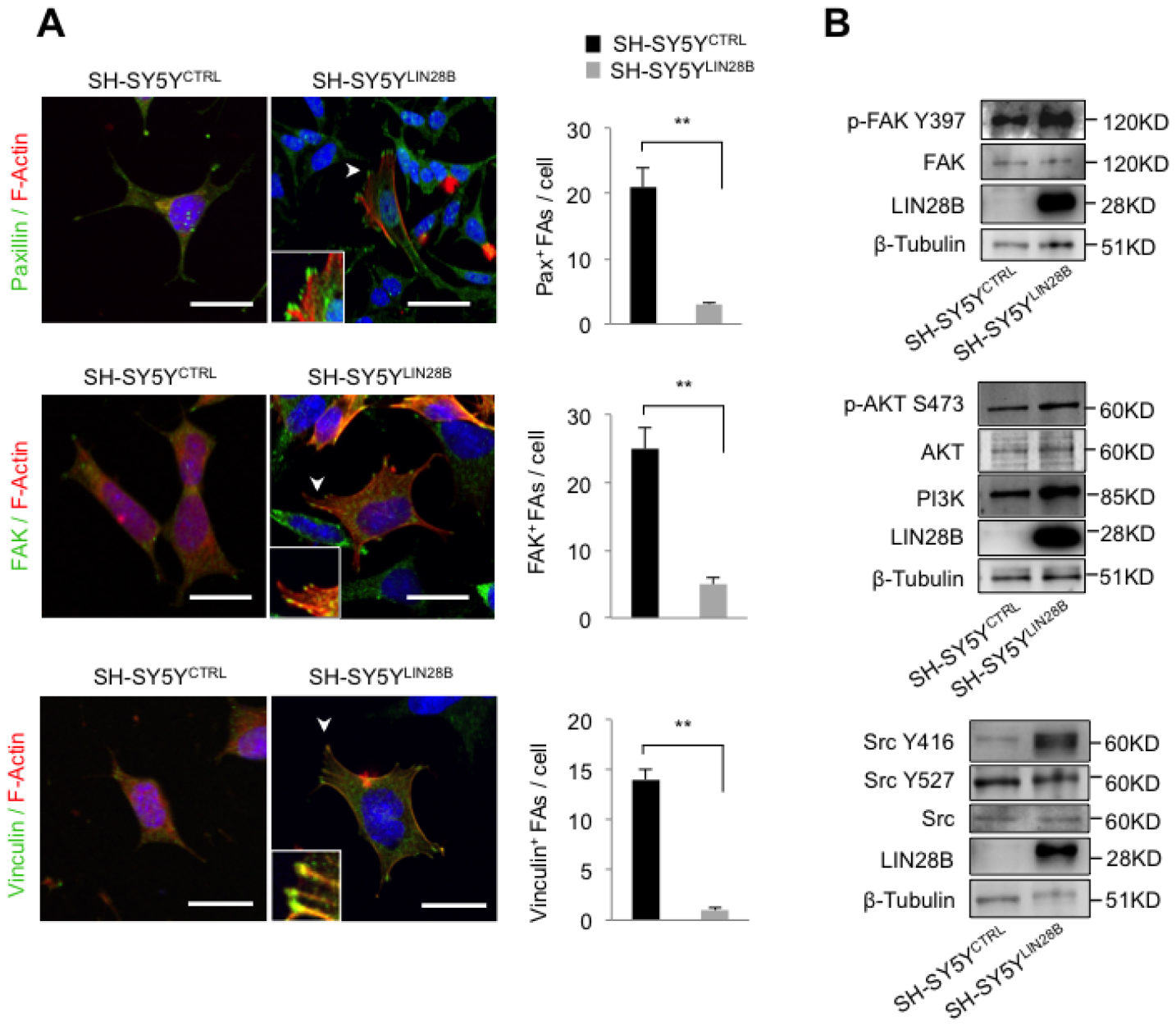

A
SH-SY5Y^CTRL
SH-SY5Y^LIN28B
Relative expression (gene/GAPDH)
18
16
14
12
10
8
6
4
2
0
**
*
n.s.
ITGA5
ITGA6
ITGA3
ITGA1
ITGB1
ITGA2
ITGB4
ITGA4
ITGA11
B
SH-SY5Y^CTRL
SH-SY5Y^LIN28B
ITGA5
ITGA5
ITGA6
ITGA6
C
SH-SY5Y^CTRL
14.5 ± 0.5 (%)
SH-SY5Y^LIN28B
28.7 ± 2.1 (%)
Count
CD49e
CD49e
D
SH-SY5Y^CTRL
66.1 ± 6.3 (%)
SH-SY5Y^LIN28B
81.2 ± 6.1 (%)
Count
CD49f
CD49f
E
SH-SY5Y^CTRL
SH-SY5Y^LIN28B
IgG
IgG
ITGA5ab
ITGA5ab
µm
µm
speed
IgG
3.1x10^-4
ITGA5ab
1x10^-1
µm/min
µm/min
SH-SY5Y^CTRL
SH-SY5Y^LIN28B
F
SH-SY5Y^CTRL
SH-SY5Y^LIN28B
IgG
IgG
ITGA6ab
ITGA6ab
µm
µm
speed
IgG
6x10^-4
ITGA6ab
8.9x10^-1
µm/min
µm/min
SH-SY5Y^CTRL
SH-SY5Y^LIN28B

Figure 7


A
SH-SY5Y^CTRL
SH-SY5Y^LIN28B
Paxillin / F-Actin
FAK / F-Actin
Vinculin / F-Actin
Pax⁺FAs / cell
FAK⁺FAs / cell
Vinculin⁺ FAs / cell
**
B
p-FAK Y397
FAK
LIN28B
β-Tubulin
120KD
28KD
51KD
p-AKT S473
AKT
PI3K
85KD
60KD
Src Y416
Src Y527
Src