Activity of the Mouse Notch ligand DLL1 is Sensitive to C-terminal Tagging in Vivo

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Research note

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Abstract

Objective

The mammalian Notch ligand DLL1 has essential functions during development. To visualise DLL1 in tissues, for sorting and enrichment of DLL1-expressing cells, and to efficiently purify DLL1 protein complexes we tagged DLL1 in mice with AcGFPHA or Strep/FLAG.

Results

We generated constructs to express DLL1 that carried C-terminal in-frame an AcGFPHA tag flanked by loxP sites followed by a Strep/FLAG (SF) tag out of frame. Cre-mediated recombination replaced AcGFP-HA by SF. The AcGFPHAstopSF cassette was added to DLL1 for tests in cultured cells and introduced into endogenous DLL1 in mice by homologous recombination. Tagged DLL1 protein was detected by antibodies against GFP and HA or Flag, respectively, both in CHO cells and embryo lysates. In CHO cells the AcGFP protein fused to DLL1 was functional. I*n vivo* AcGFP expression was below the level of detection by direct fluorescence. However, the SF tag allowed us to specifically purify DLL1 complexes from embryo lysates. Homozygous mice expressing AcGFPHA or SF-tagged DLL1 revealed a vertebral column phenotype reminiscent of disturbances in AP polarity during somitogenesis, a process most sensitive to reduced DLL1 function. Thus, even small C-terminal tags can impinge on sensitive developmental processes requiring DLL1 activity.

Introduction

The *Dll1* gene encodes one of four mammalian Notch-activating ligands and is expressed in complex patterns in numerous cell types and tissues [e.g. 1–8]. The DLL1 protein is an essential Notch ligand and critical for example during somite patterning and myogenic differentiation [9–11], during vascular development [12–14] and for establishment of left-right asymmetry [15,16], differentiation of pancreatic [17], neuronal [18], epidermal [19], marginal zone B [20] and intestinal stem cells [21].

To analyse the dynamics of DLL1 protein expression in the presomitic mesoderm and neuronal progenitors by live imaging endogenous DLL1 was previously C-terminally tagged with three luciferase proteins [1]. The fusion with red luciferase was fully functional, whereas for unknown reasons a DLL1 firefly luciferase fusion was slightly hypomorphic and a DLL1 emerald luciferase fusion was non-functional [1]. Thus, C-terminal tagging of DLL1 without compromising DLL1 function is -in principle- possible.

Here, we tagged endogenous DLL1 at its C-terminus by homologous recombination. We chose monomeric AcGFP [22] as a means to detect and isolate DLL1 expressing cells and a SF tag to affinity purify DLL1 complexes for mass spectrometric analyses.

Methods

Here, methods are briefly summarised. A detailed description of materials, primers, antibodies and methods is provided in Additional File 1.

Mouse strains

Mice expressing tagged DLL1 were generated in this study, ZP3::Cre [23] and FLPe mice [24] were described previously. Mice were maintained and analysed on a mixed (129Sv/ImJ,C57Bl/6,CD1,NMRI) genetic background.

Cell culture

Cells were cultured as described previously [25].

Generation of Constructs

A gene fragment encoding monomeric AcGFP [22] fused with an HA Tag followed by a stop codon, flanked by loxP sites followed by a SF tag [26] and a stop codon was synthesized (GENEWIZ, UK). This fragment was used to generate expression (Figure 1A) and targeting (Figure 2A) vectors by standard cloning procedures.

Cell surface biotinylation

Cell surface biotinylation was done essentially as described previously [25].

Gene targeting, and generation of mice

ES cells were electroporated as described [25], screened by PCR and validated by Southern blot hybridisations (Figure S1B Additional File 2). *Dll1*$^{AcGFPHAstopSF\text{-}Puro}$ mice were generated using correctly targeted ES cells as described [25], the Puro cassette removed by breeding to FLP-deleter mice, and the *Dll1*^{SF} allele generated by crossing *Dll1*^{AcGFPHAstopSF} mice to ZP3:Cre mice.

Skeletal preparations

Skeletal preparations were generated and documented as described [25]

Confocal imaging

Images were acquired using a Leica SP8 confocal laser microscope with a 10x or 20x air or immersion objective and optical sections of 1 µm thickness were taken using the Las X Software (Leica) and processed with Adobe Photoshop CS5.

Immunoprecipitation (IP)

IPs were done with anti-GFP or anti-HA or anti-Flag antibodies and Sepharose G beads.

Affinity purification of DLL1 complexes and Mass Spectrometry

DLL1 complexes were affinity purified and analysed by LC-MS/MS as previously described [27].

Results

Prior to tagging endogenous DLL1 we tested our strategy and functionality of the tags in cultured cells. A *Dll1* expression construct was cloned into pCMV2 using the *Dll1* cDNA with the AcGFPHAstopSF cassette fused to the C-terminus of DLL1 (Fig. 1A). This resulted in a modification identical to the one planned to tag endogenous DLL1. CHO cells were generated with stably integrated pCMV2Dll1AcGFPHAstopSF or pCMV2Dll1SF (the latter obtained by recombination of the Dll1AcGFPHAstopSF plasmid in Cre-expressing bacteria). Due to high background observed with the HA antibody in Western blots in CHO cell lysates expression of the tagged DLL1 proteins was analysed by immunoprecipitation with anti-GFP, -HA and -Flag antibodies including CHO wild type cells as negative controls. Precipitated proteins were detected by western blot analyses using the DLL1-specific monoclonal antibody 1F9, lysates of DLL1Flag overexpressing CHO cells [28] served as positive controls. DLL1AcGFPHA was detected in CHO cells carrying pCMV2Dll1AcGFPHAstopSF after IP with anti-GFP and HA antibodies (Fig. 1B a,b) but as expected not after IP with the anti-Flag antibody (Fig. 1B c). In contrast, CHO cells carrying pCMV2Dll1SF showed no detectable signal with 1F9 after IP with anti-GFP or HA antibodies (Fig. 1B a,b) but showed expression of DLL1SF after IP with the anti-flag antibody (Fig. 1B c). Thus, as planned, a differently tagged DLL1 variant was obtained after Cre-mediated recombination of pCMV2Dll1AcGFPHAstopSF that replaced AcGFPHA with the SF tag. The functionality of AcGFP fused to DLL1 was confirmed by confocal fluorescence microscopy of CHO cells stably expressing DLL1AcGFPHA (Fig. 1C). Surface presentation of the tagged DLL1 proteins was investigated by cell surface biotinylation of CHO cells stably expressing DLL1AcGFPH or DLL1SF. Both DLL1 variants were detected at the cell surface at similar levels (Fig. 1D, Figure S2A,B Additional File 3, Table S1 Additional File 4 and Table S2 Additional File 5). In addition to the tagged DLL1 proteins migrating at the expected molecular weights a shorter DLL1 protein migrating at the approximate molecular weight of wild type DLL1was detected in these assays with both variants (Figure S2A,B Additional File 3). Cell surface biotinylation followed by Avidin pull down or IP with anti-GFP or anti-Flag antibodies showed that the faster migrating DLL1 proteins lacked the C-terminal tags

(Figure S2C Additional File 3) suggesting that the tags were removed from a portion of DLL1 by proteolytical cleavage. Since “cleaved” DLL1 was less abundant in the cell lysates than in the affinity-purified fraction (Table S1 Additional File 4 and Table S2 Additional File 5) removal of the tag might at least in part occur during the purification despite the presence of protease inhibitors.

The C-terminal modifications were introduced into endogenous DLL1 by homologous recombination in ES cells. To increase the targeting frequency at the *Dll1* locus, which in our hands tended to be low, we employed the CRISPR/Cas system (see Materials and Methods for details). Targeting events were first detected by PCR and validated by Southern blot analyses, which revealed a surprising high number of off-target events and multiple integrations (Figure S1B Additional File 2). One validated correctly targeted ES cell clone transmitted the planned alteration ($Dll1^{AcGFPHAstopSF}$) through the germ line. $Dll1^{AcGFPHAstopSF}$ was recombined in the female germ line of ZP3:Cre mice to obtain the $Dll1^{SF}$ allele. Heterozygous mice carrying either allele ($Dll1^{AcGFPHAstopSF}$ n = 18 and $Dll1^{SF}$ n = 18) were phenotypically normal. Homozygous mice of both alleles and sexes were viable. Adult mice ($Dll1^{AcGFPHAstopSF}$ n = 24 and $Dll1^{SF}$ n = 46) showed short and kinky tails suggesting vertebral column defects (Fig. 2B b,c). In addition, 60% (15/25) of test-mated homozygous males carrying the $Dll1^{SF}$ allele were infertile. Skeletal preparations of E15.5 embryos ($Dll1^{AcGFPHAstopSF}$ n = 10 and $Dll1^{SF}$ n = 10) revealed misshaped vertebral bodies and ribs indicative of somite patterning defects, which appeared to be more severe in the $Dll1^{SF}$ allele (Fig. 2C b,c). Expression of tagged DLL1 was analysed by immunoprecipitations from homozygous d10.5 embryo lysates followed by detection with anti-DLL1 1F9. As observed with Dll1AcGFPHA CHO cells in $Dll1^{AcGFPHAstopSF}$ embryos DLL1 was detected after IP with anti-GFP or -HA antibodies and not after IP with anti-Flag (Fig. 2D a,b). Conversely, in $Dll1^{SF}$ embryos DLL1 was detected after IP with anti-Flag and not after IP with anti-GFP or HA (Fig. 2D c). These findings confirmed that the $Dll1^{AcGFPHAstopSF}$ allele recombined in mice as expected and the anticipated tagged DLL1 proteins were indeed generated. In contrast to DLL1AcGFPHA overexpressing CHO cells no green fluorescence was detected in homozygous d9,5 $Dll1^{AcGFPHAstopSF}$ embryos (n = 8) harbouring the $Dll1^{AcGFPHAstopSF}$ allele (Fig. 2E a). This suggests that expression levels in the transgenic CHO cells were significantly higher than expression of DLL1 from the endogenous locus and that low endogenous DLL1 levels prevented detection of AcGFP fluorescence.

To analyse whether the SF tag allows one to purify sufficient DLL1 complexes for mass spectrometry analyses we performed a pilot study using E10.5 embryo lysates and a one-step purification using anti-Flag affinity beads. Affinity complexes from wild type (control) and homozygous $Dll1^{SF}$ embryos were purified in 6 independent experiments and the material was analysed by mass spectrometry. DLL1 as well as 61 other proteins were detected specifically in the material purified from $Dll1^{SF}$ embryos (Table S3 Additional File 6) demonstrating that Flag-tagged DLL1 was specifically purified in sufficient amount from transgenic mouse tissues for analysis by mass spectrometry. The full data set has been submitted to the PRIDE database (accession number PXD024680)

Discussion

We have modified endogenous mouse DLL1 by homologous recombination in one step to tag DLL1 for observation in living cells and tissues, for sorting of DLL1 expressing cells, or for affinity purification to identify DLL1 protein complexes. The employed tags were functional in CHO cells but impinged on DLL1 function *in vivo* such that somite patterning, the process most sensitive to reduced DLL1 function [25] was affected. In addition, endogenous DLL1 tagged by AcGFP was not detected by fluorescence.

In a previous study DLL1 was C-terminally fused with three different luciferase proteins. Fusion with red luciferase was fully functional, whereas for unknown reasons a DLL1 firefly luciferase fusion was slightly hypomorphic and DLL1 fused to emerald luciferase was non-functional [1]. Our C-terminal fusions behave as hypomorphic alleles similar to but more severe than firefly luciferase.

Based on the published data [1] and our results (this paper) 4 out of 5 C-terminal tags added to DLL1 affected DLL1 function to varying degrees, although in principle C-terminal tagging is possible without impinging on DLL1 activity. Reduction of DLL1 activity does not appear to depend on the length of the tag because the long red luciferase had no effect whereas the short SF tag (plus the peptide encoded by the *loxP* site) affected DLL1 function more strongly than our longer AcGFPHA tag. Removal of a 16bp fragment in the 3´UTR might affect RNA stability and could be responsible for reduced DLL1 activity in our transgenic mice. However, this appears unlikely since mice tagged with firefly or emerald luciferase had a complete 3´ UTR [1] and also showed hypomorphic *Dll1* phenotypes. AcGFP was described as a monomeric protein [22,29]. Thus, in case of the AcGFP tag non-physiological clustering of DLL1AcGFPHA is an unlikely reason for reduced DLL1 function, although abnormal clustering and trafficking of C-terminally tagged DLL1 cannot be excluded. DLL1 carries a PDZ binding domain at its C-terminus which interacts with Acvrinp1, a MAGUK family member [30], and ARIP2, which has been implicated in stabilizing DLL1 and DLL4 [31]. For C-terminal PDZ binding domains a free C-terminus is important for interactions with PDZ proteins in many cases [reviewed in 32,33]. C-terminal extension of DLL1 with tags might interfere with such interactions but this seems unlikely to play a major role as the C-terminal extension by red luciferase had no effect on DLL1 function. It appears that sequences of the C-terminal fusion are an important contributing factor and might affect protein stability or trafficking or processing *in vivo* and thereby impinge on DLL1 protein function leading to somite patterning defects.

AcGFP fused to DLL1 in DLL1AcGFPHA over expressing CHO cells was detected by direct fluorescence indicating that AcGFP in the context of the fusion protein is functional. However, even in homozygous mouse embryos we did not detect AcGFP fluorescence in any tissue. A plausible explanation could be that expression levels in CHO cells were much higher than low levels of DLL1 expressed from the endogenous locus. Thus, absence of detectable fluorescence signals likely reflect DLL1AcGFPHA levels that are below the limit of detection of our set up. Whether fluorescing proteins with other activation and excitation properties and a better quantum yield (for example ZsGreen1 [29]) fused to DLL1 are sufficient to detect expression of a DLL1 fusion protein *in vivo* remains to be addressed.

Whereas DLL1AcGFPHA in our mice turned out to be insufficient for direct DLL1 detection by fluorescence our pilot study using the $Dll1^{SF}$ allele demonstrated its usefulness for the purification of

DLL1-containing protein complexes (Table S3Additional File 6) from endogenous sources, in this case early embryos. In our pilot study components of the secretory pathway and vesicle transport were identified, which can be expected for DLL1, a transmembrane protein that undergoes endocytotic processing [34]. In addition, enzymes involved in ubiquitination copurified with tagged DLL1. Since DLL1 is modified by ubiquitin [34] also these potential interaction partners support that specific DLL1 protein complexes were affinity-purified. GO term analysis [35,36] showed a surprising enrichment of other identified proteins implicated in metabolic processes, nucleotide-binding and catalytic activity. As far as we know, these proteins have not been implicated in or related to DLL1 function as yet and their significance for DLL1 activity will require further analyses. Given that our tag impinged on DLL1 function it is possible that the tag also prevents the isolation of a subset of DLL1 complexes.

In conclusion, DLL1 activity appears to be highly sensitive to sequences added to the C-terminus of the protein. Which sequences are tolerated by DLL1 are currently not predictable and might only be determined empirically by comprehensive studies *in vitro* and *in vivo*.

Limitations

Whether cleavage of the C-terminal tag occurs also *in vivo* and affects detection and function of tagged DLL1 is unclear. Likewise, a potential effect of the peptide encoded by the loxP sequence cannot be ruled out.

Abbreviations

3´UTR 3’ untranslated region

AcGFP Aequorea coerulescens green fluorescent protein

AP anterior-posterior

Avpd Avidin pull-down

CHO Chinese Hamster Ovary

DLL1 Delta-like 1

ES embryonic stem

GO gene ontology

HA hemagglutinin

IP Immuno precipitation

LC-MS/MS Liquid-Chromatography-Mass spectometry/ Mass spectrometry

pCMV2 cloning vector containing cytomegalovirus promoter

PDZ Domain present in PSD-95, Dlg and ZO1/2

SF tag Strep/FLAG tandem affinity purification tag

wt wild type

Declarations

Ethics approval and consent to participate

Mice were handled in accordance with the German laws and regulations (Tierschutzgesetz). All procedures were approved by the ethics committee of Lower Saxony for care and use of laboratory animals LAVES (AZ 33.12-42502-04-17/2467 and 33.12-42502-04-19/3087). Mice were housed in the animal facility of Hannover Medical School (ZTL) as approved by the responsible Veterinary Officer of the City of Hannover, Germany. Animal welfare was supervised and approved by the Institutional Animal Welfare Officer.

Consent for publication

Not applicable

Availability of data and materials

All data supporting the results of this article are included in this article and its additional files, the full mass spectrometry data are available in the PRIDE database under Accession number PXD024680 (Reviewer account details: Username: reviewer_pxd024680@ebi.ac.uk; Password: USa7Ceew)

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

KSG designed experiments, generated and analysed cell and mouse data, wrote manuscript draft, KB performed the mass spectrometry and analysed data, DB analysed cells and embryos, revised the manuscript, PDH generated data, MU analysed data, acquired funding, AG designed experiments, analysed data, wrote the manuscript. All authors read, edited and approved the final manuscript.

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Figures

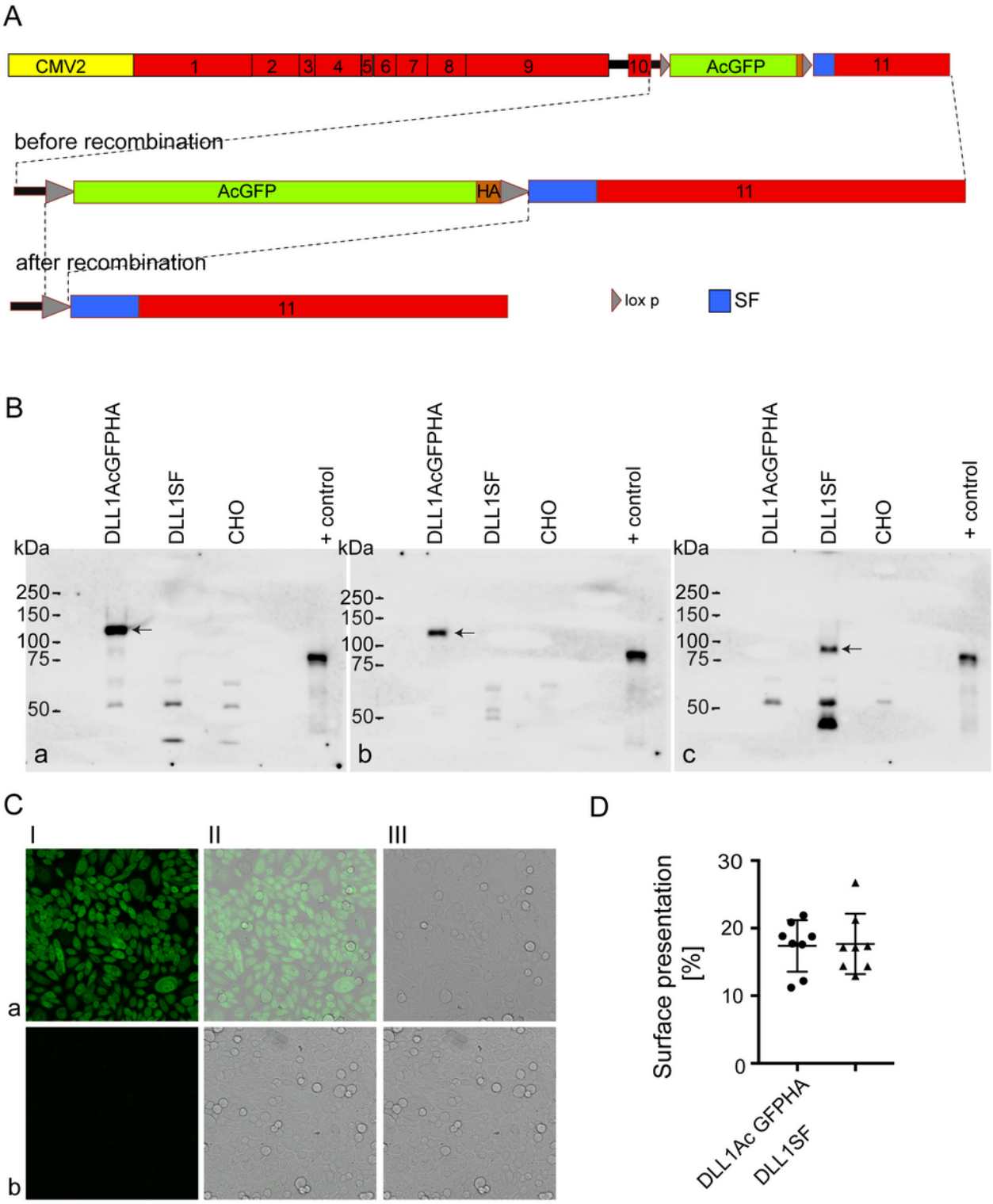

Figure 1

Analysis of DLL1AcGFPHA and DLL1SF proteins in CHO cells (A) Scheme of constructs used to express DLL1AcGFPHA or DLL1SF in CHO cells under control of the CMV2 promoter. Full length construct (top), 3' region before (middle), and after (bottom) Cre-mediated recombination. (B) Expression of DLL1 variants in CHO cells carrying DLL1AcGFPHA or DLL1SF. DLL1 variants were immuno precipitated with anti HA (a) anti-GFP (b) or anti-Flag (c) antibodies and detected in Western blots using anti-DLL1 antibody 1F9.

+control: Lysate of CHO cells stably overexpressing DLL1. Arrows point to tagged DLL1 proteins. Photographs of the Western blot membranes are shown in Figure S3 Additional File 7. (C) AcGFP fluorescence in DLL1AcGFPHA expressing CHO cells (row a) in comparison to wild-type CHO cells (row b) ; Column I: fluorescence, II: overlay, III: bright field. (D) Surface presentation of DLL1AcGFPHA (dots) and DLL1SF (triangles) in CHO detected in cell surface biotinylation assays.

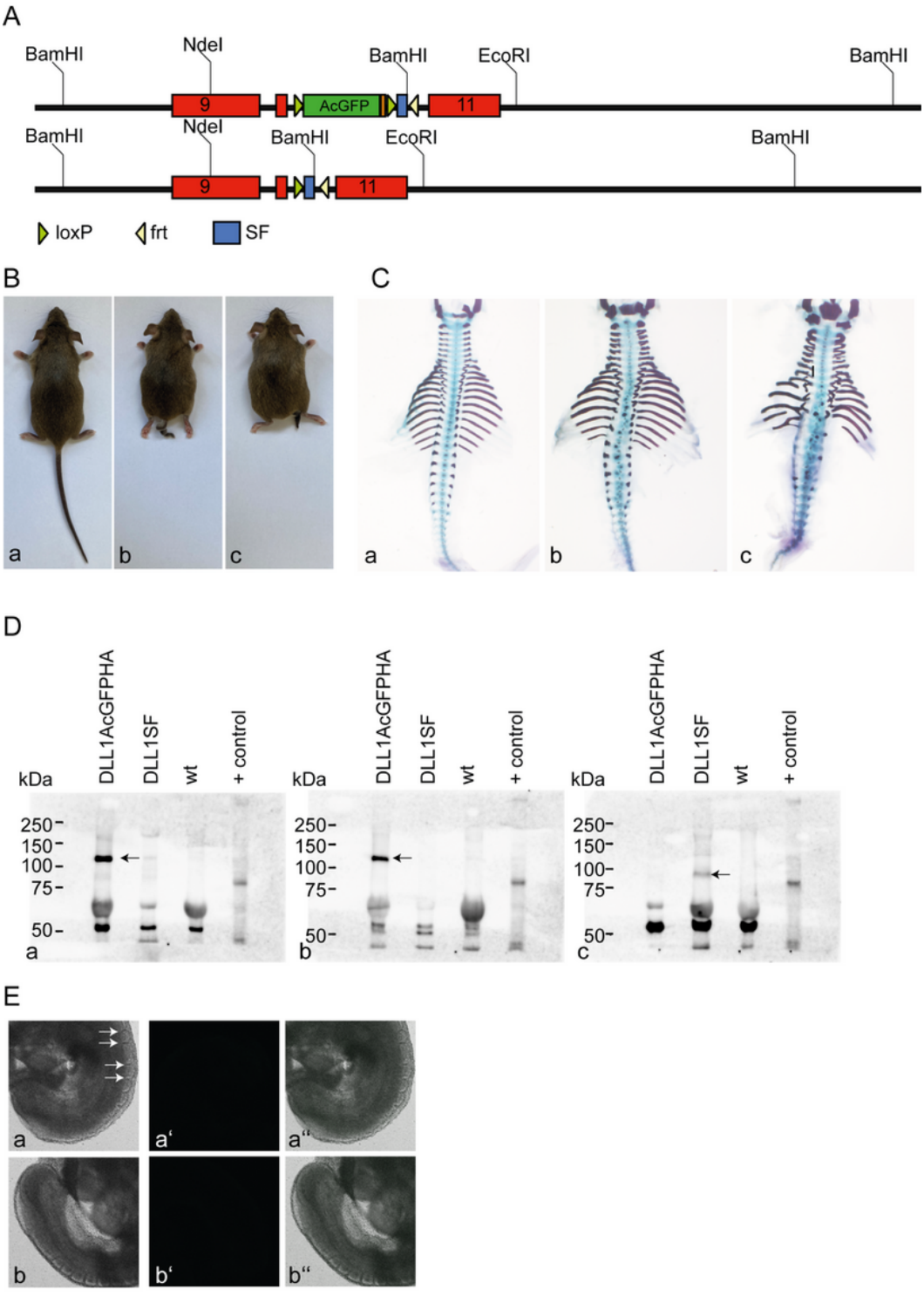

Figure 2

Analysis of Dll1AcGFPstopSF and Dll1SF mice (A) Scheme of the modified 3’ region before (top) and after (bottom) Cre-mediated recombination. (B) Phenotype of wt (a), homozygous adult Dll1AcGFPHAstopSF (b) and Dll1SF (c) mice. (C) Skeletal preparations of wt (a), homozygous Dll1GFPHAstopSF (b) and Dll1SF d15.5 fetuses. (D) Detection of DLL1 variants in homozygous Dll1AcGFPHAstopSF , Dll1SF and wild type d10.5 embryos. DLL1 variants were immunoprecipitated with anti-HA (a), anti-GFP (b) or anti-Flag (c) antibodies and detected in Western blots using anti-DLL1antibody 1F9. +control: Lysate of DLL1 overexpressing ES cells. Arrows point to tagged DLL1 proteins. Photographs of the Western blot membranes are shown in Figure S4 Additional File 8. (E) GFP fluorescence in homozygous d9,5 Dll1AcGFPHAstopSF (a-a”) and wild type (b-b”) embryos; (a, b) bright field, (a’, b’) fluorescence, (a”, b”) overlay. Arrows in (a) point to irregular somites.

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