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Graphical abstract

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Taspase1 orchestrates fetal liver hematopoietic stem cell and vertebrae fates through cleaving TFIIA

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Abstract

Taspase1, a highly conserved threonine protease encoded by TASP1, cleaves nuclear histone modifying factors and basal transcription regulators to orchestrate diverse transcription programs. Hereditary loss-of-function mutation of TASP1 has recently been reported in human resulting in a novel anomaly complex syndrome manifested with hematological, facial, and skeletal abnormalities. Here, we demonstrate that Taspase1-mediated cleavage of TFIIAα–β, rather than of MLL1 or MLL2, in mouse embryos is required for proper fetal liver hematopoiesis and correct segmental identities of the axial skeleton. Homozygous genetic deletion of Taspase1 (Tasp1−/−) disrupted embryonic hematopoietic stem cell self-renewal and quiescence states, and axial skeleton fates. Strikingly, mice carrying knockin non-cleavable mutations of TFIIAα–β (Gtf2a1nc/nc), a well-characterized basal transcription factor, displayed more pronounced fetal liver and axial skeleton defects than those with non-cleavable MLL1 and MLL2 (Mll1nc/nc;2nc/nc), two trithorax group (Trx-G) histone H3 trimethyl transferases. Our study offers molecular insights concerning TASP1-loss human syndrome and discovers unexpected role of TFIIAα–β cleavage in embryonic cell fate decisions.
Introduction

Recently, a novel human hereditary anomaly syndrome was recognized in association with loss-of-function mutations in the \textit{TASPI} gene (1-3). Those patients were characterized with microcephaly, developmental delay, distinctive facial features, and other anomalies including anemia, thrombocytopenia and lymphocytopenia (2, 3). \textit{TASPI} codes for Taspase1, which is an evolutionarily conserved threonine protease that cleaves and regulates nuclear proteins, most notably MLL (KMT2A, also known as MLL1) and TFIIA$\alpha$–$\beta$ (4-6). Taspase1 is a 50kD $\alpha$-$\beta$ proenzyme which undergoes intramolecular autoproteolysis to produce mature, active $\alpha28/\beta22$ heterodimeric protease (4, 6-8). Cleavage of Taspase1 substrates occurs distal to the aspartate residue within the conserved IXQL(V)D/G motif (4, 9).

The bona fide Taspase1 substrates are MLL1, MLL2 (KMT2B), TFIIA$\alpha$-$\beta$ (GTF2A1), ALF (TFIIA-Like Factor, GTF2A1L) and \textit{Drosophila} HCF (dHCF) (4, 5, 10-12). MLL1, a member of the trithorax group (Trx-G) epigenetic modifiers, is a histone methyl transferase (HMT) that trimethylates H3 at lysine 4 (H3K4me3), generating a histone mark of active transcription (13, 14). In the absence of proteolytic activation, immature MLL1 polypeptide displays reduced HMT activity, hence functions as a hypomorphic mutant (11). TFIIA, comprised of three polypeptides ($\alpha$, $\beta$ and $\gamma$), complexes with TBP (TATA Binding Protein) and constitutes an integral part of the basal transcription machinery (15-19). Precursor TFIIA$\alpha$-$\beta$, encoded by a single gene \textit{Gtf2a1}, is processed into TFIIA $\alpha$ and $\beta$ subunits by Taspase1-mediated cleavage (5). Complexes of TFIIA $\alpha$-$\beta/\gamma$ containing non-cleaved TFIIA$\alpha$-$\beta$ have transcriptional activity \textit{in vitro} and its maturation into TFIIA $\alpha/\beta/\gamma$ increases its susceptibility to regulatory degradation (5).
addition, TFIIA α-β/γ and TFIIA α/β/γ are different in the pattern of interactions with TFIID, an important factor for promoter recognition (20). Since TBP-like protein was reported to be a negative regulator of the Taspase1-mediated processing of TFIIA, Taspase1 may fine-tune the transcription of genes through these factors (21). Previous studies revealed that the proteolytic cleavage of TFIIA is critical for male spermiogenesis (22) and craniofacial development (23).

Taspase1-mediated cleavage of each substrate may account for various phenotypes of Taspase1 deficiency, or alternatively, protease-independent biological activities of Taspase1 may exist. Here, we perform mouse genetic studies to interrogate the biological significance of cleavage of MLL1, MLL2, and TFIIA. Taspase1-mediated cleavage of TFIIA has the most prominent effects on fetal hematopoiesis as well as on the specification of axial skeleton, offering molecular insights concerning novel human Taspase1 loss syndrome.
Results

To investigate the physiological function of Taspase1, we generated Tasp1 deficient mice (Tasp1<sup>−/−</sup>). Our initial report demonstrated that these animals displayed marked homeotic transformation of the axial skeleton and a decrease in overall body size (11). Furthermore, Tasp1<sup>−/−</sup> mice die shortly after birth, partly due to feeding defects (11). Here, we further examined the role of Taspase1 in embryogenesis and initiated studies by comparing these Tasp1<sup>−/−</sup> and wild-type (WT) littermate embryos. Tasp1<sup>−/−</sup> E14.5 (embryonic day 14.5) embryos show a severe developmental retardation, reflected by a ~40% reduction in overall weight in comparison to WT embryos (Figure 1A, left). Furthermore, Tasp1<sup>−/−</sup> embryos have significantly smaller fetal livers (Figure 1B). The decrease in fetal liver cell number was significant even after normalizing for reduced body weight (p<0.001) (Figure 1A, right).

We next analyzed the fetal liver for mature blood cell lineages and the hematopoietic stem and progenitor cell compartments using multicolor flow cytometry. The analysis of E14.5 fetal livers showed that Tasp1<sup>−/−</sup> and WT fetal livers had little difference in the populations of B220<sup>+</sup> B-cells, Gr-1<sup>+</sup> granulocytes, and TER-119<sup>+</sup> red blood cells (Figure 1C). In contrast, the stem and progenitor cell populations, marked by Lin<sup>−</sup>Sca-1<sup>−</sup>c-Kit<sup>+</sup> staining (so-called LSK cells), were reduced in Tasp1<sup>−/−</sup> fetal livers (Figure 1, D and E). LSK cells can be further specified as multipotent progenitor cells (MPPs; Lin<sup>−</sup>Sca-1<sup>−</sup>c-Kit<sup>−</sup>CD150<sup>−</sup>) or HSCs (Lin<sup>−</sup>Sca-1<sup>−</sup>c-Kit<sup>−</sup>CD150<sup>+</sup>) (24, 25). In Tasp1<sup>−/−</sup> fetal livers, the abundance of HSC was found to be reduced to approximately half that of the WT fetal liver (Figure 1E, left), resulting in a ~78% decrease in the absolute number of HSCs (Figure 1F). No difference in the frequency of Lin<sup>−</sup>Sca-1<sup>−</sup>c-Kit<sup>+</sup> myeloid progenitors was observed whereas the absolute numbers were decreased. Specifically, in Tasp1<sup>−/−</sup> fetal livers, the
relative abundance of common myeloid progenitors (CMPs), granulocyte-monocyte progenitors (GMPs), and megakaryocyte-erythrocyte progenitors (MEPs) were normal (Figure 1, D and E). Together, our data indicate that in the mouse fetal liver, Taspase1 may be required for the development and maintenance of the HSC compartment.

Accordingly, we investigated how Taspase1 loss leads to impaired fetal liver HSC renewal. Loss of Taspase1 activity could lead to increased cell death and/or aberrant cell cycle control. Cell death analysis of LSK cells and HSCs showed no increase of apoptosis in Tasp1−/− fetal livers (Figure 2A). On the other hand, cell cycle analysis on MPPs and HSCs yielded a marked difference between WT and Tasp1−/− cells. Most notably staining of DNA and RNA with 7-AAD and Pyronin Y, respectively, showed that more Tasp1−/− fetal liver HSCs exited stem cell quiescence, with decreased number of cells in G0 phase, whereas the majority of WT HSCs were in G0 phase with fewer cells at G1 and S/G2/M (Figure 2, B and C). In contrast, Tasp1−/− fetal liver MPPs displayed similar cell cycle profile as WT MPPs. Strikingly, the cell cycle profile observed of Tasp1−/− fetal liver HSCs is in resemblance to that of MPPs (Figure 2B).

To elucidate by which substrate(s) cleavage Taspase1 regulates fetal liver hematopoiesis, we first focused on the MLL family proteins MLL1 (KMT2A) and MLL2 (KMT2B). MLL1 is the best characterized Taspase1 substrate, controls Hox and Cyclin genes expression, and plays a critical role in both fetal and adult hematopoiesis (26-28). Homozygous knockin of non-cleavable (nc) Mll1 mutant alleles (Mll1nc/nc) at the endogenous Mll1 locus did not reduce the overall fetal liver cellularity (Figure 3A), but incurred a minor decrease in frequency and number of HSCs (Figure 3B). Since published data suggest a partial redundancy between Mll1 and Mll2 (11), we analyzed E14.5 fetal
livers of $Mll1^{nc/nc};2^{nc/nc}$ embryos bearing homozygous knockin of non-cleavable $Mll1$ and non-cleavable $Mll2$ mutant alleles at their native genomic loci, and did not detect reduced fetal liver cellularity or body weight (Figure 3C). Similarly, the reduced abundance or absolute number of HSCs in $Mll1^{nc/nc};2^{nc/nc}$ fetal livers was not statistically significant (Figure 3D).

TFIIA family proteins TFIIAα-β and ALF are the only bona fide mammalian Taspase1 substrates known thus far besides MLL1 and MLL2. As expression of ALF is restricted to the mammalian testis (29), we focused on the non-cleavage of TFIIAα-β. We have created a $Gtf2a1^{nc/nc}$ mouse model in which the endogenous D/G cleavage residues of TFIIAα-β are replaced with non-cleavable A/A, and reported that $Gtf2a1^{nc/nc}$ males are infertile (22). Remarkably, $Gtf2a1^{nc/nc}$ embryos were smaller than their $WT$ littermates, and their livers were disproportionately smaller (Figure 4, A and B). Furthermore, the frequencies of HSC in $Gtf2a1^{nc/nc}$ fetal livers were also reduced (Figure 4D, left). In fact, the absolute HSC number of $Gtf2a1^{nc/nc}$ fetal livers was at ~30% of their wild-type littermates (Figure 4E). Similar to $Tasp1^{-/-}$ embryos, there were no significant changes at the frequency of myeloid progenitors, CMPs, GMPs, or MEPs in $Gtf2a1^{nc/nc}$ fetal livers (Figure 4, C and D). Cell cycle analysis of $Gtf2a1^{nc/nc}$ fetal liver stem and progenitor cells demonstrated decreased number of $Gtf2a1^{nc/nc}$ HSCs in G0 phase, which is reminiscent to that of $WT$ and $Gtf2a1^{nc/nc}$ MPPs (Figure 5, A and B). The decrease in G0 phase and the coinciding increases in non-G0 phases of the $Gtf2a1^{nc/nc}$ fetal liver HSCs indicated a stem cell quiescence defect similar to that observed in $Tasp1^{-/-}$fetal liver HSCs (Figure 2, B and C). These striking similarities between $Gtf2a1^{nc/nc}$ and $Tasp1^{-/-}$ fetal livers highlight the importance of the Taspase1-TFIIA axis in the maintenance of fetal liver HSCs.
To further investigate this novel regulation, we employed stem cell transplant assays to evaluate the capacity of Tasp1\(^{-/-}\) and Gtf2a1\(^{nc/nc}\) fetal liver HSCs in long-term hematopoietic reconstitution. Competitive repopulation assays were performed by transplanting 150 CD45.2\(^+\) HSCs from WT, Tasp1\(^{-/-}\) or Gtf2a1\(^{nc/nc}\) E14.5 fetal livers along with CD45.1\(^+\) competitor cells into lethally irradiated CD45.1\(^+\) mice (Figure 6A). Twelve weeks after transplantation, peripheral blood was analyzed for the contribution of donor derived HSCs to mature blood lineages, including B220\(^+\) for B-cells, CD3\(^+\) for T-cells, and Gr-1\(^+\)Mac-1\(^+\) for myeloid cells (Figure 6, B and C). More than 5% of CD45.2\(^+\) donor-derived cells in all three lineages were detected in 13 of 19 mice transplanted with WT HSCs, whereas multi-lineage reconstitution was detected in neither of the 9 mice transplanted with Tasp1\(^{-/-}\) HSCs nor of the 8 transplanted with Gtf2a1\(^{nc/nc}\) HSCs (Table 1). Thus, the Taspase1-TFIIA axis appears to be required for long-term reconstitution of hematopoietic cells from fetal livers, suggesting its role in long term HSC self-renewal.

The phenotypic similarities between Gtf2a1\(^{nc/nc}\) and Tasp1\(^{-/-}\) in fetal liver HSCs indicate that TFIIA is the principal Taspase1 substrate conferring Taspase1-orchestrated fetal liver hematopoiesis. As non-cleaved TFIIA\(\alpha-\beta^{nc}\) is more stable than cleaved TFIIA (5, 22) and no apparent abnormalities were detected in Gtf2a1\(^{nc/nc}\) fetal livers (data not shown), TFIIA\(\alpha-\beta^{nc}\) is unlikely to function as a dominant negative mutant in fetal liver hematopoiesis. Instead, data favors cleaved TFIIA positively regulating fetal liver hematopoiesis.

*Hox* genes of the vertebrates and *homeotic* genes of the invertebrates play critical roles in implementing body plan and their deregulation results in the loss of segmental identities, i.e. homeotic transformation. As our previous studies demonstrated that Tasp1\(^{-/-}\)
mice exhibit homeotic transformation, we examined the axial skeleton of $Gtf2a1^{nc/nc}$ newborn pups (n=17) and detected overt homeotic transformations including abnormal anterior arch of atlas (a.a.a.) (82%), split of C2 (cervical vertebra) (18%), fusion of C3 to C5 (29%), posterior transformation of C7 (65%), anterior transformation of T8 (thoracic) (12%), incomplete ossification of sternabra 4 (65%), incomplete segmentation of sternabrae 3 and 4 (24%), and posterior transformation of L6 (lumbar) (53%) (Figure 7 and Table 2). Unexpectedly, homeotic defects of $Gtf2a1^{nc/nc}$ newborns were more profound than those of $Mil1^{nc/nc}, 2^{nc/nc}$ and highly reminiscent to those of $Tasp1^{-/-}$ newborns (Figure 7 and Table 2). Homeotic transformation of the axial skeletons is the defining feature of Hox gene deregulation (30, 31), and these extensive homeotic defects in $Gtf2a1^{nc/nc}$ mice indicate a novel regulation of Hox genes by TFIIA, a basal transcription factor, through a site-specific proteolytic process.
Discussion

Unlike most reversible post-translational protein modifications, such as phosphorylation, acetylation, and methylation, proteolysis through either degradation or site-specific cleavage renders permanent structural changes, and thereby potentially results in long-lasting functional consequences. In metazoans, site-specific proteolysis regulates critical aspects of biology, such as the activation of blood coagulation factors for hemostasis, the activation of caspases for cell death execution, the cleavage of Notch intracellular domain for cell fate determination, the release of SREBP for cholesterol homeostasis (32), the maturation of HCF and MLL1 for cell cycle progression (11, 12), and the assembly of mature TFIIAα/β/γ for male germ cell maturation (5, 22). Indeed, the identification and functional characterization of proteases and their cognate substrates have been instrumental in unraveling the underlying mechanisms concerning diverse biological processes.

Taspase1 is a highly conserved protease that orchestrates a plethora of genetic programs through cleaving nuclear transcription regulators, MLL1, MLL2, TFIIA, ALF, and dHCF (6, 33). Given that TFIIA is a basal transcription factor and Hox genes are highly specialized transcription factors, the connections between Taspase1-mediated proteolytic processing of TFIIA and transcriptional regulation of Hox genes were completely unexpected, adding an additional layer of complexity to the ever-discovering intricate control of Hox gene expression through upstream transcription factors, epigenetic regulators, and long intergenic noncoding (linc) RNA (34, 35) for constructing segmental body plan and specifying cell lineages including stem cells.

The paradigm of Taspase1 function provides a new opportunity to investigate how
proteases interconnect diverse genetic programs via site-specific proteolysis. In the absence of Taspase1-mediated cleavage, MLL1, MLL2, and TFIIAα–β retain partial activity and animals bearing non-cleaved MLL1, MLL2, and/or TFIIAα–β manifest hypomorphism in several biological settings (11, 22, 36, 37). Consequently, the alleles encoding non-cleavable MLL1, MLL2, and TFIIAα–β differ from null alleles and thus provide invaluable insight in cases where genetic ablation results in early embryonic lethality. Likewise, Taspase1 deficiency appears to offer a unique opportunity to uncover signaling pathways controlled by developmentally essential genes. Taspase1 regulates the cell cycle, axial skeletal formation, fetal hematopoietic stem cell homeostasis, male germ cell development (11, 22, 36, 37), and craniofacial development (23). As TFIIA complexes with not only TBP but also TRF2 (a male germ cell enriched TBP variant) and TRF3 (a vertebrate-specific TBP variant), the Taspase1-TFIIA axis is positioned to orchestrate the assembly of diverse tissue-specific or context-dependent transcription machinery that are crucial for organismal development.
Methods

Mice.

*Tasp1*<sup>−/−</sup>, *Mll1<sup>nc/nc</sup>, *Mll2<sup>nc/nc</sup>, and *Gtf2a1<sup>nc/nc</sup> mice were previously described (11, 22). *Tasp1*<sup>+/−</sup> and *Gtf2a1<sup>nc/+</sup> mice were backcrossed to the wild-type C57Bl6 strain for six generations. Further backcross of these mice with C57Bl6 strain reduces male fertility (Oyama et al. unpublished data). *Mll1<sup>nc/nc</sup> and *Mll2<sup>nc/nc</sup> mice were similarly backcrossed to the wild-type C57Bl6 strain for ten generations.

Flow cytometric analyses of hematopoietic cells.

To obtain single cell suspensions of hematopoietic cells, fetal livers were removed from E14.5 mouse embryos and homogenized by three passages through a 21 gauge needle. Freed cells were filtered through a nylon mesh with pore size of 40 µm. Collected cells were treated with Red Blood Cell Lysing Buffer (Sigma), then washed and filtered through nylon mesh. Automated blood cell counting was performed using a Hemavet Blood Analyzer (Drew Scientific). For mature blood cell analyses, one million cells were stained using the following antibodies: B220 APC (RA3-6B2), Gr-1 PE (RB6-8C5), and TER-119 Pacific Blue (TER-119). For analyses of stem and progenitor cells, one million cells were stained using a combination of following antibodies: Lin (Lineage antibody cocktail; CD3 (17A2), CD4 (GK1.5), CD8α (53–6.7), B220 (RA3-6B2), TER-119 (TER-119) and Gr-1 (RB6-8C5)) Pacific Blue, Sca-1 PE (D7), c-Kit APC/Alexa750 (2B8), CD34 FITC (RAM34), CD16/32 Alexa647 (93), and CD150 PE/Cy7 (TC15-12F12.2). To identify and exclude dead cell, 7-AAD was added in the final suspension. Cells positive for Sca-1, CD34 or CD150 were determined by fluorescence-minus-one controls. Antibodies were supplied by eBioscience, PharMingen or BioLegend. Cells were analyzed with an
LSRFortessa flow cytometer (BD Biosciences). Dot plots and histograms were made with FlowJo software. Graphs and statistical analyses were prepared with GraphPad Prism 6.

**Sorting of hematopoietic stem and progenitor cells.**

Single cell suspensions were prepared from fetal livers of E14.5 embryos or adult bone marrow, and stained as described above. Fetal liver cells from multiple littermate embryos of the same genotype were pooled to ensure sufficient number of cells for staining. Stained cells were subsequently double-sorted using a FACSria II (BD Biosciences) to a final purity of more than 95%.

**Competitive reconstitution assay.**

C57Bl6-CD45.1 mice were used as recipients and competitors. The recipient mice were irradiated by 10 Gy divided into 2 fractions on the day -1. Competitor cells were prepared from E14.5 fetal liver of the C57Bl6-CD45.1 mice and frozen in advance. On the day 0, LSK CD150⁺ HSCs were sorted from E14.5 fetal liver of donor embryos that express CD45.2. Each recipient mouse was transplanted with 150 donor HSCs and 3 x 10⁵ competitor fetal liver cells by tail vein injection. After 12 weeks, peripheral blood and bone marrow cells of recipients were analyzed for the presence of CD45.1 and CD45.2-positive cells by a LSRFortessa. Peripheral blood mononucleic cells were stained with the following antibodies for analyzing myeloid lineage: CD45.1 PE (A20), CD45.2 FITC (104), Mac-1 Ax647 (M1/70), and Gr-1 Pacific Blue (RB6-8C5). The following antibodies were used for lymphoid lineage: CD45.1 PE (A20), CD45.2 FITC (104), B220 APC (RA3-6B2), and CD3 Pacific Blue (17A2).

**Cell cycle analysis.**

Suspensions of sorted hematopoietic stem and progenitor cells were fixed with ethanol
overnight at 4°C, washed, and then resuspended in the Nucleic Acid Staining Solution (38). Cells were stained with 7-AAD at room temperature for 20 minutes, followed by Pyronin Y staining on ice for 15 minutes. Stained cells were analyzed by a LSRFortessa flow cytometer.

*Annexin V assay.*

One million fetal liver cells were stained with the following antibodies: Lin Pacific Blue, Sca-1 PE (D7), c-Kit APC/Ax750 (2B8), and CD150 PE/Cy7 (TC15-12F12.2). The cells were subsequently washed, stained with Annexin V FITC and Propidium Iodide, and then analyzed by flow cytometry.

*Skeletal studies.*

P1 newborns were sacrificed and stained using Alizarin red and Alcian blue for bone and cartilage, respectively, as described (39).

*Statistics.*

Statistical significance was evaluated using the Mann-Whitney U-test for continuous variables, unless otherwise specified. Chi-square testing was performed on cell cycle analyses, and p-values of independent experiments were combined by the Fisher’s method. p-values of less than 0.05 were considered significant.

*Study approval.*

All animal work was performed in accordance to a protocol approved by the Institutional Animal Care and Use Committee of Memorial Sloan-Kettering Cancer Center.
Author contributions

JJH designed the study. HN, ACS, and ST performed experiments, acquired and analyzed data. SAA, CYP, EHC, and JJH supervised experiments and analysis. HN and JJH wrote the manuscript.
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References


Figure 1. Taspase1 deficiency results in fetal liver hematopoietic stem cell defects.

(A) Body weight (left) and cell numbers of fetal livers (FL) normalized to body weight (right) of embryonic day E14.5 embryos of the indicated wild-type (WT) and Taspase1 knockout (Tasp1⁻⁻) embryos. Boxes contain the 25th to 75th percentiles of datasets with 50th percentile center lines, and whiskers mark the 5th and 95th percentiles. Outliers are shown by dots. (B) Photos of E14.5 FL of the indicated genotypes. White bar = 2 mm. (C)
Frequency of E14.5 FL cells committed to erythroid (TER-119⁺), myeloid (Gr-1⁺), and B-cell (B220⁺) lineages. (D) Progenitor and stem cell analyses of E14.5 FL cells by flow cytometry. Lin⁻Sca-1⁻c-Kit⁺ cells are defined as LSK, and Lin⁻Sca-1⁻c-Kit⁺ cells as myeloid progenitors (left panels). Myeloid progenitors are subdivided into CMP, GMP and MEP by CD34 and CD16/32 (center panels). LSK CD150⁺ cells are defined as HSCs (right panels). (E) Quantification of stem and progenitor cells of 15 WT and 12 Tasp1⁻/⁻ FLs. (F) HSCs per E14.5 fetal liver of the indicated genotypes. Number is calculated by multiplying FL cellularity and HSC frequency. All data are presented as mean ± standard error of the mean (SEM). * p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001 by Mann-Whitney U-test. HSC, hematopoietic stem cell; CMP, common myeloid progenitor; GMP, granulocyte-monocyte progenitor; and MEP, megakaryocyte-erythrocyte progenitor.
Figure 2. Tasp1^{-/-} fetal liver hematopoietic stem cells (HSC) exhibit an aberrant cell cycle profile reminiscent to multipotent progenitor cells (MPP).

(A) Cell death analysis quantified by Annexin V staining. The frequency of Annexin V^+ cells in FL HSC and LSK cells of the indicated genotypes were displayed (WT, n = 3; Tasp1^{-/-}, n = 5) Data are presented as mean ±SEM. (B, C) Cell cycle analysis of FL HSCs and MPPs. Cells were stained with 7-AAD and Pyronin Y to assess DNA and RNA contents, respectively. (B) The quadrant gates of the representative plots based on DNA and RNA contents are outlined as G0, G1 and S/G2/M phases. (C) Bar charts denote the frequency of HSCs and MPPs in G0 and non-G0 (G1 and S/G2/M) phases. Data presented are mean ± SEM (n = 4 for all samples). p-values of 4 independent experiments by Chi-square testing were combined by the Fisher’s method and *** denotes p-value < 0.001.
Figure 3. HSC quantification of \textit{Mll1}^{nc/nc}, and \textit{Mll1}^{nc/nc, 2nc/nc} fetal livers.

(A, B) Embryos obtained by intercrossing \textit{Mll1}^{nc/+} mice were analyzed. (A) Bar graphs present body weight (left) and normalized E14.5 FL cellularity (right). (B) Frequency for HSC (left) and number of HSCs per FL (right) are shown (\textit{WT}, n = 8; \textit{Mll1}^{nc/nc}, n = 8).

(C, D) Embryos obtained by intercrossing \textit{Mll1}^{nc/nc, 2nc/+} mice were analyzed. (C) Body weight (left) and normalized E14.5 FL cellularity (right) are shown. (D) Frequency for HSC (left), and number of HSCs per FL (right) are shown (\textit{Mll1}^{nc/nc, 2+/-}, n = 6; \textit{Mll1}^{nc/nc, 2nc/nc}, n = 10). All data are shown as mean ± SEM. p-values by Mann-Whitney U-test are displayed on each chart.
Figure 4. *Gtf2a1*nc/nc HSCs exhibit overt fetal liver hematopoiesis defects.

(A) Body weight and normalized FL cellularity of 19 WT and 27 *Gtf2a1*nc/nc E14.5 FLs. Boxes contain the 25th to 75th percentiles of datasets with 50th percentile center lines, and whiskers mark the 5th and 95th percentiles. Outliers are shown by dots. *** denotes p-value < 0.001 by Mann-Whitney U-test. (B) Photos of E14.5 FLs of the indicated WT and *Gtf2a1*nc/nc embryos. White bar = 2 mm. (C, D) Quantification of hematopoietic stem and progenitor cells of E14.5 FLs by flow cytometry. (C) Representative dot plots. (D) Bar charts summarize the frequency of stem and progenitor cells in FLs. (E) Number of HSCs per FL. All data shown as mean ± SEM. ** denotes p-value < 0.01 and *** denotes p-value < 0.001 by Mann-Whitney U-test (WT, n = 6; *Gtf2a1*nc/nc, n = 8).
**Figure 5. Aberrant cell cycle profile of** $Gtf2a1^{nc/nc}$ **HSCs.**

Cell cycle analysis of $Gtf2a1^{nc/nc}$ E14.5 FL HSCs and MPPs. (A) The quadrant gates defining G0, G1 and S/G2/M phases. (B) Bar charts denote the frequency of HSCs and MPPs in G0 and non-G0 (G1 and S/G2/M) phases. Data presented are mean ± SEM ($n = 4$ for all samples). p-values of 4 independent experiments by Chi-square testing were combined by the Fisher’s method and ** denotes p-value < 0.01.
Figure 6. *Tasp1*<sup>−/−</sup> and *Gtf2a1*<sup>nc/nc</sup> fetal liver HSCs fail to reconstitute lethally irradiated recipient mice.

(A) Outline of the experimental design of long-term competitive repopulation assays. (B) Scatter plots showing the frequency of donor-derived B-cell (B220<sup>+</sup>), T-cell (CD3<sup>+</sup>) and myeloid (Gr-1<sup>+</sup>Mac-1<sup>+</sup>) lineages reconstituted at 12 weeks after transplant. Bars denote the average and SEM. *WT*, n = 19; *Tasp1*<sup>−/−</sup>, n = 9; *Gtf2a1*<sup>nc/nc</sup>, n = 8. (C) Representative plots show reconstitution by donor HSCs of the indicated genotypes in B-cell, T-cell and myeloid lineages, assessed 12 weeks after transplant. In each lineage, CD45.2<sup>+</sup> and CD45.1<sup>+</sup> cells were derived from donor (*WT, Tasp1*<sup>−/−</sup> or *Gtf2a1*<sup>nc/nc</sup>) and competitor cells, respectively. See also Table 1.
Figure 7. Homeotic transformation of axial skeletons in *Tasp1*<sup>−/−</sup>, *Gtf2a1*<sup>nc/nc</sup> and *Mll1*<sup>nc/nc;2nc/nc</sup> newborns.

Lateral views of cervical (C1-C7) and upper thoracic (T1) regions demonstrate deformed anterior arch of atlas (C1) and fusion of C3-C4 (star) in *Tasp1*<sup>−/−</sup> and *Gtf2a1*<sup>nc/nc</sup> skeleton, and posterior transformation of C7 with an additional rib (arrowhead) in *Tasp1*<sup>−/−</sup>, *Gtf2a1*<sup>nc/nc</sup> and *Mll1*<sup>nc/nc;2nc/nc</sup> skeleton (top). Anterior views of the chest indicate incomplete ossification of sternebrae 4 (arrowhead) in *Tasp1*<sup>−/−</sup> and *Gtf2a1*<sup>nc/nc</sup> newborns, and incomplete segmentation of sternebrae 3-4 (star) in *Mll1*<sup>nc/nc;2nc/nc</sup> newborns (middle). Anterior views of lower thoracic and lumbar vertebrae (L1-L6) demonstrate anterior transformation of L1 with an additional rib (arrowhead) in *Tasp1*<sup>−/−</sup> newborn, and posterior transformation of L6 in *Gtf2a1*<sup>nc/nc</sup> newborn (bottom). See also Table 2.
Table 1. Multi-lineage reconstitution from WT, Tasp1⁻/⁻ or Gtf2a1<sup>inc/nc</sup> donor HSCs.

<table>
<thead>
<tr>
<th>Donor HSC Genotype</th>
<th>Multi-lineage Reconstitution</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>WT</em></td>
<td>13/19</td>
</tr>
<tr>
<td>Tasp1⁻/⁻</td>
<td>0/9 ***</td>
</tr>
<tr>
<td>Gtf2a1&lt;sup&gt;inc/nc&lt;/sup&gt;</td>
<td>0/8 **</td>
</tr>
</tbody>
</table>

** denotes p-value < 0.01 and *** denotes p-value < 0.001 by the Fisher’s exact test (vs WT).
Table 2. Skeletal defects observed in *Tasp1*<sup>−/−</sup>, *Gtf2a1<sup>nc/nc</sup>* and *MLL1<sup>nc/nc;2nc/nc</sup>* newborns.

<table>
<thead>
<tr>
<th>Defect</th>
<th>WT (n=14)</th>
<th><em>Tasp1</em>&lt;sup&gt;−/−&lt;/sup&gt; (n=17)</th>
<th>Gtf2a1&lt;sup&gt;nc/nc&lt;/sup&gt; (n=17)</th>
<th>MLL1&lt;sup&gt;nc/nc;2nc/nc&lt;/sup&gt; (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a.a.a., tilted, broadened, and deformed</td>
<td>14% (2)</td>
<td>47% (8)</td>
<td>82% (14)</td>
<td>20% (2)</td>
</tr>
<tr>
<td>C2, split neural arch</td>
<td>0% (0)</td>
<td>12% (2)</td>
<td>18% (3)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>C3-5, fusion</td>
<td>0% (0)</td>
<td>76% (13)</td>
<td>29% (5)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>C7, posterior transformation</td>
<td>0% (0)</td>
<td>94% (16)</td>
<td>65% (11)</td>
<td>50% (5)</td>
</tr>
<tr>
<td>T8, anterior transformation</td>
<td>0% (0)</td>
<td>59% (10)</td>
<td>12% (2)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>Sternebrae 4, incomplete ossification</td>
<td>0% (0)</td>
<td>41% (7)</td>
<td>65% (11)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>Sternebrae 3 and 4, incomplete segmentation</td>
<td>0% (0)</td>
<td>29% (5)</td>
<td>24% (4)</td>
<td>50% (5)</td>
</tr>
<tr>
<td>L1, anterior transformation</td>
<td>0% (0)</td>
<td>82% (14)</td>
<td>0% (0)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>L6, posterior transformation</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>53% (9)</td>
<td>0% (0)</td>
</tr>
</tbody>
</table>