Essential Role for the d-Asb11 cul5 Box Domain for Proper Notch Signaling and Neural Cell Fate Decisions In Vivo

Maria A. Sartori da Silva1, Jin-Ming Tee1, Judith Paridaen1, Anke Brouwers1, Vincent Runtuwene1, Danica Zivkovic1, Sander H. Diks2, Daniele Guardavaccaro1, Maikel P. Peppelenbosch3*

1 Hubrecht Institute-KNAW and University Medical Center Utrecht, Utrecht, The Netherlands, 2 Department of Cell Biology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands, 3 Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center Rotterdam, Rotterdam, The Netherlands

Abstract

ECS (Elongin BC-Cul2/Cul5-SOCS-box protein) ubiquitin ligases recruit substrates to E2 ubiquitin-conjugating enzymes through a SOCS-box protein substrate receptor, an Elongin BC adaptor and a cullin (Cul2 or Cul5) scaffold which interacts with the RING protein. In vitro studies have shown that the conserved amino acid sequence of the cullin box in SOCS-box proteins is required for complex formation and function. However, the in vivo importance of cullin boxes has not been addressed. To explore the biological functions of the cullin box domain of ankyrin repeat and SOCS-box containing protein 11 (d-Asb11), a key mediator of canonical Delta-Notch signaling, we isolated a zebrafish mutant lacking the Cul5 box (Asb11Cul). We found that homozygous zebrafish mutants for this allele were defective in Notch signaling as indicated by the impaired expression of Notch target genes. Importantly, asb11Cul fish were not capable to degrade the Notch ligand DeltaA during embryogenesis, a process essential for the initiation of Notch signaling during neurogenesis. Accordingly, proper cell fate specification within the neurogenic regions of the zebrafish embryo was impaired. In addition, Asb11Cul mRNA was defective in the ability to transactivate a her4::gfp reporter DNA when injected in embryos. Thus, our study reporting the generation and the characterization of a metazoan organism mutant in the conserved cullin binding domain of the SOCS-box demonstrates a hitherto unrecognized importance of the SOCS-box domain for the function of this class of cullin-RING ubiquitin ligases and establishes that the d-Asb11 cul box is required for both canonical Notch signaling and proper neurogenesis.

Introduction

The ubiquitin-proteasome system plays a fundamental role in the control of numerous cellular processes, including cell cycle progression, gene transcription, signal transduction, proliferation and differentiation [1]. In this system, ubiquitin is first activated by an E1 ubiquitin-activating enzyme. Activated ubiquitin is then transferred to the active-site cysteine of an E2 ubiquitin-conjugating enzyme. Subsequently, an E3 ubiquitin ligase mediates the transfer of ubiquitin from E2 to a lysine residue on the protein substrate. Multiple rounds of these reactions lead to the formation of polyubiquitylated substrates that are targeted to the 26S proteasome [2]. There are two major classes of E3 ubiquitin ligases, proteins with a HECT (homologous to E6-AP carboxyl terminus) domain and proteins with a RING (Really Interesting New Gene)-like motif. Within this class, cullin-RING E3s are multisubunit ubiquitin ligases composed of a scaffold protein known as cullin, a RING finger protein, which mediates the interaction with the E2, a variable substrate-recognition subunit and an adaptor that links the cullin-RING complex to the substrate recognition subunit [3]. Among the cullin-RING E3s, the group collectively denominated as ECS (Elongin BC-Cul2/ Cul5-SOCS-box protein) ubiquitin ligases has recently attracted special attention [4]. This group of E3 ligases has been implicated in transduction of extracellular cues to altered gene transcription. Many details of its modus operandi remain, however, obscure. Specifically, there is remarkably little insight into the in vivo relevance of the different components of ECS ubiquitin ligases. In vitro studies have shown that in ECS ubiquitin ligases the SOCS-box protein works as the substrate recognition subunit. SOCS-box proteins are composed of two distinct protein-protein interaction domains, a substrate binding domain and a SOCS-box domain. The SOCS-box motif is found at the C-terminus of over 70 human proteins in nine different families. In vitro studies show that SOCS boxes act as substrate recognition modules of the ECS type E3 ubiquitin ligase complex (Fig. 1A) [2]. The SOCS-box domain is divided into two sub-domains: the BC box, which links SOCS-box proteins to the cullin-Rbx module and a motif termed cul box, located immediately downstream of the BC box. The cul box is proposed to determine whether a given SOCS-box protein

* E-mail: M.Peppelenbosch@erasmusmc.nl

Citation: Sartori da Silva MA, Tee J-M, Paridaen J, Brouwers A, Runtuwene V, et al. (2010) Essential Role for the d-Asb11 cul5 Box Domain for Proper Notch Signaling and Neural Cell Fate Decisions In Vivo. PLoS ONE 5(11): e14023. doi:10.1371/journal.pone.0014023

Editor: Marc Vooijs, University Medical Center Maastricht, Netherlands

Received April 8, 2010; Accepted October 24, 2010; Published November 19, 2010

Copyright: © 2010 Sartori da Silva et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: MASdS and JMT are paid by Algemene Levens Wetenschappen Grant #817.02.002 and #81502006 respectively (www.nwo.nl) and SHD and MPP receive financial support from Top Institute Pharma grant T1-215 and T3-103 (http://www.tifpharma.com/). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: M.Peppelenbosch@erasmusmc.nl

PLOS ONE | www.plosone.org 1 November 2010 | Volume 5 | Issue 11 | e14023
Assemblies into either a Cul2-Rbx1 or a Cul5-Rbx2 module to recruit and activate E2 ubiquitin-conjugating enzymes for substrates ubiquitylation [5–8]. In vivo evidence that the cullin box is involved in mediating the biological action of any SOCS-box protein has not been provided hitherto.

Ankyrin repeat and SOCS-box-containing proteins (ASB) constitute the largest subclass of the SOCS-box protein family. ASB members (ASB1-ASB18) are structurally characterized by a variable number of N-terminal ankyrin repeats, which mediate the association with the substrate [9]. ASB proteins in general participate in various important biological processes [10–17], but association with the substrate [9]. ASB proteins in general participate in various important biological processes [10–17], but their role in vivo remains largely unknown. We have recently showed that Danio rerio Asb11 (d-Asb11) regulates compartment size in the endodermal and neuronal lineages [10] via ubiquitylation of DeltaA, leading to the activation of the canonical Notch pathway [11]. Thus, d-Asb11 is an attractive protein to assess the elusive functions of the cullin box motif in the SOCS-box holodomain. All ASB proteins share, with slight divergences, the consensus sequences of BC box and Cul5 box in their C-terminal portion (Fig. 1B). Hence, elucidation of the in vivo mode of action of d-Asb11 should also provide important clues for this family in its entirety. Together, these considerations prompted us to explore the function of the d-Asb11 cullin box in vivo.

Here, we describe the isolation of a zebrafish carrying a mutant allele in the conserved LPφP sequence of the d-Asb11 cullin box. This mutant represents the first meta-zebrafish harboring a mutated cullin box. While the wild-type (WT) d-Asb11 protein is expressed at the head, a truncated d-Asb11 protein encoded by the mutated allele was identified (Fig. 1C), and the predicted residual fragment and the position of the identified mutation. The different domains are indicated. (right) The T→A mutation changes a leucine into a stop codon.

Results and Discussion

Generation and characterization of d-asb11 mutants

The consensus sequence φX1LPφPXXφX,(Y/F) corresponds to the Cul5-box in the C-terminal portion of the canonical SOCS-box proteins, and is highly conserved in vertebrates [5,9] (Fig. 1B). We performed a TILLING screen on an F1 ENU-mutagenized zebrafish library for d-asb11 mutations mapping to the conserved consensus sequence [18]. A premature stop codon corresponding to amino acid 281 in the conserved LPφP sequence of the d-Asb11 was identified (Fig. 1C), and the homozygous allele was designated asb11<sup>mut</sup>. To our knowledge, this is the first report of a zebrafish mutant presenting a mutation in the consensus sequence of any SOCS-box protein, allowing for the first assessment of the in vivo function of the cullin box.

Morphological analysis of asb11<sup>mut</sup> revealed a slight hyperplacodalutum at 48 and 72 hours post-fertilization (hpf) (Fig. 2A). This corresponds to the Asb11 knockdown morphant phenotype we described previously, although with less severity [10].

Next, to further identify the functional consequences of the mutated allele, we performed whole-mount in situ hybridization (WISH) with d-asb11 probe on 10 hpf embryos. Strikingly, d-asb11 transcripts were enhanced in asb11<sup>mut</sup> mutants compared to wild type, showing expanded expression in the polster, a U-shaped structure that surrounds the head [19], and along the margins of the neural plate (Fig. 2B). Quantitative RT-PCR (qPCR), confirmed the increase of mRNA transcripts in asb11<sup>mut</sup> (Fig. 2C). Accordingly, higher protein expression levels were detected by immunoblotting on 12 hpf lysates from asb11<sup>mut</sup> embryos (Fig. 2C). No significant quantitative differences between wild type and heterozygous embryos confirmed the recessive nature of the mutation. The higher mRNA transcripts and protein levels suggest a compensatory effect of a hypomorphic mutation in the asb11<sup>mut</sup> embryo (we
can exclude that this works through reduced Notch signaling as DAPT treatment reduces Asb-11 and forced Notch signaling increases Asb-11 expression [11], implying that the cullin box mutation has consequences for d-Asb11 function.

**Cullin box is required for correct expression of Notch target genes**

Morpholino-mediated knockdown of d-asb11 causes repression of specific Delta-Notch elements and their transcriptional targets, whereas misexpression of d-asb11 induces Delta-Notch activity [11]. To test whether the cullin box mutation has comparable consequences for d-Asb11 function in regulating Delta-Notch signaling pathway, we first explored the capacity of the cullin box-deleted protein to activate, upon its overexpression, Notch-dependent transcription in vitro. We observed that overexpression of wild type d-Asb11 in human neuronal precursor cell line, NTERa2 [20] led to a strong activation of the Notch target gene hes1, however, overexpression of the mutant protein was not capable of doing so (Fig. 3). Because Notch signaling induces activation of hes1 gene through the CSL transcriptional complex [21], we used a hes1 reporter lacking the conserved CSL-binding site (hes1-RBP) to confirm Notch-specificity for this transactivation. However, neither d-Asb11 nor Asb11 Cul were capable of transactivating hes1-RBP. These results showed that the Cul5 box of d-Asb11 is essential for its function to activate the Notch target gene hes1 through Notch pathway.

Next, we investigated the expression of Notch target genes in vivo by performing WISH for the Hairy/E(spl)-related transcription factors, her1, her4 and her5 on 12 hpf asb11+/− and wild type embryos. At this time point, the expression of her1 and her4 was considerably reduced in asb11+/− embryos (Fig. 4A–B). As her1 and her4 are known to be activated by the Notch signaling [22], this result suggests that the Notch signaling pathway is disrupted in embryos lacking the cullin box domain of Asb11. In contrast, asb11+/− showed a significant increase in the expression of her5 (Fig. 4C), which is known to be downregulated by the Notch1A-intracellular domain [23]. Consistently, we observed downregulation of notch3 (Fig. 4D), which has been shown to repress hes5, a mammalian homologue of zebrafish her5 [24] (although Notch inhibition does not expand the her5 expression domain per se [25], and thus the exact status of her5 as a Notch target gene remains uncertain). The other Notch genes have not been reported to change expression of her5 at this stage of zebrafish embryogenesis,
Role of the d-Asb11 cul5 Box
thus we did not attempt to assess their expression levels in the context of the analysis of her5 expression patterns. Next, we analyzed expression of the Notch ligands DeltaA and DeltaD in asb11c4 embryos. deltaA transcripts showed increased expression in asb11c4 embryos (Fig. 4F), whereas deltaD remained unaffected (Fig. 4E). Detailed examination of the WISH expression patterns of deltaA revealed a change in distribution of mRNA in the neural plate (Fig. 4G). Wild type embryos exhibit a distinct “salt and pepper” aspect of deltaA mRNA distribution whereby some cells have stronger expression than their neighbors, consistent with the notion of Delta-Notch lateral signaling [26]. In contrast, asb11c4 embryos showed a smear of deltaA mRNA transcript across the neural plate, indicating an impaired Notch-mediated lateral inhibition. Thus, the mutation in the d-asb11 cullin box results in the disruption of canonical Delta-Notch signaling.

The cullin box domain of Asb11 is a bona-fide promoter of Notch-mediated her4 induction expression

It was reported that Hairy/E(Spl) expression and activity can be independent of Notch signaling in vivo [27]. Hence, to determine whether the altered regulation of Hairy/E(spl)-related transcription factors in asb11c4 embryos was mediated by Notch activity, we co-injected her4::gfp reporter DNA with d-asb11 or asb11c4 mRNA in zebrafish embryos, which were then treated with DAPT, a γ-secretase inhibitor that blocks Notch signaling [28]. her4 transactivation was determined as a summation of all green fluorescent protein (GFP) present in the embryo. Confocal microscopy was used to trimonolaurically classify transactivation of the her4 promoter as weak, medium or strong (Fig. 5A). When her4::gfp was injected with myc tag (MT) mRNA as a control, embryos presented 80%, 12% and 8% of weak, medium and strong GFP signals, respectively. Upon DAPT treatment, the number of medium and strong signal expressing embryos decreased to 8% and 4%, respectively, showing that Notch signaling was disrupted in response to DAPT treatment.

We performed WISH to investigate the expression of ngn1, a bHLH transcription factor, which is expressed in neuronal precursors and differentiated neural cells [30] and is negatively regulated by Notch signaling [31]. As expected, wild type embryos at 12 hpf displayed the typical clustered expression of ngn1 (Fig. 5B). However, asb11c4 embryos expressed ngn1 at a uniform high level with less evidence of clustering. The increase in ngn1 mRNA expression was confirmed by qPCR. d-Asb11 morphants showed a similar phenotype [10], confirming that the higher expression of ngn1 is caused by loss of d-Asb11 function in the mutant.

Some studies have shown that her4 is also expressed in Islet1/2-positive sensory neurons and its expression is not involved in canonical Notch signaling [29]. Consistently, islet1, detected by WISH, was also increased in zebrafish mutants at 16 hpf. Interestingly, islet1 expression was higher in the polster region where asb11c4 were significantly increased in mutants (Fig. 5C).

All together our data suggest that the cullin box domain of d-Asb11 is essential to regulate Notch targets genes although d-Asb11 lacking the cullin box may yet affect protein expression independently of Notch, via proneural genes.

The cullin box is essential for DeltaA degradation and regulation of neural committed cells

We have previously shown that d-Asb11 affects Delta-Notch signaling by targeting DeltaA for ubiquitylation and subsequent degradation. This effect, strictly dependent on the presence of the SOCS-box [11], establishes the lateral inhibition gradients between DeltaA and Notch facilitating canonical Notch signaling. To study the role of the cullin box domain in d-Asb11-mediated degradation of DeltaA, we injected zebrafish embryos with Myc-tagged deltaA (MT-deltaA) and d-asb11 or asb11c4 mRNA at one-cell stage. Embryos were analyzed for the presence of MT-DeltaA protein at 12 hpf. Wild type embryos injected with full-length d-asb11 displayed substantial DeltaA degradation. In contrast, injected asb11c4 was not capable of degrading DeltaA when compared to control (Fig. 6A; p<0.05).

Thus, we show that the cullin box domain of d-Asb11 is essential for degradation of Notch ligand DeltaA in zebrafish embryos, providing the first in vivo (but not in vitro, e.g. [32]) evidence that absence of a cullin box interferes with a protein degradation function of a SOCS-box-protein. Moreover, the expression of deltaA in the three longitudinal domains of zebrafish neural plate corresponds to regions that express elevated levels of ngn1 and in which the earliest neurons are born [33]. Asb11c4 was unable to degrade DeltaA and acted as a dominant negative increasing the quantity of DeltaA protein in mutant embryos, we propose that the premature neuronal commitment in asb11c4 embryos, assessed by the increased expression of the proneural gene ngn1, is a consequence of DeltaA accumulation in the neural plate.

Absence of the cullin box alters proliferation patterns

As Notch signaling drives (or maintain) precursor cell proliferation within the neurogenic regions of the embryo, a prediction from our findings would be that the loss of d-Asb11 cullin box would impair such proliferation. Indeed, fluorescent whole-mount antibody labeling with the mitotic marker anti-
phosphohistone-3 (PH 3) antibody showed a significant decrease in the rate of cellular proliferation of asb11cul embryos at 24 hpf (Fig. 6B, green label), indicating that the d-Asb11 cullin box is necessary for proper cell proliferation. Alternatively, the premature differentiation of precursor cells in d-asb11 mutants led to diminished number of proliferating cells.

In summary, here we show that the Cul5 domain of d-Asb11 is necessary for proper Notch signaling in vitro and in vivo. Zebrafish embryos lacking the cullin box of d-Asb11 displayed alterations in the expression of Notch pathway components and defective neurogenesis. Thus, our in vivo study reveals a novel role of cullin boxes previously unrecognized in in vitro experiments.
Materials and Methods

Fish and embryos

Zebrafish were kept at 27.5°C. Embryos were obtained by natural matings, cultured in embryo medium and staged according to methods previously described [34].

Plasmid construction

Plasmids were constructed and/or provided as previously described [10,11]. The pCS2-MT-DeltaA construct was provided by B. Appel (Vanderbilt University, Nashville TN) [35]. The her4:gfpl reporter was provided by S. Yeo (Kyungpook National University, Korea) [2]. For asb11cul, mutant zebrafish cDNA was isolated and cloned into the EcoRI and XhoI sites of pCS2-MT and pCS2+

mRNA synthesis, mRNA and DNA microinjections

Capped mRNAs were synthesized using the mMESSAGE mMACHINE kit (Ambion). Fig. 6A, embryos were injected with 600 pg MT-deltaA and 350 pg d-asb11 or 350 pg asb11cul mRNAs. Fig. 5A, embryos were injected with 5 pg her4:gfpl DNA or 5 pg her4:gfpl +300 pg d-asb11 or asb11cul mRNA. Total volume of the injection was set at 1 nL.

DAPT treatment

Half of each injected group (n = 50) (Fig. 5A) was incubated in 100 μM DAPT diluted in 1% DMSO in embryo-medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, 0.00005% Meth Blue). The other half was incubated in 1% DMSO in embryo-medium. The embryos were incubated from 1.5hpf till 14hpf, fixed with 4% PFA overnight at 4°C and analyzed for GFP expression.

In situ hybridization

Whole mount in situ hybridizations were performed according to methods previously described [36]. All probes used in this study are previously described [10,11].

Immunoblotting

Whole mount in situ hybridizations were performed according to methods previously described [36]. At 12hpf, chorion and yolk were removed. Embryos were lysed in cell lyses buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Na-deoxycholate, 1% NP-40, 10 u, 1% protease inhibitor (ROCHE), 2 μl/embryo. Primary antibodies were diluted in PBS containing 5% milk (fig. 2: rabbit anti-asb11 1:100, fig. 5: rabbit anti-MT 1:1000, Bioker) and used for immunoblotting as previously described [37]. As loading control an anti-actin body was used in addition to coomassie staining of the membrane. For densitometric analysis all bands were measured with a GS-800 Densitometer (Biorad), and total area counts (OD x mm2) were corrected for back ground (equivalent area on a non-relevant place on the blot). Subsequently samples were corrected for loading using the control band and finally values were expressed relative, defining the intensity of the wild type sample as 1.

RNA isolation and qRT-PCR

Total RNA was extracted from whole wild type and mutant embryos at 10 or 12 hpf. Total RNA extraction, cDNA synthesis and qPCR quantification were performed according to previously described methods [36].

Whole mount immunolabelling, microscopy and image quantification

Whole-mount immunohistochemistry and picture capture and analysis was performed as described [13,39]. For figure 6B anti-
Leica software (Application Suite 1.8.0) was used to create a two times in triplicate. Values were normalised with TAL-luc [11]. Scintillation Counter (Packard). The experiments were performed seeded in a 96-well plate and transfected using IBAfect and MA-enhancer (IBA Biosciences, GmbH) using the suppliers protocol. Luciferase was measured on a Packard TOPCOUNT Microplate Scintillation Counter (Packard). The experiments were performed two times in triplicate. Values were normalised with TAL-luc [11].

Luciferase reporter assay

nTera2/d1 cells were maintained in DMEM containing 10% FCS. The culture media were supplemented with 5 mM glutamine and antibiotics/antimycotics. Cells were transfected at 5% CO₂ in a humidified incubator at 37°C. nTera2/d1 cells were seeded in a 96-well plate and transfected using IBAfect and MA-enhancer (IBA Biosciences, GmbH) using the suppliers protocol. Luciferase was measured on a Packard TOPCOUNT Microplate Scintillation Counter (Packard). The experiments were performed two times in triplicate. Values were normalised with TAL-luc [11].

References

1. Gao M, Karin M (2005) Regulating the regulators: control of protein ubiquitination and ubiquitin-like modifications by extracellular stimuli. Mol Cell 19: 581–583.
2. Kelle BT, Schulman BA, Alexander WS, Nicola NA, Martin HM, et al. (2002) The SOCS box: a tale of destruction and degradation. Trends Biochem Sci 27: 215–219.
3. Deshaies RJ, Rojozeiro CA (2009) RING domain E3 ubiquitin ligases. Ann Rev Biochem 78: 399–434.
4. Piessevaux J, Lavens D, Peelman F, Tavernier J (2008) The many faces of the SOCS box. Cytokine Growth Factor Rev 19: 371–381.
5. Kamata T, Maenaka K, Kotakiba S, Matsumoto M, Kodha D, et al. (2004) VHL-box and SOCS-box domains determine binding specificity for Cul2-Rbx1 and Cul3-Rbx2 modules of ubiquitin ligases. Gene Dev 18: 3055–3065.
6. Kohroki J, Nishiyama T, Nakamura T, Masuho Y (2005) ASB proteins interact with Cul5 and Rbx2 to form E3 ubiquitin ligase complexes. FEBS Lett 579: 6796–6802.
7. Krebs DL, Hilton DJ (2000) SOCS: physiological suppressors of cytokine signaling. J Cell Sci 113: 2013–2019.
8. Mahour R, Redwine VB, Florens I, Swanson SK, Martin-Brown S, et al. (2008) Characterization of Cul5-box sequences that direct recruitment of Cul2-Rbx1 and Cul5-Rbx2 modules to E3 ubiquitin C ubiquitin ligases. J Biol Chem 283: 8085–8093.
9. Hilton DJ, Richardson RT, Alexander WS, Vinay EM, Willson TA, et al. (1998) Twenty proteins containing a C-terminal SOCS box form five structural classes. Proc Natl Acad Sci U S A 95: 114–119.
10. Diks SH, Bink RJ, van de Water S, Joore J, van Rooijen C, et al. (2006) The Notch-dependent HES1, HES5, HEY1, HEY2, HEYL transcription in fetal tissues, adult tissues, or cancer. Int J Oncol 31(2): 461–6.
11. Takai C, Campos-Ortega JA (1999) her1, a zebrafish pair-rule like gene, acts downstream of notch signaling to control somite development. Development 126: 3005–14.
12. Hans S, Scheer N, Reedl I, V Weizacker E, Blader P, et al. (2004) her1, a zebrafish member of the hairy-E(spl) family, is repressed by Notch signalling. Development 131: 2957–2969.
13. Beatus P, Lundkvist J, Oberg C, Lundahl U (1999) The notch 3 intracellular domain represses notch 1-mediated activation through Hairy/Enhancer of split (HES) promoters. Development 126: 3925–3935.
14. Geling A, Pleasy C, Rastegaev S, Strahle U, Bally-Cuif L (2004) Her5 acts as a prepattern factor that blocks neurogenin1 and coe2 expression upstream of Notch to inhibit neurogenesis at the midbrain-hindbrain boundary. Development 31: 1993–2006.
15. Skeath JB, Thor S (2003) Genetic control of Drosophila nerve cord development. Curr Opin Neurobiol 13: 8–15.
16. Ye SY, Kim M, Kim HS, Huh TL, Chimis AB (2005) Fluorescent protein expression driven by her4 regulatory elements reveals the spatiotemporal pattern of Notch signaling in the nervous system of zebrafish embryos. Dev Biol 301: 555–567.
17. Geling A, Steiner H, Willem M, Bally-Cuif L, Haass C (2002) A gamma-secretase inhibitor blocks Notch signaling in vivo and causes a severe neurogenic phenotype in zebrafish. EMBO Rep 3: 608–694.
18. Nij HJ, Chuan HS, Bas YK, Kim HS, Park YM, et al. (2008) Her4 is necessary for establishing peripheral projections of the trigeminal ganglia in zebrafish. Biochem Biophys Res Commun 379: 22–26.
19. Ma Q, Chen Z, del Barco Barrantes I, de la Pompa JL, Anderson DJ (1998) neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. Neuron 20: 469–482.
20. Blader P, Fischer N, Gradwohl G, Guillet M, Strahle U (1997) The activity of neurogenin1 is controlled by local cues in the zebrafish embryo. Development 124(22): 4557–4569.
21. Chung AS, Guan VJ, Yuan ZL, Albina JE, Chin YV (2005) Ankyrin repeat and SOCS box 3 (ASB3) mediates ubiquitination and degradation of tumor necrosis factor receptor II. Mol Cell Biol 25: 4716–4726.
22. Appel B, Eisen JS (1998) Regulation of neuronal specification in the zebrafish spinal cord by Delta function. Development 125: 371–380.
23. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the zebrafish. Dev Dyn 193(3): 253–310.
24. Appel B, Fritz A, Westerfeld M, Grunwald DJ, Eisen JS, et al. (1999) Delta-mediated specification of midline cell fates in zebrafish embryos. Curr Biol 12: 247–56.
25. Oxtoby E, Jowett T (1993) Cloning of the zebrafish krox-20 gene (krox-20) and its expression during hindbrain development. Nucleic Acids Res 21: 1087–109.
26. Versteege HH, Sorensen BB, Sofirotah SH, Van den Brande JH, Stam JC, et al. (2002) HER3/tissue factor interaction results in a tissue factor cytoplasmic domain-independent activation of protein synthesis, p70, and p90 S6 kinase phosphorylation. J Biol Chem 277: 27065–72.
27. Braat H, Stokkers P, Hommes T, Cohn D, Vogels E, et al. (2003) Consequence of functional Not2 and Not3 mutations on gene transcription in Crohn’s disease patients. J Mol Med 81: 603–611.
28. Peppelenbosch M, Boone E, Jones GE, van Deventer SJ, Hargeman G, et al. (1999) Multiple signal transduction pathways regulate TNF-induced actin reorganization in macrophages: inhibition of Cdc42-mediated filopodia formation by TNF. J Immunol 162: 837–45.

Statistical testing

Each value with a standard deviation is the average of at least two independent experiments performed in triplicate. Statistical tests were performed using two-tailed t-test. All bars in graphs depict mean values with error bars depicting standard deviations. Statistical X²-test was performed for Fig. 5A.

Acknowledgments

We thank Dr. Paula van Tijn for helpful discussions.

Author Contributions

Conceived and designed the experiments: MSdS, JMT, DZ, MP. Performed the experiments: MSdS, JMT, JP, AB, SHD. Analyzed the data: MSdS, JMT, VR, DZ, MP. Contributed reagents/materials/analysis tools: DG. Wrote the paper: MSdS, JMT, DG, MP.