Expression of *rag1* at 82 hpf in the thymus of *man* embryos was completely absent (Figure 16A-D), although normal expression of *rag1* was observed in the olfactory epithelium at this time-point (Figure 16D), showing that despite the significant brain and facial abnormalities seen in *man* by 82 hpf, *rag1* could still be expressed normally outside the haematopoietic system. Expression of *foxn1*, a marker of thymic epithelial cells (Schorpp et al. 2002) was markedly reduced or absent in *man* embryos (Figure 16F). This may indicate a role for the *man* gene in both T cell specification and thymus formation, alternatively the effects seen may be secondary to the structural defects seen in this region of *man* embryos by this timepoint. B lymphocytes were not able to be analysed as the *man* mutation is embryonic lethal before the onset of *ighm* expression. Expression of both thrombocyte markers *mpll* and *itga2b* was completely absent from the PBI of 82 hpf *man* embryos (Figure 16G-J).

### 3.2.2.6 Effect on erythroid development

The effect of *man* on both primitive and definitive erythropoiesis was examined. Normal expression of the erythroid-specific transcription factor *gata1* at the 5-somite stage indicated primitive erythropoiesis could commence. In addition, *gata1* was normally expressed in the ICM at 20 somites (19 hpf) (Figure 14A). Expression of the embryonic β-globin *hbbe3* was normal at 28 hpf and 48 hpf, indicating mature erythrocytes could be produced and enter circulation (Figure 14B-D). Erythrocytes in the DVA were morphologically normal (Figure 14E,F). Finally *man* erythrocytes in the circulation were morphologically normal (Figure 14G,H). Together these data indicate the *man* mutation does not affect erythropoiesis in the first 48 hpf. At 82 hpf in WT embryos globin expression (*hbae3*) decreases, with expression only seen in circulating erythrocytes in the heart (Brownlie et al. 2003). In *man* embryos, strong expression was seen in erythrocytes in circulation over the yolk and in the DVA/PBI regions (Figure 14I,J). Although this demonstrated that there was no loss of erythrocytes at 82 hpf in *man*, the distribution of erythrocytes was abnormal. Normally, by 3.5 dpf circulation occurs only through the heart itself and not over the surface of the
yolk (Isogai et al. 2001). The distribution of globin expression seen in man at 82 hpf resembled that of WT embryos at 48 hpf. The persistent circulation of erythrocytes over the yolk and its detection in trunk erythrocytes may either reflect a delay in late erythrocyte maturation, or be a consequence of alterations in vascular development in man embryos.

3.2.3 Overview of other tissues effected by the man mutation

3.2.3.1 The man mutation causes neuronal cell death via apoptosis

By 48 hpf, man embryos had brain and eye abnormalities with reduction in eye size, opacification of the forebrain and increased brain ventricle size (hydrocephalus). man embryos at 48 hpf had striking abnormalities in the nervous system throughout the brain, eye and spinal cord (Figure 17A-F). Neuronal cells within the brain and eye were undergoing apoptosis with cellular shrinkage, nuclear condensation and membrane abnormalities (Cohen et al. 1992) (Figure 17D). Consistent with this, the vital dye acridine orange, which marks apoptotic but not necrotic cells (Furutani-Seiki et al. 1996), demonstrated an increase in apoptotic cells at 48 hpf in man, particularly in the midbrain and forebrain but also throughout the rest of the brain and eye (Figure 17G, H).

The eye of man embryos showed complete loss of normal structure. The lens was smaller with disruption of the surrounding epithelial layer. The retinal ganglion cell layer, inner plexiform layer, amacrine cell layer, bipolar cell layer, outer plexiform layer, photoreceptor layer and pigmented epithelial layers of the retina could not be distinguished (Figure 17E, F). The marked enlargement of the brain ventricles compared to WT embryos was confirmed by rhodamine-dextran ventriculography (Figure 18).

3.2.3.2 Other affected tissues in man

By 100 hpf, man embryos exhibited slowing of the heart rate, with sluggish circulation of erythrocytes and marked oedema overlying the heart. The defect underlying the circulation was not due to an abnormality in the vasculature
Figure 16. Analysis of expression of other genes expressed in haematopoiesis in man

Gene expression analysis by WISH in embryos phenotypically WT or man.

A-D Expression of *rag1* was seen in the bilateral thymi of WT embryos (closed arrowheads, A, C) and in the olfactory epithelium (open arrowheads, C). In contrast in *man* embryos the thymic expression was completely lost, however the olfactory epithelial expression was retained (open arrowheads, D).

E, F Normal expression of *foxn1* in epithelial cells within the thymus was seen in WT embryos (closed arrowhead, E). Weak expression was seen in 2/5 *man* embryos (closed arrowhead, F). Expression was absent in 3/5 *man* embryos.

G, H Expression of *mpll* was seen in the PBI region of WT embryos (closed arrowhead, G). Expression was absent in *man* embryos (H).

I, J Expression of *igta2b* showed the same pattern as *mpll*.

DVA= Ventral wall dorsal aorta, PBI= Posterior blood island. WISH= Whole mount in-situ hybridisation.
Figure 16

WT  man

rag1

E  F

foxn1

G  H

mpll

I  J

igta2b
Figure 17. Apoptosis in the nervous system of *man* embryos

Light microscopy on Haematoxylin & Eosin stained tissue sections unless otherwise stated on phenotypically WT or *man* embryos.

A In WT embryos the retina showed normal ordered structure (open arrowhead) and a visible but not expanded 4th ventricle (closed arrowhead).

B In contrast, *man* embryos showed loss of the ordered structure of the retina (open arrowhead) and expansion of the 4th ventricle (closed arrowhead).

C, D Neuronal cells in *man* showed morphological features of cells undergoing apoptosis including cellular shrinkage and nuclear condensation (open arrowheads, D).

E WT eye, with normal lens (closed arrowhead) and easily distinguishable retinal layers (i- retinal ganglion cell layer, ii- inner plexiform layer, iii- amacrine cell layer/bipolar cell layer, iv- outer plexiform layer, v- photoreceptor layer, vi- pigmented epithelial layer).

F In *man* embryos, the lens structure was abnormal and the lens epithelial layer was lost (closed arrowhead). The retinal layers were completely disrupted (open arrowhead) and the pigment was dispersed in the pigmented epithelial layer (arrow).

G, H Increased acridine orange staining was seen in the nervous system of *man* embryos (H) compared to WT embryos (G). This was most prominent in the midbrain/forebrain (open arrowhead, H) and the eye (closed arrowhead, H).

All embryos are 48 hpf. Acridine orange staining (G,H).
**Figure 18. Enlargement of the brain ventricles in \textit{man} embryos**

Light microscopy on phenotypically WT or \textit{man} embryos.

A In WT embryos, injection of rhodamine dextran into the hindbrain ventricle outlined the hindbrain ventricle (closed arrowhead) and the forebrain ventricle (open arrowhead).

B In \textit{man} embryos, the injected rhodamine outlined a markedly enlarged hindbrain ventricle (closed arrowhead). In addition the midbrain ventricle, not easily seen in WT embryos, was expanded (arrow).
Figure 18

WT

man

Rhodamine dextran
structure itself as examination of man embryos also carrying the fli1a:GFP transgene demonstrated that the man mutation had no effect on the formation of the major trunk vessels, nor the intersegmental vessels (Figure 19). Heart development did not proceed normally, expression of the cardiac gene nkh2.5 in man embryos was seen in an elongated domain reminiscent of the primitive heart tube, suggesting the complex cardiac morphogenetic movements needed to form a multi-chambered heart did not occur (Figure 20A, B).

Formation of the gastrointestinal system was also abnormal. Expression of foxA1 at 48 hpf, which is expressed in the developing gut and digestive organs, showed that only a primitive liver bud had formed and no pancreas (Figure 20C, D).

The man mutation did not cause a global defect in embryo development. At 48 hpf, despite the embryo exhibiting significant abnormalities in several tissues, other tissues remained unaffected. In the trunk, despite marked degeneration in the spinal cord with apoptosis, muscle tissue appeared normal (Figure 20F). The pronephric duct, a mesoderm-derived tissue like haematopoietic tissue, also appeared normal (Figure 20F).

### 3.2.4 Temperature-sensitivity of the man phenotype, a unique aspect of the zebrafish model

man was recovered from a phase of the ENU screen conducted at 33°C, to increase the chances of recovering temperature sensitive alleles, which might be incompletely penetrant and potentially missed in the screen, at a lower (permissive) temperature, but fully penetrant at a higher (restrictive) temperature (Johnson et al. 1995). Among the mutants recovered from the mutagenesis screen done at 33°C, only man demonstrated a clear temperature-sensitive phenotype.

For comparable developmental ages, man embryos reared at 21°C showed ameliorated gross phenotype, in those reared at 37°C, it was more severe (Figure
21). The size of the eye, one easily quantified aspect of the man phenotype, was directly related to temperature. For the comparison of Figure 21, the WT sibling embryos used for comparison were selected for similar developmental age, as assessed by embryo length. In developmentally age-matched embryos, the mpx deficiency was also temperature-sensitive (Figure 22). The number of mpx expressing cells per embryo at 21°C was significantly higher than at 28.5°C and closer to WT numbers, demonstrating a partial rescue of the phenotype at the permissive temperature. In contrast, at 33°C mpx expressing cell numbers were significantly reduced compared to 28.5°C.

3.3 Discussion

3.3.1 Summary of results

The characterisation of man demonstrated a defect within haematopoiesis primarily affecting definitive haematopoiesis. This included a marked reduction in myeloid cell numbers and a complete lack of T lymphocytes and thrombocytes. man also displayed marked abnormality in the nervous system with brain and eye degeneration due to apoptosis of neural cells. Although some ectodermal tissues (e.g. brain), and endodermal tissues (e.g. liver and pancreas) were markedly abnormal and mesoderm derived haematopoietic tissue showed defects, other mesodermal tissues (e.g. muscle and pronephric duct) appeared normal. This suggested that the requirement for the man gene during development is greater in some tissues than others.

3.3.2 Limitations of the analysis of the man phenotype

3.3.2.1 Haematopoiesis

EM of man neutrophils provided only a single cell for analysis. One method to increase the number of neutrophils in a tissue area, to maximise visualisation by EM studies, would be to transect the embryo tail in order to attract neutrophils to the site of tissue injury (Lieschke et al. 2001). However it is not known if this has
Figure 19. Trunk vascular development was normal in man embryos

Fluorescence microscopy on phenotypically WT or man embryos.

A The major trunk vessels marked by GFP expression in Tg (fli:GFP) transgenic embryos were normal in embryos WT for man.
B The intersegmental vessels (open arrowhead) were also normal in WT embryos.
C The major trunk vessels were also normal in man embryos carrying the fli:GFP transgene.
D Intersegmental vessels (open arrowhead) were also normal in man embryos.

All embryos are 48 hpf.

GFP= Green fluorescent protein.
Figure 19
Figure 20. Development of other tissues in *man* embryos

Gene expression analysis by WISH, unless otherwise stated, in embryos phenotypically WT or *man*.

A, B Expression of *nkx2.5* in WT embryos defined the entire domain of the multi-chambered heart (closed arrowhead, A). In contrast in *man* embryos expression of *nkx2.5* was seen in an elongated domain (closed arrowheads, B) reminiscent of the primitive heart tube.

C, D Expression of *foxA1* in WT embryos was seen in the developing liver bud (open arrowhead, C) and the pancreas (closed arrowhead, C). The liver bud in *man* embryos was smaller (open arrowhead, D) and the pancreas failed to develop (closed arrowhead, D).

E, F Despite marked apoptosis in the spinal cord of *man* embryos (Closed arrowhead, F), adjacent muscle tissue (open arrowhead, F) and pronephric duct (arrow, F) were not different from the WT structures (E).

All embryos are 48 hpf.

Haematoxylin & Eosin staining (E,F). View in C, D is dorsal.
Figure 20

WT  \hspace{2cm} \textit{man}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure20}
\caption{Comparison of WT and \textit{man} phenotypes at 48hpf.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure20}
\caption{Expression of \textit{nkrx2.5} at 48hpf.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure20}
\caption{Expression of \textit{foxA1} at 48hpf.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure20}
\caption{E-F: Cross-sections showing detailed structures at 48hpf.}
\end{figure}
Figure 21. Temperature sensitivity of the *man* gross phenotype

Light microscopy of phenotypically WT or *man* embryos.

A At equivalent developmental ages, the phenotype of *man* at 37°C was more severe than at the standard 28.5°C rearing temperature. In contrast, at 21°C the phenotype was ameliorated such that *man* embryos were similar in appearance to WT embryos.

B Eye size, one quantifiable aspect of the *man* phenotype, was smaller in *man* than WT at all temperatures, but *man* showed an association between temperature and eye size.

C The length of the comparator WT sibling embryos from the same corresponding clutch as *man* embryos was the same in the 3 temperature groups, demonstrating the developmental ages were equivalent.
Figure 21

A

33hpf

37°C

28.5°C

21°C

man

man

man

WT

B

C

n=10

n=2

n=10

n=11

p<0.0001

p=0.11

p=2

n=2

Temperature

Size (pixel area)

Phenotype

Temperature

WT

28.5°C

21°C

28°C
Figure 22. Temperature sensitivity of the mpx phenotype of man

Fluorescence microscopy on phenotypically WT or man embryos.

A Compared to the phenotype at 28.5°C, the reduction in mpx expressing cells by WISH in man was less severe at 21°C.

B The number of mpx expressing cells, assessed by WISH, in man was partially rescued at 21°C compared to 28°C.

C The number of mpx expressing cells, assessed in the Tg (mpx:GFP) strain, was reduced further at 37°C compared to 28.5°C.
Figure 22

A

WT

28.5°C

21°C

man

48hpf

B

C

p<0.0001

p=0.03

Number of positive cells

Number of positive cells
any effect on activation or morphology of man neutrophils. In addition it is still possible that if neutrophils in man are reduced due to premature apoptosis, identification of neutrophils by their characteristic granules would be difficult if they were already undergoing apoptosis. Functional inhibition of apoptosis and observation of man would be a better way of assessing the role of apoptosis in the haematopoietic phenotype.

3.3.2.2 Temperature sensitivity

A temperature-sensitive allele can act as a conditional allele, easily manipulated by changing the water temperature of developing embryos. Temperature-shift experiments can also be used to define a developmental time window during which a particular protein is required for a biological process (Tian et al. 2003).

These data demonstrated that the man phenotype was temperature-sensitive. Several mechanisms might explain temperature-sensitive mutations. The simplest model envisages that a temperature-sensitive mutation encodes an amino acid substitution at a residue critical either directly or indirectly in tertiary protein structure. In this case, when increasing the temperature of the protein, structure could be further compromised. This might occur either at a global level, or by changing the structure such that critical domains of the protein could be obstructed from interacting with DNA or other proteins (Parichy et al. 2003). However, this mechanism cannot explain all temperature-sensitive mutations as in zebrafish at least both nonsense and splice site mutations have been described that act as temperature sensitive alleles (Nechiporuk et al. 2003; Tian et al. 2003). No mechanism for the temperature sensitivity of either of these alleles was experimentally demonstrated. In the former, it was postulated that the temperature-sensitivity of the nonsense mutation was by translational read-through, or activation of an alternate start site downstream of the premature stop codon at lower temperature. With the latter, the authors were able to demonstrate that the aberrant splicing was not affected by manipulating the temperature.
Another possibility is that the developmental pathway or process is itself sensitive to either temperature or to the rate of embryo development overall. The mutant protein may have equal function at all temperatures but the pathway may be stressed by increased temperature or faster development. Against this mechanism is data showing that of two different zebrafish missense \textit{tp53} alleles, one was temperature-sensitive, the other not (Berghmans et al. 2005). Here, the data proved the temperature sensitivity was a direct effect on protein structure through a particular amino acid mutation, rather than an effect of temperature on the developmental pathway within which \textit{tp53} resides.

In order to dissect the temperature sensitivity of \textit{man} and determine if this was intrinsic to the \textit{man} protein, a comparison of the temperature sensitivity of a complete loss-of-function allele in the same gene with \textit{man} is planned. If the temperature sensitivity of \textit{man} was due to the missense mutation itself, and not an indirect effect of temperature, a null allele would be temperature-insensitive.

After \textit{man} was cloned, a \textit{zbtb11} retroviral insertion allele (ZM_00432624), predicted to be a null, was requested from the library of the commercial company Znomics. Unfortunately Znomics were not able to recover the line from cryopreserved sperm despite several attempts and the company’s financial difficulties prevented attempts at recovery of other \textit{zbtb11} insertion mutants. A null allele has been requested from the Zebrafish Mutation Resource project at the Sanger Centre (Hinxton, UK). An allele will be screened for by high-throughput sequencing of DNA corresponding to F1 library fish generated by F0 ENU mutagenesis (TILLING) (Wienholds et al. 2002).

A morpholino oligonucleotide knockdown approach could also test if the \textit{man} phenotype is intrinsic to the \textit{man} genetic lesion itself. An effective \textit{zbtb11} morpholino oligonucleotide would be predicted to transiently generate a reduction in amount of Zbtb11 protein, and thus should be temperature-
insensitive, in contrast to \textit{man}, if the temperature sensitivity of \textit{man} is due to the missense mutation itself. Limiting this approach is the potentially different effects of temperature on the speed of embryo development and degradation of the morpholino over time. At 21°C embryos take 82h to reach a developmental time-point equivalent to 48h at 28.5°C (Kimmel et al. 1995). This may be near the limits of ongoing mRNA knockdown by morpholinos if their degradation is temperature-independent, and currently no Zbtb11 antibody is available to quantitate the extent of protein knockdown. A comparison of \textit{man} and \textit{zbtb11} morphant embryos could be undertaken earlier but this would require recognition of an earlier \textit{man}-dependant phenotype then those that are currently described.

### 3.3.3 Conclusions

This initial characterisation of the \textit{man} mutant suggested the \textit{man} gene was dispensable for primitive haematopoiesis and initiation of definitive haematopoiesis, but with an essential role in the early development of myeloid, and possibly thrombocyte and lymphoid lineages. This suggested identification of the \textit{man} gene could provide new insights into the molecular regulation of haematopoiesis.

The temperature sensitivity of the \textit{man} phenotype provided a unique reagent with which to dissect the temporal requirements for the \textit{man} gene during development.
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