Genetic targeting and anatomical registration of neuronal populations in the zebrafish brain with a new set of BAC transgenic tools

Dominique Förster1, Irene Arnold-Ammer1, Eva Laurell1, Alison J. Barker1,2, António M. Fernandes1, Karin Finger-Baier1, Alessandro Filosa1,2, Thomas O. Helmbrecht1, Yvonne Kölsch1, Enrico Kühn1, Estuardo Robles1,3, Krasimir Slanchev1, Tod R. Thiele1,4, Herwig Baier1 & Fumi Kubo1

Genetic access to small, reproducible sets of neurons is key to an understanding of the functional wiring of the brain. Here we report the generation of a new Gal4- and Cre-driver resource for zebrafish neurobiology. Candidate genes, including cell type-specific transcription factors, neurotransmitter-synthesizing enzymes and neuropeptides, were selected according to their expression patterns in small and unique subsets of neurons from diverse brain regions. BAC recombineering, followed by Tol2 transgenesis, was used to generate driver lines that label neuronal populations in patterns that, to a large but variable extent, recapitulate the endogenous gene expression. We used image registration to characterize, compare, and digitally superimpose the labeling patterns from our newly generated transgenic lines. This analysis revealed highly restricted and mutually exclusive tissue distributions, with striking resolution of layered brain regions such as the tectum or the rhombencephalon. We further show that a combination of Gal4 and Cre transgenes allows intersectional expression of a fluorescent reporter in regions where the expression of the two drivers overlaps. Taken together, our study offers new tools for functional studies of specific neural circuits in zebrafish.

Deciphering the circuitry of the brain requires experimental strategies that allow monitoring and manipulating the function of individual neurons within larger, genetically defined populations. To this end, numerous genetically encoded sensors and actuators are available to neurobiologists1, 2, but their utility strongly depends on the precision by which these effectors can be genetically targeted to small subsets of cells. Binary expression systems, like the Gal4/UAS and the Cre/lox system, are to date the most popular tools to target gene expression to defined cell populations, while retaining flexibility of driver and effector combinations3, 4. Further, engineering a combined Gal4-plus-Cre system in which a UAS-linked reporter is flanked by loxP sites followed by a second transgene on the same construct allows to restrict the expression of that second transgene to cells in which Gal4 and Cre expression patterns overlap. While this intersectional strategy was first implemented in zebrafish by Satou et al5, a related approach was pioneered for single-cell labeling by Sato et al6.

Several thousand transgenic zebrafish lines have been generated using short enhancer/promoter sequences from known genes or by unbiased enhancer trap screens7–12. As single enhancer sites typically do not regulate the complex spatial and temporal aspects of expression, these lines often do not faithfully recapitulate the expression pattern of the endogenous gene13, 14. Recent advances in transgenesis techniques have made it possible to more reliably reproduce the endogenous expression of the gene of interest by using the larger gene regulatory

1Max Planck Institute of Neurobiology, Department Genes - Circuits - Behavior, Am Klopferspitz 18, D-82152, Martinsried, Germany. 2Present address: Max-Delbrück Center for Molecular Medicine, Berlin, Germany. 3Present address: Department of Biological Sciences, Purdue University, West Lafayette, USA. 4Present address: Department of Biological Sciences, University of Toronto Scarborough, Toronto, Canada. Correspondence and requests for materials should be addressed to H.B. (email: hbaier@neuro.mpg.de) or F.K. (email: fumikubo@neuro.mpg.de)
regions (50–300 kb) contained in bacterial artificial chromosomes (BACs). State-of-the-art protocols for efficient application of BAC recombineering and transgenesis in zebrafish have been established15,16, paving the way for larger-scale approaches to generate reliable tools for targeted gene expression. Here we report the generation, characterization and comparison of 58 BAC-derived Gal4 and Cre constructs. This screen has led to the production of 22 new transgenic zebrafish lines, each of which allows genetic access to unique neuronal subpopulations.

Results and Discussion

Transgenesis by BAC recombineering yields 22 stable zebrafish lines. To generate an array of transgenic lines that label diverse subsets of neuronal populations, we selected candidate genes primarily based on their published spatiotemporal expression patterns (Fig. 1a and Table 1). A large fraction of these genes had previously been shown by RNA in situ hybridization to be transcribed in small populations of retinal ganglion cells (RGCs) and/or tectal neurons. In addition, we were interested in neurons that share the transmitter GABA or acetylcholine across the brain and therefore included genes that encode for transmitter-synthesizing enzymes. A third category was genes encoding neuropeptides, since these are some of the most specific markers of defined cell types in the central nervous system17,18.

For each gene, we chose a BAC clone that contains both upstream and downstream sequences of the translation start site to include transcription regulatory elements on both sides of the coding sequence. BAC recombineering was performed according to the protocol of Bussmann and Schulte-Merker, which is a plasmid-based technique employing an arabinose-inducible homologous recombination11. The method was slightly modified by adding a stable fluorescent marker to the construct, which labels cardiac muscle cells for ease of screening and subsequent re-identification. The red-fluorescent marker “bleeding heart” (BH, cmlc2:mCherry) was linked to Gal4VP16 constructs, and “cold heart” (CH, cmlc2:Cerulean) was linked to Cre (Fig. 1b). In addition, a small subset of Gal4VP16 lines was generated without heart marker.

Recombineered BAC constructs were injected into fertilized eggs obtained from UAS:Dendra-kras transgenic fish or from wild-type fish. Potential F0 founders were selected at larval stages for their expression of the transgene and raised to fertility. Specifically, we selected F0 larvae that expressed Dendra (for Gal4VP16 and Gal4VP16-BH), the “bleeding heart” marker (for Gal4VP16-BH) or the “cold heart” marker (for Cre-CH). When the F0 fish had reached adulthood, they were crossed to wild type, UAS:Dendra or UAS:Intersec (see below) and their F1 progeny were screened for inherited transgene expression in the expected pattern. Germline transformation and mosaicism rates were similar to other Tol2 based BAC transgenesis approaches previously reported5,15.

Out of 58 BAC constructs that were confirmed to drive expression of Gal4VP16, Gal4VP16-BH, or Cre-CH, we isolated 22 stable transgenic lines. The results are summarized in Tables 1 and 2. The success rate for obtaining stable transgenic lines was 42.9% (3/7 genes), 43.6% (17/39 genes) and 50% (6/12 genes) for Gal4VP16, Gal4VP16-BH and Cre-CH, respectively. For those stable transgenic lines, the percentage of germline transgenic founders out of all adult fish screened (“founder rate”) ranged from 1.1% to 25% (average 6.3%). In general, the founder rates of different driver constructs derived from the same BAC (Gal4VP16 only, Gal4VP16-BH or Cre-CH) were found to be similar, with one exception being grin2ab (25% and 3.4% for the Gal4-BH and Cre-CH construct, respectively). Consistent with a previous report5, we did not find a clear correlation between transgenesis efficiency and genomic insert length of the BACs (Fig. 1c).

Gal4VP16 and Cre drivers give access to defined neuronal subsets by largely recapitulating endogenous gene expression. In accord with the RNA expression patterns of the chosen genes, the established Gal4 transgenic lines label different subsets of neurons in the brain (Fig. 1d and Supplementary Video 1–11). To determine to what extent the BAC-driven Gal4 patterns recapitulate endogenous gene expression, we compared expression of a UAS-linked reporter (UAS:GFP or UAS:GCaMP6s) to the distribution of the selected genes by antibody staining. The Tg(chat:Gal4) and Tg(gad1b:Gal4) lines labeled cells in several brain areas that were positive for choline acetyltransferase (ChAT) and GABA, respectively (Fig. 2a,b). Out of all ChAT- or GABA-positive cells, a little less than half visibly expressed the UAS-linked reporter in each line (48 ± 24%, n = 105 of 231 cells out of two larvae in Tg(chat:Gal4), and 42 ± 15%, n = 150 of 355 cells out of four larvae in Tg(gad1b:Gal4)). In Tg(galn:Gal4) and Tg(sst3:Gal4) lines, antibody staining greatly overlapped with, or was identical to, the Gal4-driven reporter expression (94 ± 4%, n = 88 of 94 cells out of two larvae in Tg(galn:Gal4) line) (Fig. 2c,d). Differences in protein localizations in Tg(galn:Gal4) and Tg(sst3:Gal4) lines can be explained by the transport into axon terminals of the Galn and Sst proteins, both of which encode neuropeptide transmitters19,20 versus the cytosolic localization of GFP. It is also possible that some of the signal from the Galn and Sst antibodies is derived from extracellular localization of these secreted factors. These results with four transgenes, for which cross-reactive antibodies are available, suggest that the BAC transgenic Gal4 lines largely reproduce the endogenous gene expression pattern and thus provide genetic access to the labeled neuronal populations. Individual transgenic lines should be tested for recapitulation of endogenous gene expression patterns for each study at hand.

We also confirmed expression of our Cre constructs, either transiently or in stable transgenic lines, using a Cre reporter line named UAS:Intersec (Fig. 3a). The UAS:Intersec transgene is designed to drive the expression of the reporter gene in cells in which Gal4 and Cre overlap. This enables intersectional genetic approaches as reported previously5,21. Taken together, the BAC transgenic driver lines established here allow experimental access to small neuronal populations, either in binary (Gal4/UAS) or ternary (Cre/Gal4/UAS:Intersec) genetic configurations. We noted that Cre recombination is often incomplete in our transgenic animals (Fig. 3b–e), similarly to a previous observation reported for a different Cre transgenic line22. Specifically, even though the F0 founder fish produced offspring expressing transgenesis marker Cold Heart, Cre-mediated reporter expression was often incomplete in the Cold Heart-positive offspring. The reasons for the inefficient recombination event could be due to 1) targeted Cre expression at either transcription or translation level, 2) inefficient recombine activity of expressed Cre protein, 3) variated expression of the UAS:Intersec reporter line, or a combination of all three effects.
Image registration and initial characterization of Gal4 patterns suggest new avenues into functional studies of neural circuits. In order to determine the three-dimensional labeling pattern of our newly generated Gal4 lines, we scanned optical sections at high resolution with a confocal microscope and performed image registrations across age-matched specimens. By registering expression patterns to a standard reference brain, it is possible to compare the distribution of labeled cells with those present in other lines. This data format can be integrated in a comprehensive brain atlas. We found that the HuC:lynTagRFP-T marker is...
### Table 1.

List of Gal4VP16 and Gal4VP16-Bleeding Heart constructs and transgenic lines. Seven Gal4VP16 and 39 Gal4VP16-Bleeding Heart BAC constructs that were generated and validated to express Gal4VP16 after transient injection. Founder rate is defined as a percentage of germline founders out of total adult fish screened. Bold texts indicate the constructs from which Gal4VP16-expressing transgenic line(s) were isolated. FB, forebrain; MB, midbrain; HB, hindbrain; RGCs, retinal ganglion cells; OT, optic tectum; *lines discontinued; ×second line identified in an additional round of screening.

| Gene name | Expression pattern | BAC clone # | BAC insert size (Kb) | # Fish screened | # Positive founders | Founder rate (%) | Allele number |
|-----------|--------------------|-------------|----------------------|----------------|-------------------|-----------------|--------------|
| arl4ca    | RGCs               | DKEY-16H18  | 208                  | 22             | 0                 | 0               |              |
| cactnb3b  | RGCs               | DKEY-180E8  | 210                  | 32             | 0                 | 0               |              |
| chata     | OT, HB, spinal cord| DKEY-194K24 | 212                  | 22             | 1                 | 4.5             |              |
| chd1l     | FB, tegmentum, OT, HB| CH211-209C15 | 200                  | 22             | 0                 | 0               |              |
| ccr4b     | RGCs, FB, HB       | DKEY-260L22 | 196                  | 42             | 1                 | 2.4             |              |
| dbnxb1b   | OT, HB             | CH211-39M11 | 159                  | 9              | 2                 | 22.2            |              |
| grin2ab   | RGCs               | DKEY-90H7   | 221                  | 34             | 0                 | 0               |              |

**Gal4VP16 only**

| Gene name | Expression pattern | BAC clone # | BAC insert size (Kb) | # Fish screened | # Positive founders | Founder rate (%) | Allele number |
|-----------|--------------------|-------------|----------------------|----------------|-------------------|-----------------|--------------|
| arl4ca    | RGCs               | DKEY-16H18  | 208                  | 22             | 0                 | 0               |              |
| cactnb3b  | RGCs               | DKEY-180E8  | 210                  | 32             | 0                 | 0               |              |
| chata     | OT, HB, spinal cord| DKEY-194K24 | 212                  | 22             | 1                 | 4.5             |              |
| chd1l     | FB, tegmentum, OT, HB| CH211-209C15 | 200                  | 22             | 0                 | 0               |              |
| ccr4b     | RGCs, FB, HB       | DKEY-260L22 | 196                  | 42             | 1                 | 2.4             |              |
| dbnxb1b   | OT, HB             | CH211-39M11 | 159                  | 9              | 2                 | 22.2            |              |
| grin2ab   | RGCs               | DKEY-90H7   | 221                  | 34             | 0                 | 0               |              |

**Gal4VP16 Bleeding Heart (clmc2:mCherry)**

| Gene name | Expression pattern | BAC clone # | BAC insert size (Kb) | # Fish screened | # Positive founders | Founder rate (%) | Allele number |
|-----------|--------------------|-------------|----------------------|----------------|-------------------|-----------------|--------------|
| adcyap1a  | habenula, HB       | CH73-310J3  | 107                  | 28             | 0                 | 0               |              |
| arl4ca    | RGCs               | DKEY-16H18  | 208                  | 12             | 0                 | 0               |              |
| bcfla     | cerebellum, OT, retina| DKEY-253J8  | 174                  | 8              | 0                 | 0               |              |
| cabp7b    | OT, tegmentum, cerebellum| DKEY-87A16 | 161                  | 108            | 0                 | 0               |              |
| cactnb3b* | RGCs               | DKEY-180E8  | 210                  | 14             | 1                 | 7.1             |              |
| cadm4     | retina, FB, MB, HB  | DKEY-178J22 | 204                  | 71             | 0                 | 0               |              |
| cart2a*   | FB, HB, tegmentum   | DKEY-114P5  | 175                  | 91             | 1                 | 1.1             |              |
| chata*    | OT, HB, spinal cord | DKEY-194K24 | 212                  | 85             | 1                 | 1.2             |              |
| chd1l     | FB, tegmentum, OT, HB| CH211-209C15 | 200                  | 56             | 0                 | 0               |              |
| coch      | unknown             | CH211-29J20 | 154                  | 13             | 0                 | 0               |              |
| dbx1a     | FB, MB, HB         | CH211-271F10 | 128                  | 45             | 1                 | 2.2             |              |
| drd1b     | FB, hypothalamus, HB| CH211-148O10 | 147                  | 63             | 0                 | 0               |              |
| drd2a     | FB, HB, OT, spinal cord| DKEY-50H16  | 325                  | 41             | 0                 | 0               |              |
| drd2a*    | FB, HB, OT, spinal cord| CH211-335E14 | 103                  | 24             | 1                 | 4.2             |              |
| foxb1a    | FB, HB, OT         | CH211-2C17  | 144                  | 50             | 2                 | 4.0             |              |
| foxb1b    | OT, HB              | CH211-92B21 | 177                  | 59             | 0                 | 0               |              |
| gad1b     | FB, HB, OT         | CH211-24M22 | 168                  | 54             | 5                 | 9.3             |              |
| gad2a*    | FB, hypothalamus, pretectum| CH211-37E10 | 167                  | 88             | 1                 | 1.1             |              |
| galn      | hypothalamus, preoptic area| CH211-103A19 | 78                  | 10             | 1                 | 10.0            |              |
| grin2aa   | retina              | DKEY-255O2  | 202                  | 29             | 0                 | 0               |              |
| grin2ab*  | RGCs               | DKEY-90H7   | 221                  | 4              | 1                 | 25.0            |              |
| id2b      | retina, OT, HB      | CH211-175H7 | 137                  | 28             | 1                 | 3.6             |              |
| lbx2b     | FB, MB, HB, retina  | CH211-159C13 | 173                 | 57             | 2                 | 3.5             |              |
| lbx9      | FB, MB, HB         | DKEY-121A9  | 200                  | 56             | 4                 | 7.1             |              |
| lrtrm4l2  | MHB, HB, retina     | CH73-359G19 | 102                  | 45             | 0                 | 0               |              |
| nos1      | MB, spinal cord     | CH73-385P12 | 112                  | 34             | 0                 | 0               |              |
| opn4b     | retina, FB          | DKEY-156P15 | 223                  | 58             | 0                 | 0               |              |
| ottx1b    | FB, MB, OT         | DKEY-209N21 | 173                  | 48             | 2                 | 4.2             |              |
| pcps4a    | FB, HB              | CH211-231M12 | 149                  | 57             | 0                 | 0               |              |
| penka     | FB, MB, HB, spinal cord| CH211-189N20 | 149                 | 28             | 0                 | 0               |              |
| rorb      | retina, MB         | DKEY-196E24 | 80                   | 12             | 0                 | 0               |              |
| sdk2b     | unknown             | DKEY-172F4  | 169                  | 10             | 0                 | 0               |              |
| scla6a4a  | pretectum, raphe    | DKEY-263M15 | 157                  | 8              | 0                 | 0               |              |
| slit1a    | retina, OT, HB      | DKEY-118N13 | 114                  | 74             | 3                 | 4.1             |              |
| sp5l      | OT                  | DKEY-156F14 | 187                  | 19             | 0                 | 0               |              |
| st3       | FB, MB              | DKEY-265F18 | 163                  | 44             | 1                 | 2.3             |              |
| tac1      | FB, HB              | CH211-51C11 | 143                  | 28             | 0                 | 0               |              |
| lbx20     | RGCs, tegmentum, HB | CH211-132C11 | 179                 | 117            | 2                 | 1.7             |              |
| tmem200a  | FB, OT, MB, RGCs    | DKEY-252D12 | 139                  | 20             | 0                 | 0               |              |
Our analysis now shows that this developmental patterning not only holds true for glutamatergic, GABAergic, and glycinergic groups of neurons, as reported by Kinkhabwala et al.,

* excellently suited as a bridging template for across-line image registrations. This membrane-targeted red fluorescent cent reporter is expressed in almost all neurons and strongly labels the cell membrane-enriched neuropil areas, whose outlines in the fish brain are highly stereotyped. After crossing Gal4 carriers of selected lines, driving the expression of green-fluorescent UAS-linked reporters (UAS:Dendra, UAS:GFP, or UAS:GCaMP6s), to carriers of the HuClynTagRFP-T transgene, triple-transgenic larvae were identified and whole-brain images of red and green channels were obtained. These images were aligned with each other using the HuClynTagRFP-T pattern as a template, and the green reporter channels were superimposed using different colors to visualize the spatial relationships of the respective patterns (Fig. 4a and Supplementary Video 12).

This analysis demonstrates the utility of our new lines for functional investigations into neural circuitry. Three immediate applications come to mind. First, telencephalic inhibitory connections have to our knowledge not been studied in teleosts. Our new gad1b:Gal4 line will be particularly useful for functional studies of GABAergic cells studied in teleosts. Our new

Table 2. List of Cre-Cold Heart constructs and transgenic lines. 12 Cre-Cold Heart BAC constructs have been generated and validated to express Cre after transient injection. Expression pattern is derived from previously reported expression data. Founder rate is defined as a percentage of germline founders out of total adult fish screened. Bold texts indicate the constructs from which CH-positive transgenic line(s) were isolated. FB, forebrain; MB, midbrain; HB, hindbrain; RGCs, retinal ganglion cells; OT, optic tectum; * lines discontinued. N/A, line has not been screened for founders. otx1b:Cre BAC was generated without the Cold Heart cassette.

| Cre Cold Heart (clmc2:Cerulean) | Gene name | Expression pattern | BAC clone # | BAC insert size (Kb) | # Fish screened | # Positive founders | Founder rate (%) | Allele number |
|---------------------------------|-----------|--------------------|-------------|----------------------|----------------|-------------------|----------------|--------------|
| arl16a                           | RGCs      | DKEY-161118        | 208         | 75                   | 0              | 0                 |                |              |
| otob7                            | RGCs      | DKEY-111E19        | 181         | 10                   | 2              | 20.0  mpn221      |                |              |
| cart2                            | FB, HB, tegmentum | DKEY-114P5       | 175         | 12                   | 0              | 0                 |                |              |
| chata*                           | OT, HB, spinal cord | DKEY-194K24      | 212         | 62                   | 4              | 6.5               |                |              |
| cxcr4b                           | RGCs, FB, HB | DKEY-260L22      | 196         | 80                   | 0              | 0                 |                |              |
| gad1b                            | FB, HB, OT | CH211-24M22        | 168         | 2                    | 0              | 0                 |                |              |
| gad2                             | FB, hypothalamus, pretectum | CH211-37E10     | 167         | 34                   | 0              | 0                 |                |              |
| grin2a*b                         | RGCs      | DKEY-90H7         | 221         | 58                   | 2              | 3.4               |                |              |
| isl2b*                           | RGCs, cranial ganglia | DKEY-73M9     | 157         | 88                   | 2              | 2.3               |                |              |
| otx1b                            | FB, MB, OT | DKEY-209N21        | 173         | N/A                  | N/A            | N/A               |                |              |
| slit1a                           | retina, OT, HB | DKEY-11H8N13     | 114         | 58                   | 3              | 5.2   mpn222      |                |              |
| tbx20                            | RGCs, tegmentum, HB | CH211-132C11   | 179         | 4                    | 0              | 0                 |                |              |
| th                               | FB, hypothalamus, HB | CH211-77O7      | 166         | 36                   | 4              | 11.1  mpn223      |                |              |

**Image registration reveals new aspects of the layered architecture of the larval brain.** Our initial characterization also revealed interesting architectonic principles that invite future investigations. We identified Gal4 lines with clustered, but mutually exclusive expression in the rhombencephalon (Fig. 4c). Specifically, chat:Gal4, gad1b:Gal4 and cxcr4b:Gal4 exhibited non-overlapping expression in rostral to caudal stripes in horizontal sections (Fig. 4d,e), and dorsomedial to ventrolateral stripes in transverse sections, respectively (Fig. 4f). This observation is consistent with the previously reported striped organization of neurotransmitter and transcription factor expression in the hindbrain. Our analysis now shows that this developmental patterning not only holds true for glutamatergic, GABAergic, and glycinergic groups of neurons, as reported by Kinkhabwala et al., but also for cholinergic neurons. A multi-label image registration approach, such as the one used here, is uniquely suited to uncover principles of mesoscale spatial organization.

The tectum is an intensively studied, multisensory processing area in the vertebrate midbrain. Its densely packed neuropil is subdivided into layers, originally defined by the stratification pattern of incoming RGC axons. RGC axons project to the following layers (from superficial to deep): Stratum opticum (SO); stratum fibrosum et griseum superficiale (SFGS, with its six sublayers, SFGS1 through SFGS6); the retinorecipient stratum griseum centrale (SGC); and the boundary between stratum album centrale and stratum periventriculare (SAC/SPV). From among our collection of BAC lines, several showed expression in the tectal neuropil (Fig. 5a). Co-registration of the patterns of our newly generated lines with that of an RGC-specific Gal4 line (isl2b:Gal4) allowed us to further explore the laminar architecture of the tectum beyond RGCs (Fig. 5a,b). A densitometric analysis revealed that cxcr4b:Gal4 consistently but sparsely labeled SFGS3, SFGS4 and SAC/SPV (Fig. 5c). While the labeling of SFGS3 consistently but sparsely labeled SFGS3, SFGS4 and SAC/SPV (Fig. 5c). While the labeling of SFGS3
neurons (data not shown). Neuropil labelings in chat:Gal4, dmbx1b:Gal4 and gad1b:Gal4 do not derive from RGC axons, but from neurites of specific classes of periventricular tectal neurons and incoming axons from various other brain areas (Fig. 5d–f). The chat:Gal4 pattern showed highest fluorescence intensities in the deep stratum griseum centrale (SGC) (Fig. 5d). Fluorescence signals in gad1b:Gal4, on the other hand, are strongest in the superficial layers (Fig. 5e), including superficial interneuron (SIN) cell bodies, which are GABAergic. Neurites labeled by dmbx1b:Gal4 arborize in SFGS and SGC sublayers that are superficial to the retinorecipient SGC (Fig. 5f). As a rule, neuropil stratifications in these three lines are not restricted to single layers, but differences in labeling intensities rather reflect graded preferences for layer positioning. In summary, we isolated an array of transgenic lines, which allow targeting of different components of the layer-specific circuitry in the zebrafish tectum.

Conclusions and outlook. Progress in the neurobiology of the zebrafish preparation has recently accelerated, driven by advances in imaging technology, behavioral analysis and computational methods. Optogenetics affords the ability to manipulate circuit components in the behaving animal. We expect that our new set of BAC transgenic Gal4 and Cre lines will facilitate research that employs imaging or optogenetics in the zebrafish system and thus provide a valuable resource for the neuroscience community. In the future, breakthroughs are expected from the refinement of genetic tools that allow addressing specific subpopulations of neurons in the context of intact circuitry. Knock-in technology using CRISPR/Cas9 represents a promising approach to target endogenous loci with superior fidelity and may in the future be scaled up for the systematic generation of transgenic lines.

Figure 2. New transgenic lines largely recapitulate endogenous gene expression. (a–d) Antibody stainings of 6 dpf old larvae against GFP (green) and ChAT (a), GABA (b), Galanin (c) or Somatostatin (d), respectively (magenta). Genotypes are indicated on the left. Whole-brain images on the left show live expression pattern (lynTagRFP-T in magenta), outlining the location of the magnified regions on the right. Scale bar, 100 μm for overview, 20 μm for magnified images.
Methods

Animal care and transgenic lines. All animal procedures conformed to the institutional guidelines of the Max Planck Society and the local government (Regierung von Oberbayern). Experimental protocols were approved by Regierung von Oberbayern (55.2-1-54-2532-101-12 and 55.2-1-54-2532-31-2016).

Figure 3. Cre-expressing BAC constructs allow intersectional genetics. (a) UAS:intersec construct for intersectional genetic approach. A pan-neuronal Gal4 line (Gal4s1101t) was used in this figure. (b) Antibody staining against GFP and RFP reveals highly variegated, transgenic expression of th:cre stable transgenic line. (c–e) Live imaging showing transient expression of chat:cre (c), isl2b:cre (d) and otx1b:cre (e).
previously described transgenic lines used in this study are as follows: Tg(UAS:Dendra-kras)s1998t; Et(E1b:Gal4-VP16)s1101t; Tg(isl2b.3:Gal4-VP16)zc65; Tg(UAS:GCaMP6s)mpn101; Tg(5xUAS:EGFP)zf82. These and all of the newly generated transgenic lines are available upon request.

**Plasmids.** To generate pCR8GW-Gal4VP16-FRT-Kan-FRT-Bleeding Heart and pCR8GW-Cre-FRT-Kan-FRT-Cold Heart plasmids, the Bleeding Heart (cmlc2:mCherry, also known as myl7:m-Cherry) and Cold Heart (cmlc2:Cerulean, also known as myl7:Cerulean) cassettes (obtained from Michael Nonet, Washington University, USA) were inserted into pCR8GW-Gal4VP16-FRT-Kan-FRT and pCR8GW-Cre-FRT-Kan-FRT, in the reverse orientation relative to Gal4VP16 or Cre coding sequence. To generate the Tol2 HuC:lynTagRFP-T plasmid, lynTagRFP-T (TagRFP-T tagged with lyn kinase membrane targeting sequence) was PCR amplified and cloned downstream of the HuC promoter. For the intersectional reporter, the loxP-tdTomato-CAAX-loxP-EGFP-CAAX cassette was codon optimized and synthesized by Genscript (Piscataway, NJ), and subsequently cloned into a pTol2-14xUAS vector to obtain UAS:loxP-tdTomato_CAAX-loxP-EGFP_CAAX.

**Selection of candidate genes and obtaining BAC clones.** To identify corresponding BAC clones, we searched, using the Ensembl genome browser ([www.ensembl.org/](http://www.ensembl.org/)) or the UCSC genome browser (Zv6 assembly, [https://genome.ucsc.edu/](https://genome.ucsc.edu/)), for BAC clones that encompass both upstream and downstream of the first ATG site of the target gene. BAC clones were purchased from Source BioScience ([www.lifesciences.sourcebioscience.com/](http://www.lifesciences.sourcebioscience.com/)) and BACPAC resources center, Children’s Hospital Oakland ([https://bapacresources.org/](https://bapacresources.org/)).

**BAC recombineering.** BAC recombineering was performed as described previously. Briefly, in the first step, BAC clones were transformed with the pRedET plasmid (Gene Bridges), which enables the arabinose-inducible homologous recombination. In the second step, Tol2 arms in opposing directions flanking an ampicillin resistance cassette were PCR amplified from pTol2_amp plasmid and inserted into the BAC backbone. In the third and final step, either Gal4VP16 only, Gal4VP16-BH or Cre-CH cassettes were PCR amplified and inserted into the BAC, such that the start-ATG site of the gene of interest was replaced by that of Gal4VP16 or Cre. For th:Cre-CH line, the GFP coding sequence of the th:GFP BAC was replaced with the...
Cre-CH cassette. After we confirmed successful insertions of the cassette by PCR, the final BAC DNA was purified using NucleoBond Xtra BAC kit (Machery Nagel), and correct insertions were verified by sequencing.

Transgenesis. Tg(HuC:lynTargRFP-T)mpn404 and Tg(UAS:intersec)mpn128 were created using the standard Tol2 transposon system. BAC DNAs were injected at 100 ng/µl together with zebrafish codon–optimized Tol2 transposase mRNA (50–100 ng/µl), synthesized from pCS-zT2TP plasmid16. Gal4VP16 and Gal4VP16-BH BAC DNAs were injected into Tg(UAS:Dendra-kras)s1998t transgenic embryos. Cre-CH BAC DNAs were injected into wild-type TL embryos. After injection, embryos expressing Dendra, Bleeding Heart, or Cold Heart were screened and raised to sexual maturity. Injected fish were either incrossed or outcrossed with wild-type or Tg(UAS:Dendra-kras)s1998t transgenic adult fish to identify transgenic carriers. The germline mosaicism rate was defined as a percentage of transgenic offspring (F1) out of all offspring born from F0 founder fish. Outcrossing of F1 transgenic lines (and of following generations) revealed segregation of Gal4 expression at Mendelian ratios, suggesting that they carry a single insertion. In cases where multiple founders were identified, the founders which showed the most complete expression patterns in their F1 offspring were selected and maintained. We noted that the transgene expression of different larvae derived from the same transgenic founders was variable in some of our BAC transgenic lines, as is known for the Gal4/UAS system in general50, 51. Nevertheless, among larvae that were pre-screened for the high-level expression of the transgene, the expression patterns were largely consistent across different cell bodies.

Immunohistochemistry. Immunostaining was performed according to ref. 24, with slight modifications. Fish were fixed in 4% paraformaldehyde (PFA) in PBS overnight. For the initial antigen retrieval, fish were heated to 63 °C for 15 minutes in 150 mM Tris- HCl. The time for staining with primary antibody was increased to at least 5 days and with secondary antibody to at least 2 days. Secondary antibodies were diluted in only PBT. After staining, samples were washed with PBT and postfixed for 30 minutes in 4% PFA, thereafter briefly washed in PBT and then soaked in 85% glycerol. ChAT antibody staining was performed according to ref. 52, with the proteinase K treatment prolonged to 120 min for 6 dpf larvae. For a list of antibodies used, see Supplementary Table S1.

Image acquisition. For live imaging, 6–7 dpf larvae were anesthetized in 0.016% tricaine and embedded in 2% low-melting-point agarose. Imaging was performed on a Zeiss LSM-780 or LSM-700 confocal microscope,
using 20 × 1/0. NA water-dipping objectives. For imaging fixed samples, larvae were embedded in 85% glycerol, and imaging was performed using a 25 × /0.8 NA multi-immersion objective. Whole-brain images were acquired by tiling the brain into three individual image stacks and subsequent stitching, using ZEN software (black edition, v8.0; Zeiss). Images were corrected for fluorescence attenuation in the z-dimension using the brightness correction function in the ZEN software.

**Image registration.** Registration was performed using the Computational Morphometry Toolkit (CMTK)\(^3\). Whole-brain images of living 6 dpf zebrafish larvae were co-registered into one reference brain using expression of *HuC/CynTagRFP-T* as a template. For visualization of RGC innervation strata in the tectum, fish expressing *isl2b:Gal4 UAS:GCaMP6s* and *HuC/CynTagRFP-T* were co-registered into the reference brain.

**References**

1. Yizhar, O., Fenno, L. E., Davidson, T. J., Mogri, M. & Deisseroth, K. Optogenetics in neural systems. *Neuron* 71, 9–34 (2011).
2. Looger, L. L. & Griesbeck, O. Genetically encoded neural activity indicators. *Curr. Opin. Neurobiol.* 22, 18–23 (2012).
3. Brand, A. H. & Perrimon, N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118, 401–415 (1993).
4. Tisen, J. Z. et al. Subregion- and Cell Type–Restricted Gene Knockout in Mouse Brain. *CELL* 87, 1317–1326 (1996).
5. Satou, C. et al. Transgenic tools to characterize neuronal properties of discrete populations of zebrafish neurons. *Development* 140, 3927–3931 (2013).
6. Sato, T., Hamaoka, T., Aizawa, H., Hosoya, T. & Okamoto, H. Genetic Single-Cell Mosaic Analysis Implicates ephrinB2 Reverse Signaling in Projections from the Posterior Tectum to the Hindbrain in Zebrafish. *Journal of Neuroscience* 27, 5271–5279 (2007).
7. Bergeron, S. A. et al. Brain selective transgene expression in zebrafish using an NRSE derived motif. *Front. Neural Circuits* 6, 110 (2012).
8. Asakawa, K. et al. Genetic dissection of neural circuits by Tol2 transposon-mediated Gal4 gene and enhancer trapping in zebrafish. *Proceedings of the National Academy of Sciences* 103, 1253–1260 (2006).
9. Scott, E. K. et al. Targeting neuronal circuitry in zebrafish using GAL4 enhancer trapping. *Nat Meth* 4, 323–326 (2007).
10. Otsubo, H. et al. High-resolution analysis of central nervous system expression patterns in zebrafish Gal4 enhancer-trap lines. *Dev Dyn* 244, 785–796 (2015).
11. Scott, E. K. & Baier, H. The cellular architecture of the larval zebrafish tectum, as revealed by gal4 enhancer trap lines. *Front. Neural Circuits* 3, 13 (2009).
12. Halpern, M. et al. Gal4/UAS transgenic tools and their application to zebrafish. *Zebrafish* 5, 97–110 (2008).
13. Levine, M. & Tjian, R. Transcription regulation and animal diversity. *Nature* 424, 147–151 (2003).
14. Goto, T., Macdonald, P. & Maniatis, T. Early and late periodic patterns of even-skipped expression are controlled by distinct regulatory elements that respond to different spatial cues. *CELL* 57, 413–422 (1989).
15. Bussmann, J. & Schulte-Merkel, S. Rapid BAC selection for tol2-mediated transgenesis in zebrafish. *Development* 138, 4327–4332 (2011).
16. Suster, M. L., Abe, G., Scowu, A. & Kawakami, K. Transposon-mediated BAC transgenesis in zebrafish. *Nat Protoc.* 6, 198–201 (2011).
17. Herget, U. & Ryu, S. Coexpression analysis of nine neuropeptides in the neurosecretory preoptic area of larval zebrafish. *Front. Neuroanat.* 9, 5027 (2015).
18. Prober, D. A., Rihel, J., Onah, A. A., Sung, R. J. & Schier, A. F. Hypocretin/Orexin Overexpression Induces An Insomnia-Like Visuomotor Behavior. *Neuron* 19, 816–825 (2016).
19. Devos, N. et al. Differential expression of two somatostatin genes during zebrafish embryonic development. *Mol Dev* 115, 133–137 (2002).
20. Marquardt, G. D. et al. A 3D Searchable Database of Transgenic Zebrafish Gal4 and Cre Lines for Functional Neuroanatomy Studies. *Front. Neural Circuits* 9, 7 (2015).
21. Boniface, E. J., Lu, J., Victoroff, T., Zhu, M. & Chen, W. FlEx-based transgenic reporter lines for visualization of Cre and Flp activity in live zebrafish. *Genesis* 47, 484–491 (2009).
22. Rohlfing, T. & Maurer, C. R. Nonrigid image registration in shared-memory multiprocessor environments with application to brains, breasts, and bees. *IEEE Trans Inf Technol Biomed* 7, 16–25 (2003).
23. Randlett, O. et al. Whole-brain activity mapping onto a zebrafish brain atlas. *Nat Meth* 12, 1039–1046 (2015).
24. Wanner, A. A., Genoud, C., Masudi, T., Sikou, L. & Friedrich, R. W. Dense EM-based reconstruction of the interglomerular projection in the zebrafish olfactory bulb. *Nat Meth* 19, 816–825 (2016).
25. Kinkhabwala, A. et al. A structural and functional ground plan for neurons in the hindbrain of zebrafish. *Proc Natl Acad Sci USA* 108, 1164–1169 (2011).
26. Nevin, L. M., Robles, E., Baier, H. & Scott, E. K. Focusing on optic tectum circuitry through the lens of genetics. *BMC Biol.* 8, 126 (2010).
27. Meek, J. Functional anatomy of the tectum mesencephalon of the goldfish. An explorative analysis of the functional implications of the laminar structural organization of the tectum. *Brain Research Reviews* 6, 247–297 (1983).
28. Xiao, T., Roese, T., Staub, W. & Baier, H. A GFP-based genetic screen reveals mutations that disrupt the architecture of the zebrafish retinotectal projection. *Development* 132, 2955–2967 (2005).
29. Robles, E., Smith, S. J. & Baier, H. Characterization of genetically targeted neuron types in the zebrafish optic tectum. *Front. Neural Circuits* 5, 1 (2011).
30. Fujimoto, E., Gaynes, B., Brimley, C. J., Chien, C.-B. & Bonkowski, I. L. Gal80 intersectional regulation of cell-type-specific expression in vertebrates. *Dev Dyn* 240, 2324–2334 (2011).
31. Del Bene, F. et al. Filtering of visual information in the tectum by an identified neural circuit. *Science* 330, 669–673 (2010).
32. Ahrens, M. B. et al. Brain-wide neuronal dynamics during motor adaptation in zebrafish. *Nature* 485, 471–477 (2012).
33. Kudo, F. et al. Functional Architecture of an Optic Flow-Responsive Area that Drives Horizontal Eye Movements in Zebrafish. *Neuron* 81, 1344–1359 (2014).
34. Mirt, A. et al. Spatial gradients and multidimensional dynamics in a neural integrator circuit. *Nat Neurosci* 14, 1150–1159 (2011).
35. Baier, H. & Scott, E. K. Genetic and optical targeting of neural circuits and behavior–zebrafish in the spotlight. *Curr. Opin. Neurobiol.* 19, 553–560 (2009).
36. Portugal, R., Feierstein, C. E., Engert, F. & Orger, M. B. Whole-brain activity maps reveal stereotyped, distributed networks for visuomotor behavior. *Neuron* 81, 1328–1334 (2014).
37. Thiele, T. R., Donovan, J. C. & Baier, H. Descending control of swim posture by a midbrain nucleus in zebrafish. *Neuron* 83, 679–691 (2014).
38. Orger, M. B. The Cellular Organization of Zebrafish Visuomotor Circuits. *Curr. Biol.* 26, R377–85 (2016).
40. Arrenberg, A. B., Del Bene, E. & Baier, H. Optical control of zebrafish behavior with halorhodopsin. Proceedings of the National Academy of Sciences 106, 17968–17973 (2009).
41. Wyart, C. et al. Optogenetic dissection of a behavioural module in the vertebrate spinal cord. Nature 461, 407–410 (2009).
42. Douglass, A. D., Kraves, S., Deisseroth, K., Schier, A. F. & Engert, F. Escape Behavior Elicited by Single, Channelrhodopsin-2- Evoked Spikes in Zebrafish Somatosensory Neurons. Current Biology 18, 1133–1137 (2008).
43. Auer, T. O., Duroure, K., De Cian, A., Concordet, J.-P. & Del Bene, F. Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair. Genome Research 24, 142–153 (2014).
44. Hisano, Y. et al. Precise in-frame integration of exogenous DNA mediated by CRISPR/Cas9 system in zebrafish. Sci Rep 5, 8841 (2015).
45. Kimura, Y., Hisano, Y., Kawahara, A. & Higashijima, S.-I. Efficient generation of knock-in transgenic zebrafish carrying reporter/ driver genes by CRISPR/Cas9-mediated genome engineering. Sci Rep 4, 6545 (2014).
46. Ota, S. et al. Functional visualization and disruption of targeted genes using CRISPR/Cas9-mediated eGFP reporter integration in zebrafish. Sci Rep 6, 34991 (2016).
47. Yokogawa, T., Hannan, M. C. & Burgess, H. A. The Dorsal Raphe Modulates Sensory Responsiveness during Arousal in Zebrafish. Journal of Neuroscience 32, 15205–15215 (2012).
48. Tay, T. L., Ronneberger, O., Ryu, S., Nitschke, R. & Driever, W. Comprehensive catecholaminergic projectome analysis reveals single-neuron integration of zebrafish ascending and descending dopaminergic systems. Nat Commun 2, 171 (2011).
49. Dal Maschio, M., Donovan, J. C., Helmbrecht, T. O. & Baier, H. Linking Neurons to Network Function and Behavior by Two-Photon Holographic Optogenetics and Volumetric Imaging. Neuron 94(4), 774–789.e5 (2017).
50. Goll, M. G., Anderson, R., Stainier, D. Y. R., Spradling, A. C. & Halpern, M. E. Transcriptional silencing and reactivation in transgenic zebrafish. Genetics 182, 747–755 (2009).
51. Akitake, C. M., Macurak, M., Halpern, M. E. & Goll, M. G. Transgenerational analysis of transcriptional silencing in zebrafish. Dev Biol 352, 191–201 (2011).
52. Rath, M., Nitschke, R., Filippi, A., Ronneberger, O. & Driever, W. Generation of high quality multi-view confocal 3D datasets of zebrafish larval brains suitable for analysis using Virtual Brain Explorer (ViBE-Z) software. Protocol Exchange https://www.nature.com/protocols/exchange/protocols/2408/#/procedure (2012).
53. Ostrovsky, A., Cachero, S. & Jefferis, G. Clonal analysis of olfaction in Drosophila: image registration. Cold Spring Harbor Protocols 2013, 347–349 (2013).

Acknowledgements
We thank all the Baier lab members for participating in the screening. We thank Koichi Kawakami, Maximiliano Suster, Michael Nonet and Wolfgang Driever for sharing plasmids and BACs used in this study. Support was provided by the Max Planck Society (all authors), the European Molecular Biology Organization (EMBO ALT/F) (DF), the Toyobo Biofoundation (FK), and a Human Frontier Science Program fellowship (FK).

Author Contributions
D.F., H.B. and F.K. conceived and designed the project. I.A.A. performed BAC recombineering. D.F., A.J.B., A.M.F., A.F., T.O.H., Y.K., E.K., E.R., K.S., T.R.T., F.K. selected candidate genes and designed BAC recombineering strategies. D.F., I.A.A., E.L., A.J.B., A.M.F., K.F.B., A.F., T.O.H., Y.K., E.K., E.R., K.S., T.R.T., F.K. screened BAC transgenic founders. D.F., E.L. and A.M.F. performed antibody staining and confocal imaging. D.F. performed image registrations. T.O.H. generated HuC:lynTagRFP-T transgenic fish. D.F., H.B. and F.K. wrote the manuscript.

Additional Information
Supplementary information accompanies this paper at doi:10.1038/s41598-017-04657-x

Competing Interests: The authors declare that they have no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2017