Author contribution

SK, ST and YF designed the research study; SK, MT and AI performed experiments; MM collected plasma samples; SK analysed data; MT supported construction of assay procedure; SK and YF wrote the manuscript. All authors reviewed the manuscript.

Conflicts of interest

The authors have some conflicts of interest relevant to this manuscript submitted to *British Journal of Haematology*.

Employment: Seiji Kato and Mutsumi Tanaka are employees of Alfresa Pharma Corporation. Tomohiro Samori is an advisor of Japan Clinical Laboratories, Inc.

Patent: Alfresa Pharma corporation holds a patent (WO2006085441) for the ADAMTS13 activity assay. Seiji Kato, Masanori Matsumoto and Yoshihiro Fujimura are the inventors of the patent.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Effect of interfering substances, anti-ADAMTS13 monoclonal antibody and EDTA on the ADAMTS13 act-GPI.

Table S1. Plasma ADAMTS13 levels activity determined by three methods in four groups.

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Functional analysis of truncated forms of ETV6

The ETV6 (TEL) transcription factor has been shown to play a wide role in haematopoiesis, influencing the development of multiple lineages, while chromosomal translocations involving fusions of the *ETV6* gene occur frequently in haematological malignancies (Rasighaemi *et al*, 2014). Recurrent mutations of *ETV6* have been reported in cases of acute myeloid leukaemia (AML) (Barjesteh Van Waalwijk Van Doorn-Khosravani *et al*, 2005; Silva *et al*, 2008), childhood B cell acute lymphoblastic leukaemia (B-ALL) (Zhang *et al*, 2011) and early immature adult T-cell ALL (T-ALL) (Van Vlierberghe *et al*, 2011), along with alternative splicing of *ETV6* in myelodysplastic syndrome (MDS) (Sasaki *et al*, 2004). These

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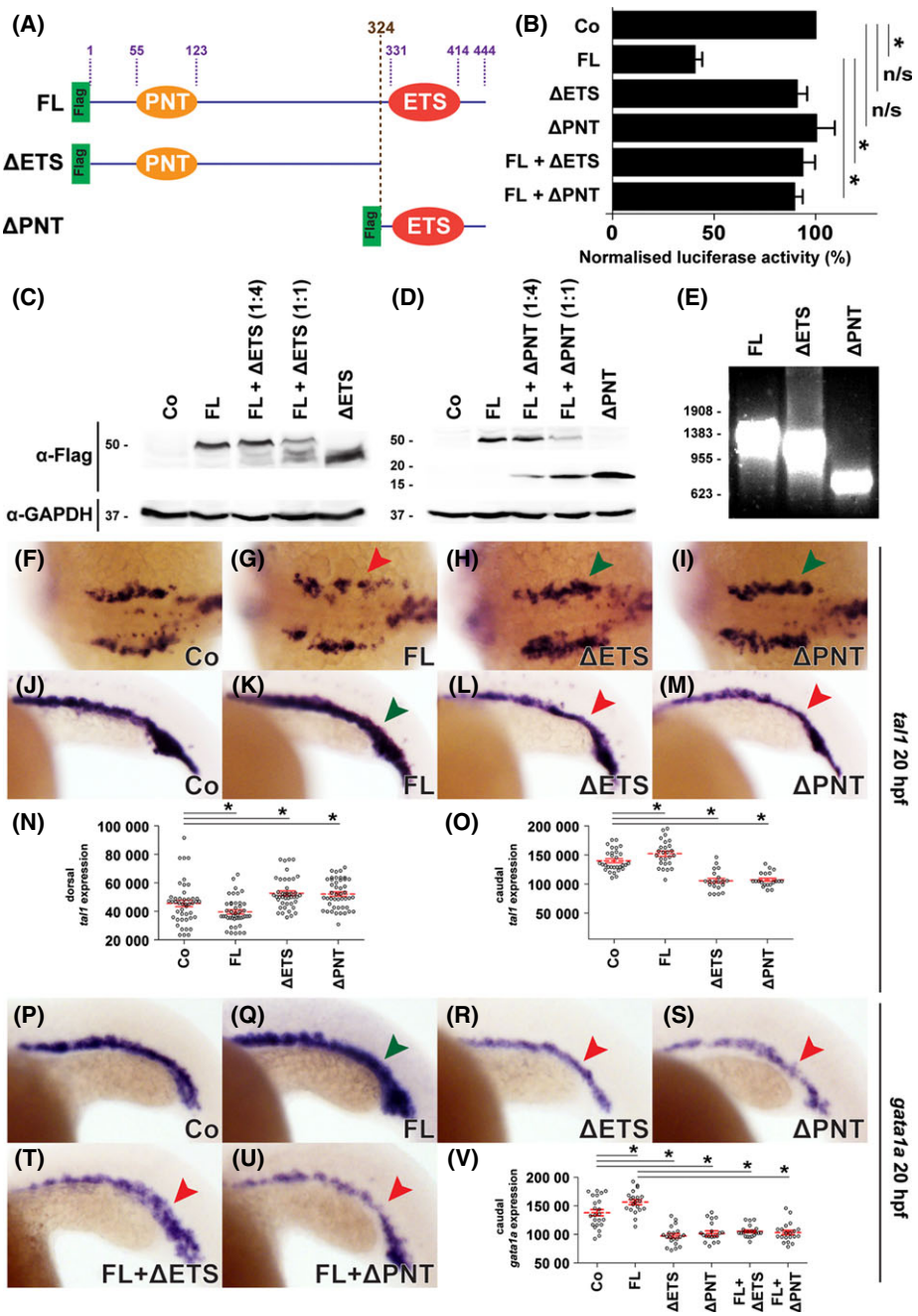

Fig 1. Truncated forms of zebrafish Etv6 disrupt primitive HSC and erythroid compartments. (A) Zebrafish Etv6 truncations. Schematic representation of constructs expressing Flag-tagged full-length Etv6 (FL) and truncated Etv6 forms lacking either the ETS (ΔETS) or PNT (ΔPNT) domain. Segments corresponding to Flag (green), PNT (orange) and ETS (red) domains and relevant amino acids numbers are indicated. (B) Transcriptional properties of truncated Etv6 forms. HEK293T cells were transfected using Fugene 6 with vectors expressing Flag-tagged FL Etv6 and the ΔETS and ΔPNT truncations alone or in combination, along with Mmp3-luciferase and CMV-Renilla. Luciferase activity was determined using a Dual Luciferase kit after 2 d, with Firefly luciferase activity normalized against Renilla luciferase and expressed relative to mock transfected control (Co) cells, presented as mean ± SD from triplicate experiments (*$P < 0{\cdot}05$; n/s, not significant). (C–D) Expression of Etv6 forms. HEK293T cells transfected with vectors expressing Flag-tagged Etv6-FL and either Etv6-ΔETS (C) or Etv6-ΔPNT (D) alone or in combination at either 1:1 or 1:4 ratio. Total cell lysates from these and mock transfected control (Co) cells were subjected to Western blot analysis with α-Flag and control α-GAPDH antibodies. (E) *In vitro* transcribed mRNA encoding the different zebrafish Etv6 forms used for injection (FL, ΔETS, ΔPNT). (F–V) Whole-mount *in situ* hybridization (WISH) analysis. Control embryos (Co) and embryos injected with mRNA encoding different forms of Etv6 (FL, ΔETS, ΔPNT) were subjected to WISH to analyse the expression *tal1* in its dorsal (F–I) and caudal (J–M) domains and *gata1a* (P–S), with quantitation of the relative area of expression dorsal *tal1* (N), caudal *tal1* (O) and *gata1a* (V) at 20 hpf, presented as mean ± SE of the mean (*$P < 0{\cdot}05$). Arrowheads indicate areas of increased (green) and decreased (red) expression relative to controls. [Correction added on 12 October 2015, after first online publication: panel (R) has been corrected].

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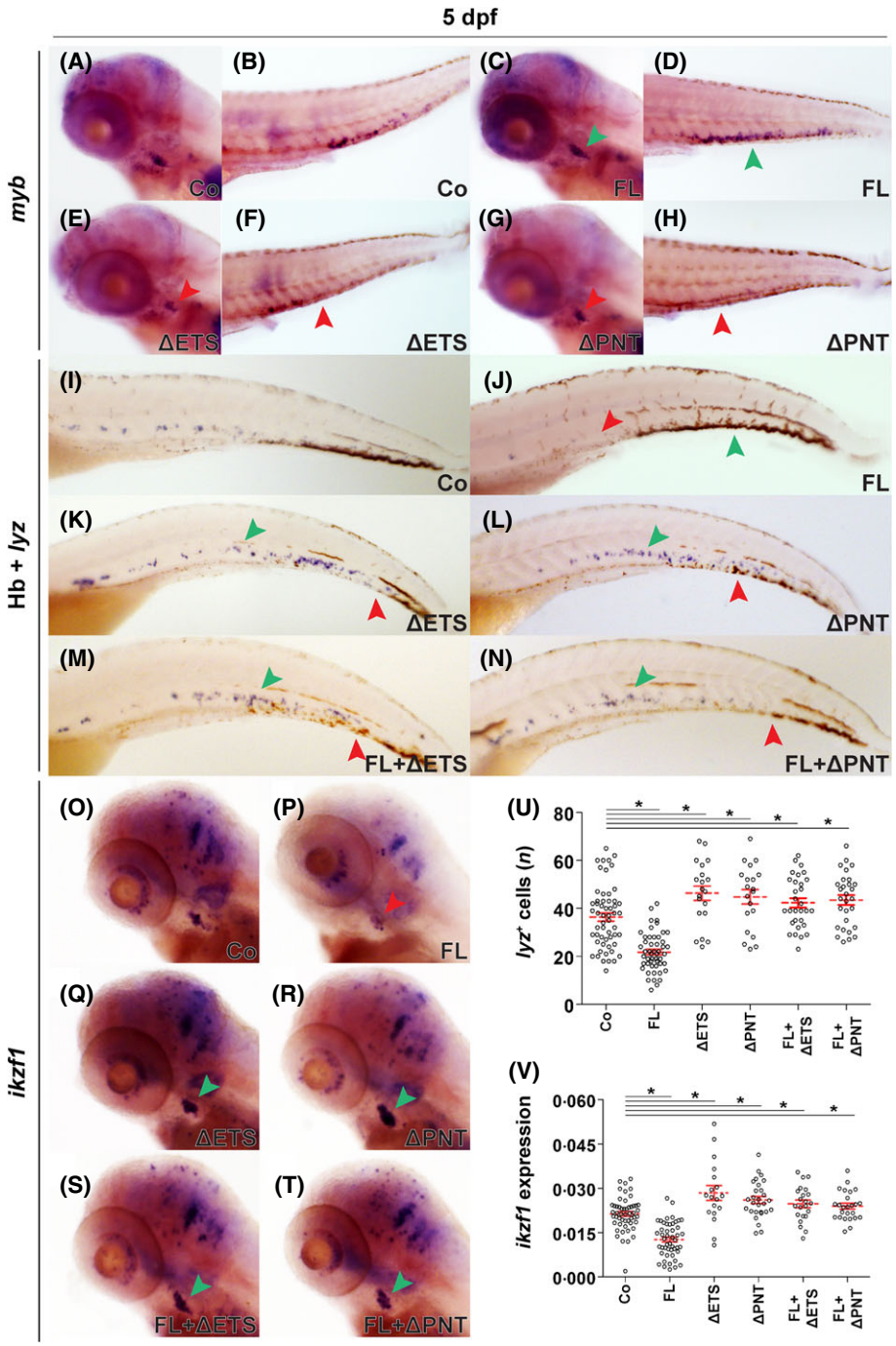

Fig 2. Truncated forms of zebrafish Etv6 disrupt definitive haematopoiesis. (A–V) Haematopoietic analysis. Control embryos (Co) and embryos injected with mRNA encoding different forms of Etv6 (FL, ΔETS, ΔPNT) were analysed for *myb* expression (A–H), haemoglobin (Hb) staining and *lyz* expression (I–N), and *ikzf1* expression (O–T) at 5 dpf. The lyz^+ cells were enumerated and are represented as mean ± SEM (U) with the area of *ikzf1* staining quantified relative to eye size, and expressed as mean ± SEM (V) (*$P < 0{\cdot}05$).

lead to expression of alternate forms of ETV6 lacking either the N-terminal PNT domain, involved in protein–protein interactions, or the C-terminal ETS DNA-binding domain, unable to repress transcription but capable of ablating the repressional activity of full-length ETV6 *in vitro* (Barjesteh Van Waalwijk Van Doorn-Khosravani *et al*, 2005; Van Vlierberghe *et al*, 2011). However, the *in vivo* role of these ETV6 truncations has not been characterized. The zebrafish represents an established model for the study of haematopoiesis and its perturbation, which has previously been used to investigate the role of ETV6 (Rasighaemi *et al*, 2015), and the consequences of enforced expression of *ETV6-JAK2* (Onnebo

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et al, 2012). This study has utilized this model to analyse the *in vivo* function of truncated ETV6 proteins.

Sequences encoding Flag-tagged versions of full-length zebrafish Etv6 (Etv6-FL) and truncated Etv6 forms corresponding to amino acids 1–324, thereby deleting the ETS domain (Etv6-ΔETS), and amino acids 325–444, thereby deleting the PNT domain (Etv6-ΔPNT) were generated (Fig 1A). Consistent with their mammalian counterparts, the truncated forms lacked the repressive properties of full-length Etv6, but interfered with the repression mediated by full-length Etv6 (Fig 1B), confirming that truncated versions of zebrafish Etv6 act in a similar dominant-negative manner *in vitro* to equivalent mammalian ETV6 proteins (Sasaki *et al*, 2004; Barjesteh Van Waalwijk Van Doorn-Khosravani *et al*, 2005). Some evidence of degradation of co-expressed full-length and truncated Etv6 protein was observed (Fig 1C–D), consistent with the absence of ETV6 protein observed in leukaemic blasts heterozygous for truncating ETV6 mutations (Patel *et al*, 2003; Sasaki *et al*, 2004; Barjesteh Van Waalwijk Van Doorn-Khosravani *et al*, 2005).

To investigate the function of truncated isoforms of ETV6 *in vivo*, zebrafish embryos were microinjected with *in vitro* transcribed mRNA encoding Etv6-FL, Etv6-ΔETS or Etv6-ΔPNT (Fig 1E), which was stable to at least 5 d post-fertilization (dpf) (data not shown). We have previously shown that ablation of zebrafish Etv6 affects embryonic haematopoiesis (Rasighaemi *et al*, 2015). Therefore, embryos injected with truncated Etv6 isoforms were examined for similar haematological perturbations using specific blood lineage markers to directly assess their *in vivo* effects.

During primitive haematopoiesis, embryos injected with Etv6-ΔETS and Etv6-ΔPNT showed increased expression of the early haematopoietic marker *tal1* in the rostral lateral plate mesoderm (LPM) compared to control embryos at 20 h post fertilization (hpf), while those injected with Etv6-FL showed decreased *tal1* expression (Fig 1F–I, N). In contrast, the caudal LPM *tal1*$^{+}$ population was significantly decreased in Etv6-ΔETS- and Etv6-ΔPNT-injected embryos but increased in those injected with Etv6-FL (Fig 1J–M, O). Expression of the early erythroid marker *gata1a* at the same time point was also decreased in Etv6-ΔETS- and Etv6-ΔPNT-injected embryos but increased in Etv6-FL embryos relative to controls (Fig 1P–S, V). Co-injection of Etv6-FL with either Etv6-ΔETS or Etv6-ΔPNT resulted in decreased *gata1a* expression compared to controls (Fig 1T–V).

Definitive haematopoiesis was analysed at 5 dpf. Embryos injected with either Etv6-ΔETS or Etv6-ΔPNT showed an overall decrease in the expression of the HSC marker *myb* and haemoglobin (Hb) staining, but an increased number of cells expressing the leucocyte marker *lyz*. In contrast, Etv6-FL embryos showed increased *myb* expression and Hb staining but reduced *lyz*$^{+}$ cells relative to controls (Fig 2A–L, U). Analysis of the lymphoid marker *ikzf1* at 5 dpf revealed increased expression in Etv6-ΔETS- and Etv6-ΔPNT-injected embryos but decreased expression in Etv6-FL-injected embryos compared to controls (Fig 2O–R, V). Co-injection of Etv6-FL with either Etv6-ΔETS or Etv6-ΔPNT resulted in increased *lyz* and *ikzf1* expression but decreased Hb staining compared to controls (Fig 2M–N, S–T, U–V).

The disruption of both primitive and definitive haematopoiesis observed following expression of both truncated ETV6 forms closely resembled that seen following Etv6 ablation (Rasighaemi *et al*, 2015). Furthermore, these effects were reversed in embryos injected with full-length Etv6, which suggests that levels of Etv6 represent a crucial determinant of haematopoietic cell development. Importantly, co-injection of Etv6-ΔPNT or Etv6-ΔETS not only ablated the effects of Etv6-FL expression, but elicited a similar phenotype to when they were injected alone, demonstrating they act in a dominant manner.

This is the first study showing a dominant-negative effect of truncated forms of ETV6 on normal ETV6 function *in vivo*. This suggests that in cases with truncating ETV6 mutations in AML (Barjesteh Van Waalwijk Van Doorn-Khosravani *et al*, 2005; Silva *et al*, 2008), B-ALL (Zhang *et al*, 2011) and T-ALL (Van Vlierberghe *et al*, 2011) or alternate splicing in MDS (Sasaki *et al*, 2004), it is likely that wild-type ETV6 function is low or indeed absent. Such a conclusion is consistent with expression data showing a similar enrichment in genes in immature adult T-ALL carrying truncating ETV6 mutations as in a T-ALL cell line in which ETV6 was knocked down, including up-regulation of *CD33*, *HOXA13*, *PTEN* and *PRDM16* (Van Vlierberghe *et al*, 2011). Collectively, this adds to our understanding of the contribution of ETV6 truncations to leukaemia aetiology at the molecular level.

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Authorship and disclosures

PR performed the bulk of the experimental work, analysed the data and assisted in manuscript preparation, while CL and SMNO provided additional input. ACW conceived the project and contributed to data analysis and preparation of the manuscript and takes overall responsibility for the manuscript. None of the authors have any competing interests to declare.

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Feasibility and efficacy of dose-dense and dose-intense ABVD for high-risk patients with advanced Hodgkin lymphoma

A recent report highlighted the promising results of a phase II trial of dose-dense (DD) and dose-intense (DI) ABVD (doxorubicin, bleomycin, vinblastine, dacarbazine; $ABVD_{DD\text{-}DI}$) in advanced classical Hodgkin lymphoma (HL) (Russo *et al*, 2014). In this study, a dose-powered ABVD variant was administered to 82 patients on a three-weekly basis without encumbering toxicities and with remarkable response and event-free survival rates. The authors concluded that an early treatment intensification, exploiting the 'first hit' principle of the BEACOPP (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, prednisone) escalated programmes (Diehl *et al*, 2003; Skoetz *et al*, 2013), could be safely and effectively accomplished within the ABVD platform (Corazzelli *et al*, 2012; Russo *et al*, 2014). In addition, they omitted consolidation radiotherapy (RT) in cases of complete response (CR) also in patients with mediastinal bulky disease (Corazzelli *et al*, 2012; Russo *et al*, 2014).

Based on the promising early results of such intensified ABVD version (Russo *et al*, 2009), 16 consecutive patients with advanced HL were treated at our Institution with the $ABVD_{DD\text{-}DI}$ programme between June 2011 and November 2013. According to the published $ABVD_{DD\text{-}DI}$ schedule, cycles were administered every 21 d and supported with granulocyte-colony stimulating factor; the drugs were given on days 1 and 11 at the same doses of standard ABVD except for doxorubicin, which was escalated to 35 mg/m^2 in the first four of six courses (Russo *et al*, 2009, 2014).

Patient characteristics are detailed in Table I. The median age was 39 years (range, 23–67 years) and unfavourable risk factors, such as erythrocyte sedimentation rate >50 mm/h, ≥3 nodal areas and International Prognostic Score ≥3 were present in 69%, 75% and 50% of patients, respectively. Adherence to treatment was high: 100% of patients completed the planned six courses of $ABVD_{DD\text{-}DI}$ without any treatment delay or dose reduction (Table II). World Health Organization Grade 4 neutropenia and anaemia were both observed in 13% of patients. Other relevant toxicities were Grade 2 mucocutaneous changes (19%), consisting primarily of skin hyperpigmentation and

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