A murine Zic3 transcript with a premature termination codon evades nonsense-mediated decay during axis formation

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SUMMARY

The ZIC transcription factors are key mediators of embryonic development and ZIC3 is the gene most commonly associated with situs defects (heterotaxy) in humans. Half of patient ZIC3 mutations introduce a premature termination codon (PTC). In vivo, PTC-containing transcripts might be targeted for nonsense-mediated decay (NMD). NMD efficiency is known to vary greatly between transcripts, tissues and individuals and it is possible that differences in survival of PTC-containing transcripts partially explain the striking phenotypic variability that characterizes ZIC3-associated congenital defects. For example, the PTC-containing transcripts might encode a C-terminally truncated protein that retains partial function or that dominantly interferes with other ZIC family members. Here we describe the katun (Ka) mouse mutant, which harbours a mutation in the Zic3 gene that results in a PTC. At the time of axis formation there is no discernible decrease in this PTC-containing transcript in vivo, indicating that the mammalian Zic3 transcript is relatively insensitive to NMD, prompting the need to re-examine the molecular function of the truncated proteins predicted from human studies and to determine whether the N-terminal portion of ZIC3 possesses dominant-negative capabilities. A combination of in vitro studies and analysis of the Ka phenotype indicate that it is a null allele of Zic3 and that the N-terminal portion of ZIC3 does not encode a dominant-negative molecule. Heterotaxy in patients with PTC-containing ZIC3 transcripts probably arises due to loss of ZIC3 function alone.

INTRODUCTION

The gene most commonly associated with congenital situs defects, known as heterotaxy, in humans encodes the X-linked transcription factor ZIC3 (MIM 306955). Mouse models of Zic3 dysfunction also result in heterotaxy, indicating conserved mammalian function of this protein. Deletion of the entire ZIC3 locus in humans, or in the classical mouse mutant bent tail (Bn), results in heterotaxy, indicating that loss-of-function is the most likely pathogenic mechanism (Garber, 1952; Ferrero et al., 1997; Gebbia et al., 1997; Carrel et al., 2000; Klootwijk et al., 2000). To date, 12 ZIC3 variant sequences have also been identified in heterotaxy-affected families: six missense, five nonsense and one frameshift [caused by a two-nucleotide insert, which results in a premature termination codon (PTC) 182 nucleotides upstream from the wild-type transcription termination codon] [Gebbia et al., 1997; Mégarbané et al., 2000; Ware et al., 2004; Chhin et al., 2007]. The functional significance of the 12 variant sequences has also been investigated in vitro using mutant proteins expressed from ZIC3 full-length cDNAs containing each relevant mutation (Ware et al., 2004; Chhin et al., 2007). The in vivo consequence of the mutations that produce a PTC-containing transcript is, however, hard to predict from these analyses. In vivo these mutant transcripts might be subjected to nonsense-mediated decay (NMD), whereas the cDNA variants used to model these mutations would typically evade NMD, which, in mammalian cells, appears dependent upon mRNA splicing (Neu-Yilik et al., 2001).

NMD is a model of gene regulation and surveillance that recognizes and rapidly decays PTC-containing transcripts (Frischmeyer and Dietz, 1999; Maquat, 2004). One purpose of NMD is to limit the formation of C-terminally truncated polypeptides that might possess deleterious gain-of-function or dominant-negative activity. The mechanism by which a normal stop codon is distinguished from a premature one appears dependent upon the position of the PTC; a transcript will be committed to decay if a PTC is situated more than about 50-55 nucleotides upstream of an exon-exon junction (Nagy and Maquat, 1998). Because the rules regarding PTC recognition are not completely understood, the NMD sensitivity of PTC-containing transcripts needs to be assessed on a case-by-case basis (Holbrook et al., 2004). Moreover, not only do transcripts differ in their intrinsic sensitivity to NMD, the efficiency of NMD with respect to a given transcript can vary between tissues. To determine the functional significance of a nonsense mutation, RNA and/or protein levels must therefore be documented in the tissue and stage of development relevant to the particular disorder (Bateman et al., 2003). For heterotaxy cases, this requires assessing mRNA or protein levels at gastrulation (the time of left-right axis formation); a task not possible for human cases of heterotaxy. If the identified ZIC3 PTC-containing transcripts evade NMD, they might code for a ZIC3 molecule with a hypermorphic, hypomorphic or dominant-negative effect.

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murine five genes show extensive homology throughout the DNA binding Aruga et al., 1996b; Furushima et al., 2000; Ali et al., 2012). The Zic gene family (sequence homology with four other members of the mammalian or the wild-type ZIC3 function. At the time of left-right axis formation, the mutant transcript is not subjected to NMD, predicting the production of a mutant protein during embryogenesis. When the analogous mutant CDNA is expressed in cultured mammalian cell lines, a stable protein that is truncated just two amino acids upstream of the zinc finger domain is produced. The truncated protein lacks the zinc finger domain and should fail to undergo nuclear import (Bedard et al., 2007); however, it accumulates within the nucleus as a result of passive diffusion. Despite its presence in the nucleus, the katun protein is unable to elicit transcription of a ZIC3 target promoter and does not compete with the wild-type trans-activation ability of the ZIC3, ZIC2 or ZIC5 proteins, indicating that the N-terminal portion of ZIC3 does not possess dominant-negative activity. Additionally, the katun protein fails to inhibit Wnt-mediated transcription, unlike wild-type ZIC3 protein. The phenotype of katun mutant embryos replicates that of a targeted null allele of Zic3 and confirms that the Ka mutant phenotype results from a loss of Zic3 activity alone.

The relative ability of a transcript to undergo NMD is influenced by sequences within the transcript that promote RNA splicing, such as splice donor and acceptor sites (Gudikote et al., 2005). The murine Zic3 and human ZIC3 transcripts contain identical splice sites so the finding that the murine Zic3 transcript is relatively insensitive to NMD during axis formation and early organogenesis suggests that the human transcript also evades NMD at these stages of embryogenesis. This prompted the examination of other truncated ZIC3 proteins predicted in human heterotaxy cases and none were found to possess dominant-negative activity. The data suggest that ZIC3-associated heterotaxy caused by nonsense or frameshift mutations affects ZIC3 function alone. The katun mutant is useful not only for investigations into the molecular mechanisms underlying ZIC3-associated heterotaxy but also for studies of Zic protein interaction and transcriptional surveillance mechanisms.

RESULTS
The Zic3 gene is mutated in the katun mouse strain
The katun allele arose as a spontaneous, X-linked mutation during an N-ethyl-N-nitrosourea (ENU) mutagenesis experiment (Bogani et al., 2004), indicating that it might be a new allele of Zic3, the gene deleted in the classical, X-linked mouse mutant bent tail (Bn) (Carrel et al., 2000; Klootwijk et al., 2000) and mutated in targeted mouse strains (Purandare et al., 2002; Zhu et al., 2007). The coding region of Zic3 was amplified from the genomic DNA of a heterozygous female, a hemizygous male, a C3H mouse and a BALB/c mouse and sequenced. This revealed a single base change of guanine (G) to thymine (T) at nucleotide position 1283 (1283G >T) of accession number NM_009575 in the affected animals (Fig. 1A–C). The colony has subsequently been maintained via
The katun transcript evades nonsense-mediated decay

The katun transcript conforms to the rule for NMD (Nagy and Maquat, 1998) and is predicted to be absent from hemizygous null embryos. To determine whether the katun transcript is subjected to NMD we performed whole mount in situ hybridization (WMISH) to Zic3 in wild-type and Ka/Y embryos (7.0, 8.5, and 9.5 days post-conception; dpc). As shown in Fig. 2A-E, Zic3 transcript levels were indistinguishable from wild-type levels in the hemizygous null embryos at all stages examined, suggesting that NMD of the Ka transcript is deficient. To confirm this, allele-specific RT-PCR was performed at each of the embryonic stages examined by WMISH from embryos of all three genotypes (+/+, Ka/X and Ka/Y). The absence of genomic DNA in each sample was confirmed by amplification of an intron spanning fragment from the Zic3 gene. The 162-bp cDNA-specific product was only amplified from cDNA samples synthesized in the presence of reverse transcriptase, whereas the 1006-bp genomic DNA-specific product was absent in all samples (Fig. 2F and data not shown). Allele-specific RT-PCR products were then produced (using exon 1 primers) from each RNA sample and analysed by high resolution melt analysis. In each case, the melt profile of the products obtained from cDNA samples was compared with positive control profiles obtained from genomic DNA of animals of known genotype (Fig. 2G). At 7.5, 8.5 and 9.5 dpc, embryos containing only the normal Zic3 allele (Zic3+/+) exclusively express the wild-type transcript whereas embryos with one mutant and one wild-type allele (Zic3−/−/+) express a mixture of the two transcripts (Fig. 2G-I). The hybridization signal seen in Zic3−/−/Y mutant embryos is therefore due to the accumulation of the Ka transcript. Together, these data indicate that the Ka transcript is not rapidly subjected to NMD during axis formation (the stage at which the Zic3-associated heterotaxy phenotype emerges).

The mutant protein is stable and accumulates in the nucleus via passive diffusion

The finding of incomplete NMD raises the possibility that the message could be translated into a short-lived, truncated protein with some function. Detection of Zic3 protein in wild-type and katun mutant embryos using SDS-PAGE and western blotting was not successful due to failure of the antibodies to specifically detect endogenous Zic3 (data not shown). Therefore, to determine whether this mutant Zic3 transcript can be translated into a stable protein, either a N-terminal V5-epitope tagged version of ZIC3 (V5-ZIC3-wt) or ZIC3-katun variant (V5-ZIC3-katun) were expressed in cultured mammalian cell lines. Following transfection of COS-7, HEK293T or NIH 3T3 cells a wild-type protein of ~60 kDa and a katun protein of ~35 kDa were detected with western blotting using either an anti-Zic3 antibody or an anti-V5 antibody. Both proteins were detected in lysates made 24, 42 and 72 hours post-transfection, indicating that the katun protein is stable (Fig. 3A and data not shown). The predicted sizes of the wild-type and katun ZIC3 proteins are 52 kDa and 27 kDa, respectively, with the difference corresponding to the size of the V5 tag and spacer fragment within the pcDNA3.1/nV5-DEST vector.

The Zic3 protein does not contain a canonical nuclear localization signal but is transported into the nucleus via the importin pathway through cryptic nuclear localization signals within the zinc finger domain (Bedard et al., 2007; Hatayama et al., 2008). The absence of the zinc finger domain in the katun protein is therefore expected to prevent accumulation of V5-ZIC3-katun protein.
within the nucleus. As shown in Fig. 3A, subcellular fractionation of transfected cell lysates revealed that a significant proportion of the katun protein was found in the nucleus. To confirm this result and quantify the extent of katun nuclear accumulation, the subcellular localization of V5-ZIC3-katun was compared with that of V5-ZIC3-wt by immunofluorescent staining following transfection into HEK293T cells (Fig. 3B,D). Consistent with previous studies on the subcellular location of Zic proteins (Ware et al., 2004), 88.5% of the wild-type V5-ZIC3-wt protein was found within the nucleus. Strikingly, 61.4% of the mutant V5-ZIC3-katun
protein accumulated within the nucleus. This raised the possibility that the N-terminal portion of Zic3 contains sequences sufficient for directed nuclear transport. It is, however, also possible that the small size of the katun protein enables it to diffuse into the nucleus because proteins smaller than ~40 kDa can diffuse into the nucleus (Wei et al., 2003). To distinguish between these possibilities, the size of the katun protein was artificially increased by fusion with enhanced green fluorescent protein (EGFP). The subcellular localization of EGFP-ZIC3-katun was compared with that of EGFP-ZIC3-wt following transfection into HEK293T cells using western blot and immunofluorescent staining (Fig. 3B-D). Western blot analysis detected EGFP-ZIC3-wt in the nuclear fraction at ~85 kDa (predicted size, 79 kDa) and EGFP-katun predominately in the cytoplasmic fraction at ~55 kDa (predicted size, 54 kDa). Immunofluorescent localization analysis found that 88.9% of the EGFP-ZIC3-wt protein was within the nucleus, whereas only 10.3% of the mutant EGFP-ZIC3-katun protein accumulated within the nucleus. The difference between the localization of the V5-ZIC3-katun protein and the EGFP-ZIC3-katun protein suggests that the katun protein accumulates in the nucleus by passive diffusion.

The mutant protein is transcriptionally inactive and does not compete with wild-type ZIC3

Given that the katun protein is stable and localizes to the nucleus, it is possible that the protein exerts some effect. The zinc finger domain is crucial for the trans-activation ability of Zic proteins because deletion of the domain or point mutations within the zinc fingers that disrupt DNA binding leads to proteins that are unable to stimulate transcription (Brown et al., 2005). The ability of V5-ZIC3-katun to stimulate transcription was evaluated using a well-established cell-based Apoe promoter luciferase reporter assay (Salero et al., 2001; Brown et al., 2005). Co-transfection of the Apoe reporter construct with either the V5-ZIC3-wt construct or the V5-ZIC3-katun construct into HEK293T cells followed by quantification of luciferase activity demonstrated that the truncated protein was unable to elicit transcription (Fig. 4A). The comparable expression of each ZIC3 construct was confirmed by SDS-PAGE and western blot (Fig. 4A, Blot). When V5-ZIC3-katun was placed in competition with wild-type ZIC proteins (V5-ZIC3-wt, V5-ZIC2-wt or V5-ZIC5-wt) the trans-activation abilities of the wild-type proteins were not significantly altered (Fig. 4B).

The mutant protein does not inhibit β-catenin-mediated transcription

The human ZIC2 and Xenopus zic1-5 proteins have recently been shown to act as cofactors that inhibit Wnt-dependent β-catenin-mediated transcription (Pourerbrahim et al., 2011; Fujimi et al., 2012). Upon Wnt stimulation, β-catenin enters the nucleus and interacts with the TCF transcription factors to stimulate transcription of target genes (Behrens et al., 1996). A luciferase reporter construct containing consensus TCF binding sites (TOPflash) or mutated TCF sites (FOPflash) is routinely used to assess Wnt-dependent transcription (Korinek et al., 1997). Co-transfection of the TOPFlash construct with one encoding a stabilized form of β-catenin (β-catenin-ΔN89) into HEK293T cells drove high levels of luciferase activity (Fig. 4C) but this level was not attained in the presence of V5-ZIC3-wt. This indicates that human ZIC3, like ZIC2, is able to inhibit β-catenin-mediated transcription of Wnt target genes in cultured HEK293T cells. In addition, when ZIC3 is expressed, lower levels of β-catenin-ΔN89 are detected, which is consistent with the enhanced β-catenin degradation previously seen with the expression of Xenopus zic3 (Fujimi et al., 2012). In contrast to wild-type ZIC3 protein, co-
transfection with V5-ZIC3-katun does not decrease luciferase activity, indicating that the katun protein is unable to inhibit Wnt-dependent β-catenin-mediated transcription.

**Ka phenocopies a targeted null allele of Zic3**

The most definitive test of allele type involves placing the new allele in *trans* to a known null allele, but this test cannot be performed...
for X-linked genes. To determine whether the Ka allele of Zic3 behaves as a null mutation in vivo we therefore recovered embryos at gastrulation stages and examined the associated phenotype. The allele of Zic3 that is best characterized at these stages is the targeted null allele of Zic3 (Zic3tm1Bca) (Purandare et al., 2002). Embryos from this strain that are hemizygous or homozygous null have a variable phenotype, with mutant embryos being assigned to three classes. Type I and Type II embryos display aberrant morphology at early and mid-gastrula stages, whereas the Type III embryos are not morphologically abnormal until the end of gastrulation. The Type I and Type II embryos are characterized by defects in the endoderm and mesoderm formed during gastrulation (Ware et al., 2006b). When embryos hemizygous for the Ka allele (Zic3Ka/y) were recovered at gastrulation a highly variable phenotype was apparent. A proportion of the recovered embryos displayed defects of the distal egg cylinder or of the extra-embryonic/embryonic junction that are characteristic of the Type I and Type II embryos, as described previously (Ware et al., 2006b). 100% of these embryos were of the Zic3Ka/y genotype.

To confirm that the phenotype observed in the Zic3Ka/y embryos is analogous to that described for embryos from the targeted null allele, WMISH to markers of endoderm and mesoderm formation was performed (a minimum of four embryos were examined per genotype class and probe). Embryos that had morphological defects characteristic of type I mutants were analysed by hybridization to probes that mark the primitive streak and emerging wings of embryonic mesoderm (Fgf8 and Foxa2). Consistent with previous analysis (Ware et al., 2006b), Type II embryos exhibited mesoderm abnormalities that included a protrusion of the primitive streak into the amniotic cavity (Fig. 5C) and ectopic expression of mesoderm markers (arrows in Fig. 5D,E). Additional embryos (n=65 Zic3+/y and n=27 Zic3Ka/y) were recovered at 9.5 dpc and the heart examined for looping defects indicative of heterotaxy. Consistent with previous analysis of a Zic3 null allele (Ware et al., 2006a), 52% of null embryos exhibited normal hearts (dextral looping, Fig. 5F), 19% exhibited a leftward curve of the heart tube (sinistral looping, Fig. 5G) and the remaining 30% had a heart tube that looped forward (ventral looping) or did not loop at all.

PTC-containing ZIC3 mutant transcripts produce proteins that do not compete with wild-type ZIC3

The magnitude of NMD is dependent upon the strength of the splice donor and acceptor sites within an intron, with stronger sites (i.e. those that more closely match the consensus sequence) subject to increased NMD (Gudikote et al., 2005). To assess whether inefficient NMD of the human ZIC3 can be predicted, the mouse Zic3 and human ZIC3 splice sites were compared. As shown in Table 1, the

Table 1. Zic3/ZIC3 splice site homology and scores

| Gene     | Intron   | Donor site  | Acceptor site | Donor site | Acceptor site |
|----------|----------|-------------|---------------|------------|--------------|
| Mouse Zic3 | Intron 1/2 | CAGgtaagg | tgcctttgcagGT | 98.07 | 92.14 |
| Human ZIC3 | CAGgtaagg | tgcctttgcagGT | 86.73 | 82.17 |
| Mouse Zic3 | Intron 2/3 | AAGgtaatt | tgtaatttagGT | 90.41 | 89.80 |
| Human ZIC3 | AAGgtaatt | tgtaatttagGT | 86.73 | 92.14 |
| Mouse Zic3 | Intron 2/4 | AAGgtaatt | ttcctttgcagTG | 90.41 | 89.80 |
| Human ZIC3 | AAGgtaatt | ttcctttgcagTG | 86.73 | 92.14 |

Splice site consensus values (CV) were calculated using Human Splicing Factor (http://www.umd.be/HSF/4DACTION/input_SSF). Upper case letters indicate exon sequence; lower case letters indicate intron sequence.
sites are identical (intron 1/2 and 2/3) or nearly so (intron 2/4). Intron 2/3 has the weakest splice signals, as indicated by a lower consensus score. The absolute conservation of Zic3 and ZIC3 splice sites prompted the examination of the predicted proteins from each of the ZIC3-associated heterotaxy mutations that generate a PTC. Six different ZIC3-associated heterotaxy mutations with a PTC have been previously documented and the protein stability, subcellular localization and transcriptional ability of each of these has been assessed using cell-based assays. Two of these mutations generate unstable proteins (S43X and Q249X) whereas the remaining mutations generate stable proteins that can either be found exclusively in the cytoplasm (C268X and Q292X) or in both cytoplasmic and nuclear compartments (1507insTT and K408X) (Ware et al., 2004). To test whether any of these proteins possess dominant-negative properties, the analogous mutant proteins were expressed in HEK293T in competition with wild-type ZIC3. Expression of each ZIC3 construct was verified by SDS-PAGE and western blot of lysates containing both nuclear and cytoplasmic fractions. Note that the Q249X mutation was excluded from this analysis because it is probably well-modelling by experiments with the V5-ZIC3-katun construct. Our results confirm the finding of Ware and colleagues that the K408X protein retains some trans-activation ability (Ware et al., 2004). We show here that the K408X protein confirmed, however, that it lacks activities associated with wild-type ZIC3.

**DISCUSSION**

**The katun mouse strain carries a null allele of Zic3**

The katun mouse strain arose spontaneously during an ENU-mutagenesis experiment (Bogani et al., 2004). We show here that the identified phenotype (a bent tail) is caused by mutation of the X-linked Zic3 gene. Several lines of evidence indicate that the identified mutation is responsible for the phenotype: (i) the identified base pair change is not a commonly occurring polymorphism; (ii) the variant segregates with the phenotype, being linked through over 1000 meioses to date during maintenance of the colony; (iii) the identified nonsense mutation generates an inactive protein; and (iv) the phenotype precisely recapitulates that documented for a pre-existing null allele of Zic3.

The katun mutation introduces a nonsense codon into the Zic3 transcript that conforms to the rule for PTC recognition in mammalian cells. This provides the first opportunity to investigate the fate of such Zic3 transcripts during mammalian axis formation. WMISH to Zic3 in Ka/Y embryos at gastrulation and early organogenesis stages showed no decrement in Zic3 mRNA accumulation. Allele-specific RT-PCR confirmed that the only transcript expressed in Ka/Y embryos carries the nonsense mutation and that both wild-type and mutant transcripts are present in Ka/Y embryos. These data indicate that the Zic3 transcript is a poor substrate for NMD at the time of left-right axis formation and raise the possibility that the Ka mutation does not represent a null allele. Investigations into the molecular properties of the katun mutant protein confirmed, however, that it lacks activities associated with wild-type ZIC3. The katun protein is truncated just two amino acid residues upstream of the zinc finger domain that is required for nuclear localization (Bedard et al., 2007; Hatayama et al., 2008), trans-activation of target gene expression (Koyabu et al., 2001; Mizugishi et al., 2001; Ware et al., 2004) and inhibition of β-catenin-mediated transcription (Fujimi et al., 2012). Although katun can accumulate in the nucleus to appreciable levels (due to passive diffusion rather than active transport), it is transcriptionally inert and does not inhibit β-catenin-mediated transcription.

These data establish that the katun protein is null for the known Zic3 molecular activities, but *Xenopus* experiments have implied that a similar zic3 protein interferes with the function of wild-type zic3 during axis formation (Kitaguchi et al., 2000). This possibility has not previously been biochemically tested. When the katun mutant protein is placed in competition with either wild-type ZIC3 or the other ZIC molecules coexpressed at the time of axis formation (ZIC2 and ZIC3), it does not interfere with their ability.
ZIC3 mutant evades nonsense-mediated decay

The possibility of incomplete NMD influences the interpretation of ZIC3 PTC-inducing mutations

The finding that the murine Zic3 transcript is a poor substrate for NMD at axis formation suggests that this is also the case for the human ZIC3 transcript. Direct assessment of the fate of human ZIC3 PTC-containing transcripts during axis formation is not possible and predictions regarding the likely NMD behaviour based on the murine transcript provide one alternative. The features that render a transcript a poor target for NMD are not fully characterized. There is, however, evidence that the position of the PTC within the transcript and RNA splicing influences NMD amplitude (Nagy and Maquat, 1998; Gudikote et al., 2005). The genomic arrangement of the murine (Zic3) and human (ZIC3) genes is nearly identical and the splice donor and acceptor sites are completely conserved. It is likely that the human ZIC3 transcript is similarly able to avoid mRNA surveillance mechanisms, which needs to be considered when interpreting the probable effect of ZIC3 PTC-inducing mutations. If PTC-containing transcripts are translated, the two most likely effects are: (i) proteins that truncate downstream of crucial domains might be hypomorphic; and (ii) proteins that do not produce crucial domains might encode dominant-negative molecules.

Six mutations that introduce a PTC into the human ZIC3 transcript have been associated with congenital defects. Four of these adhere to the position rule for NMD (i.e. the PTC is sited more than about 50-55 nucleotides upstream of an exon-exon junction (Nagy and Maquat, 1998). The most 5′-ward of the mutations (C633A) would encode a severely truncated molecule (S43X) if not degraded. Previous examination of this putative protein has shown that it is not stably produced in cell lines (Ware et al., 2004) and is therefore likely to generate a null allele regardless of NMD amplitude. The C1250T mutation would encode the Q249X protein. An expression construct incorporating this mutation into the ZIC3 cDNA has previously been reported to produce no protein (Ware et al., 2004). This PTC lies very close to that generated here in order to mimic the katun protein (E250X), but the katun mutation results in a stable protein. Whatever the reason for the discrepancy between these results, the studies of the katun protein suggest that even if the Q249X protein is generated in vivo it would encode a protein with neither transcriptional Wnt inhibition nor dominant-negative activity. The remaining two mutations (C1338A and C1408T) that conform to the NMD PTC position rule would generate proteins that contain part of the zinc finger domain (C268X and Q292X, respectively). Previous studies indicate that these proteins are transcriptionally inactive and here we confirm and extend this analysis to show that these proteins also fail to dominantly interfere with ZIC trans-activation ability. It seems likely that each of these four mutations would generate a null allele of ZIC3 regardless of NMD amplitude and not result in a composite ZIC phenotype. The remaining two mutations (1507insTT and A1741T) introduce a PTC close to the last intron and, regardless of ZIC3 transcript sensitivity to NMD, are likely to be translated into a stable protein. Here, we confirm that the frameshift protein is transcriptionally inert whereas the K408X protein (corresponding to the A1741T mutation) retains some trans-activation ability. Neither protein is able to interfere with the trans-activation ability of wild-type ZIC. The frameshift mutation therefore appears to generate a null allele whereas the K408X mutation is predicted to be hypomorphic. This conclusion is consistent with the finding of incomplete penetrance for the A1741T mutation (Mégarbané et al., 2000).

In summary, the work presented here confirms the notion that loss of function (partial or complete) of ZIC3 alone is the probable mode of pathology in ZIC3-associated heterotaxy cases that involve PTC-containing transcripts. It implies, however, that ZIC3 PTC-inducing mutations cannot, a priori, be considered to encode a null allele. It is possible that the ZIC3 transcript, like Zic3, is a poor substrate for NMD at the axis formation stage of embryogenesis. Instead, each putative protein needs to be evaluated for its trans-activation ability, co-repressor ability and potential dominant-negative effects. Analysis of the phenotype associated with a murine PTC-containing transcript suggests that the N-terminal portion of the Zic3 protein that lies upstream of the zinc finger does not possess dominant-negative activity and does not interfere with the function of other coexpressed Zic proteins.

Materials and Methods

Mouse strains and husbandry

Mice were maintained according to Australian Standards for Animal Care under protocol A2011/63 approved by The Australian National University Animal Ethics and Experimentation Committee for this study. The katun (Ka) allele (MGI:3043027) of Zic3 (Zic3Ka) was maintained by continuous backcross to C57BL/6J inbred mice; animals from backcross 10 and beyond were used for analysis. Mice were maintained in a 12-hour light-dark cycle, the midpoint of the dark cycle being 1 am. For the production of staged embryos, 1 pm on the day of appearance of the vaginal plug was designated 0.5 dpc. Genomic DNA was prepared for genotyping as previously described (Arkell et al., 2001) and amplified for high resolution melt analysis using IMMOLASE™ DNA polymerase with TD60 PCR thermal cycling conditions (Thomsen et al., 2012). The Ka mice and embryos were genotyped using the primers Arki1085_F 5′-CCTTCTTCGGTACATCG-3′ and Arki1086_R 5′-CTGAGCCTCCTGATCC-3′ and sexed using primers Arki1002_F 5′-GAGGTCATGAAGTCCAG-3′ and Arki1003_R 5′-GGGCATAAGTCTTTCCAG-3′.

Mutation detection

Mutation detection was performed by direct sequencing of PCR amplicons from genomic DNA isolated from affected animals and
the appropriate parental strains (C3H/HeH and BALB/c OlhaSd) (Bogani et al., 2004). All primers correspond to intron sequence such that coding sequence and intron/exon boundaries were examined for mutations. The oligonucleotides used for sequencing Zic3 were: fragment 1, Ark207_F 5’-TGGAAAGTGTCACTGCC-3’ and Ark208_R 5’-ATAGTTAGGGAAACTGGC-3’; fragment 2, Ark209_F 5’-CTACTTGCTCTTCTG-3’ and Ark210_R 5’-TGGTACTGAAGGCTCCG-3’; fragment 3, Ark211_F 5’-CAGATGCTATGTCGTC-3’ and Ark212_R 5’-TCTAAGGTGTCAGTGTCG-3’; fragment 4, Ark213_F 5’-CTAGGGGTATCATTCTCG-3’ and Ark214_R 5’-CTGAGAAAAGGCATTAGC-3’.

Whole mount in situ hybridization and cDNA clones

All embryos were dissected from the maternal membranes in PBS with 10% newborn calf serum and staged using morphological criteria (Downs and Davies, 1993). Embryos were fixed overnight at 4°C in 4% paraformaldehyde in PBS. WMISH was carried out according to standard procedures (Wilkinson, 1992; Rosen and Beddington, 1994). Probes for WMISH were as previously described: Zic3 (Elms et al., 2004), Lhx1 (Shawlot and Behringer, 1995), Cer1 (Belo et al., 1997), Foxa2 (Sasaki and Hogan, 1993) and Fgf8 (Mammoth et al., 1995). After completion of the WMISH, embryos were de-stained in PBT (PBS with 0.1% Tween-20) for 48 hours and post-fixed in 4% paraformaldehyde in PBS for 1 hour at room temperature. Embryos were processed for photography through a glycerol series (50%, 80% and 100%) and photographed on a glass slide.

RT-PCR

Embryos, dissected and staged, for RT-PCR were individually frozen in 96-well plates on dry ice and stored at −70°C. Upon geno- and sex-typing of the corresponding embryo tissue, the embryos were pooled in three genotype classes (Zic3+/+, Zic3+/−, and Zic3−/−). Each pool consisted of ten embryos at 7.5 dpc, four embryos at 8.5 dpc and two embryos at 9.5 dpc. Genomic DNA-free RNA was extracted from each sample using the Ambion RNAqueous 4 PCR kit (Life Technologies). RNA concentration was quantified by Nanodrop spectrophotometry and 500 ng of RNA template included in a random primed cDNA first strand synthesis reaction (Superscript VILO cDNA synthesis kit; Life Technologies). A RT negative control synthesis reaction was performed in parallel from each RNA sample. To confirm absence of contaminating genomic DNA in the original RNA samples, amplification from each cDNA sample was performed using primers Ark364 and Ark311 (located in exons 2 and 3, respectively, of Zic3) using Abgene DNA polymerase (with buffer: 10 mM Tris-HCl, pH 8.3, at 25°C, 50 mM KCl, 1.5 mM MgCl2) and TD60 PCR thermal cycling conditions with an extension time of 45 seconds (Thomsen et al., 2012). PCR products were analysed by agarose gel electrophoresis. Amplification from genomic DNA produces a 1006-bp product whereas that from cDNA results in a 162-bp product. For allele-specific PCR, each cDNA (0.5 µl) was used in the genotyping assay.

Plasmids

All plasmid construction used standard molecular biology procedures. The Gateway® Recombination Cloning Technology (Life Technologies®) was used to generate V5-epitope-tagged expression plasmids. The appropriate cDNA was transferred to the pENTR™3C vector that had been linearized with EcoRI (NEB) and dephosphorylated with Antarctic Phosphatase (NEB) to produce an ‘entry’ clone. Four entry clones were generated as follows.

pENTR™3C-ZIC3-wt: a full length ZIC3 cDNA was recovered from the HA-ZIC3-wt expression plasmid (a gift from Stephanie Ware, Cincinnati Children’s Hospital Medical Center, OH) (Ware et al., 2004) by PCR with oligonucleotides Ark1152_F 5’-ACCCGGATCCGAGATTTGTCTGCA-3’ and Ark1153_R 5’-GTGGCCCAGATTCTCCTCTGAC-3’ and Ark1154_R 5’-CAGATGCTATGTCGTC-3’ and Ark1155_F 5’-ATGAGAAAAGGCATTAGC-3’. This amplicon was cloned using In-Fusion™ Dry-Down PCR Cloning System (Clontech) into pENTR™3C (Life Technologies).

pENTR™3C-ZIC3-katun: the HA-ZIC3-wt plasmid was subjected to site-directed mutagenesis with the QuikChange® II Site-Directed Mutagenesis Kit (Stratagene) using oligonucleotides Ark1006_F 5’-GGCTATTAAAGTCCGTAGTGACAGTG-3’ and Ark1007_R 5’-CACTTGACAGACGTACTGTCCTG-3’. This plasmid was amplified and cloned as ZIC3-wt into pENTR™3C.

pENTR™3C-ZIC5-wt: a full length ZIC5 cDNA was PCR amplified from pcDNA-ZIC2 (a gift from Maral Mouradian, Robert Wood Johnson Medical School, NJ) (Yang et al., 2000), using oligonucleotides: Ark1150_F 5’-ATCGCGTATCCGAAATTCACTG-3’ and Ark1168_R 5’-GTGCAGGGCAGATGGAAGGCAGTGTTAGG-3’ and Ark1120_F 5’-ATGTAGGTTCATGAATCTCAA-3’ and Ark1121_R 5’-CTAGGGGGGCCCAGGTAGAACAACACAGCTGC-3’ and Ark1140_R 5’-GGCGGTCATGACTTACAGTCGCC-3’ and Ark1401_F 5’-GAAGGTCTGCTTCTTCTTCTCCG-3’ and Ark1402_R 5’-ATGAGAAAAGGCATTAGC-3’. This amplified ZIC5 cDNA was cloned into pENTR™3C using the In-Fusion™ Dry-Down PCR Cloning System.

pENTR™3C-ZIC5-wt: a full length ZIC5-wt cDNA was excised from pCMV6-XL5-ZIC5 (Origene) via EcoRI digestion and ligated into pENTR™3C using T4 DNA ligase (NEB). Entry clones for all other ZIC3 mutant plasmids were created via site-directed mutagenesis of pENTR™3C-ZIC3-wt with the QuikChange® II Site-Directed Mutagenesis Kit (Stratagene). Primers used to introduce mutations were as follows: pENTR™3C-ZIC3-C268X, Ark1399_F 5’-GGCGCCCAAGAGAGAGCTAGGAGGGCCGACTGCTGC-3’ and Ark1400_R 5’-TGGGAAGGTCGGTCGTACCTGCTGGCC-3’ and Ark1401_F 5’-GGCGCCCAAGAGAGAGCTAGGAGGGCCGACTGCTGC-3’ and Ark1402_R 5’-ATGAGAAAAGGCATTAGC-3’. This plasmid was amplified and cloned as ZIC3-wt into pENTR™3C.

pENTR™3C-ZIC3-1507insTT: a full length ZIC3-wt cDNA was recovered from pENTR™3C-ZIC3-wt via Gateway® LR Clonase reaction (as per manufacturer’s instructions; Life Technologies) via a Gateway® LR Clonase reaction (as per manufacturer’s instructions; Life Technologies) to produce the following plasmids: V5-ZIC3-wt, V5-ZIC3-katun, V5-ZIC3-C268X, V5-ZIC3-Q292X, V5-ZIC3-1507insTT, V5-ZIC3-K408X, V5-ZIC2-wt and V5-ZIC5-wt. To generate the EGFP-tagged ZIC3 constructs, ZIC3 was amplified from HA-ZIC3-wt or HA-ZIC3-katun using the following primers: Ark1208_F 5’-GAGCTCAAGCTCTGAAATTCTACCC-3’ and Ark1209_R 5’-TGGCAAGATTCAGATTTCACTCCTTCCG-3’. This plasmid was excised from pCMV6-XL5-ZIC5 (Origene) via EcoRI digestion and ligated into pENTR™3C using T4 DNA ligase (NEB). Entry clones for all other ZIC3 mutant plasmids were created via site-directed mutagenesis of pENTR™3C-ZIC3-wt with the QuikChange® II Site-Directed Mutagenesis Kit (Stratagene). Primers used to introduce mutations were as follows: pENTR™3C-ZIC3-C268X, Ark1399_F 5’-GGCGCCCAAGAGAGAGCTAGGAGGGCCGACTGCTGC-3’ and Ark1400_R 5’-TGGGAAGGTCGGTCGTACCTGCTGGCC-3’ and Ark1401_F 5’-GGCGCCCAAGAGAGAGCTAGGAGGGCCGACTGCTGC-3’ and Ark1402_R 5’-ATGAGAAAAGGCATTAGC-3’.
To detect protein bands, blots were incubated with SuperSignal being immunoblotted using standard western blotting techniques. Tween 20 (Sigma Aldrich) (western blot blocking buffer) before transfer at 15 V for 16 hours. Membranes were blocked overnight Proteins were transferred to PVDF membranes (Millipore) via wet loaded onto 8%, 10% or 12% SDS-PAGE gels and run at 100 V. and the samples heated for 5 minutes at 90°C. Samples were then transacted with either 0.4-0.6 μg or 3-4 μg of the mammalian expression plasmid DNA using Lipofectamine™ 2000 (Life Technologies) according to the manufacturer’s guidelines. Cell culture and transfection Mammalian cell lines COS-7, NIH3T3 and HEK293T were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies), supplemented with 10% (v/v) fetal bovine serum (Life Technologies), 2 mM L-glutamine (Life Technologies) and 0.1 mM non-essential amino acid solution (Life Technologies) at 37°C in humidified air. For transient transfections, cells were transfected with either 0.4-0.6 μg or 3-4 μg of the mammalian expression plasmid DNA using Lipofectamine™ 2000 (Life Technologies) according to the manufacturer’s guidelines. Immunofluorescence staining, microscopy and quantification of subcellular localization Cells were prepared for immunofluorescence microscopy as previously described (Kovacs et al., 2002) and viewed using the LSM 5 Pascal (ZEISS) confocal microscope. At least 100 transfected cells per experiment were scored blind as follows. For each cell, the nuclear and cytoplasmic compartment was traced using the Intuos® 2 graphics tablet (Wacom) and the average fluorescence intensity for each compartment of the cell was measured with ImageJ analysis software (NIH). The values from the 100 scored cells were averaged to give the percentage nuclear and percentage cytoplasmic localization for the experiment. Three independent experiments were conducted and the percentage localization in each cellular compartment averaged across the three experiments. For statistical analysis, GenStat (VSN International) was used to perform a non-orthogonal factorial ANOVA. Images were assembled in Adobe Photoshop CS7. Subcellular fractionation, SDS-PAGE and western blotting HEK293T cells grown on 35-mm tissue culture dishes or 12-well tissue culture plates (Corning®) were lysed and fractioned into nuclear and cytoplasmic fractions using the NE-PER kit (Pierce) according to the manufacturer’s protocol. CERI and NERI lysis buffers were supplemented with protease inhibitors (complete, EDTA-free protease inhibitor cocktail, Roche). Then, 2 mM DTT (Sigma Aldrich) and 1x NuPAGE LDS Sample Buffer (Life Technologies) were added to nuclear and cytoplasmic fractions and the samples heated for 5 minutes at 90°C. Samples were then loaded onto 8%, 10% or 12% SDS-PAGE gels and run at 100 V. Proteins were transferred to PVDF membranes (Millipore) via wet transfer at 15 V for 16 hours. Membranes were blocked overnight at 4°C with a solution of 5% skim milk powder, PBS and 0.2% Tween 20 (Sigma Aldrich) (western blot blocking buffer) before being immunoblotted using standard western blotting techniques. To detect protein bands, blots were incubated with SuperSignal West Pico reagent (as per manufacturer’s guidelines; Pierce) then exposed to film (Amersham Hyperfilm MP, GE Life Sciences). Developed films were scanned and assembled in Adobe Illustrator CS5.1. Antibodies Primary antibodies used were: goat polyclonal anti-Zic3 N-19 (1:500 dilution for western blot; Santa Cruz Biotechnology, sc-28154), mouse monoclonal anti-HA (1:100 dilution for immunofluorescence, 1:4000 dilution for western blot; Sigma, H3663), mouse monoclonal anti-V5 (1:200 dilution for immunofluorescence, 1:3000 dilution for western blot; Life Technologies, R960-25), rabbit polyclonal anti-GFP (1:300 dilution for immunofluorescence, 1:1000 dilution for western blot; Cell Signaling, 2555), rabbit polyclonal anti-Lamin B1 (1:1000 dilution for immunofluorescence; Abcam, ab16048), mouse monoclonal anti-β-tubulin (1:1000 dilution for western blot; Abcam, ab7792), mouse monoclonal anti-TATA binding protein (TBP) (1:2000 dilution for western blot; Abcam, ab818) and goat polyclonal β-catenin C-18 (1:500 dilution for western blot; Santa Cruz Biotechnology, sc-1496). Secondary antibodies used for immunofluorescence (all at 1:500 dilution) were Alexa-Fluor-594-and Alexa-Fluor-488-conjugated donkey anti-mouse, anti-goat and anti-rabbit (Molecular Probes, Life Technologies). Secondary antibodies used for western blot (all at 1:5000 dilution) were horseradish peroxidase-conjugated rabbit anti-mouse, rabbit anti-goat and goat anti-rabbit (Zymed, Life Technologies). All antibodies were diluted in blocking buffer. Luciferase reporter assays HEK293T cells grown in 12-well tissue culture plates were transfected with the relevant combination of constructs. For ZIC trans-activation assays, a total of 1.2 μg of DNA was added per well: 0.6 μg of the Apoe reporter construct and either 0.6 μg of the expression construct or the negative control construct, pcDNA3.1/nV5-DEST™. For ZIC competition assays, a total of 1.2 μg of DNA was added per well: 0.4 μg of the Apoe reporter construct, 0.4 μg of the wild-type ZIC expression construct and 0.4 μg of either the competing katun expression construct or the pcDNA3.1/nV5-DEST™ vector when required to equalize the amount of transfected DNA. For the Wnt inhibition assays, a total of 1.5 μg of DNA was transfected per well: 0.5 μg of the TOPflash or FOPflash reporter vectors, 0.5 μg β-catenin-ΔN89 construct and 0.5 μg of the appropriate ZIC3 construct or pcDNA3.1/nV5-DEST™. To assess background Wnt activation levels, one well was transfected with 0.5 μg of the TOPflash or FOPflash reporter vectors and 1 μg of the pcDNA3.1/nV5-DEST™ vector. Either 8 hours (for the ZIC trans-activation and competition assays) or 5.5 hours (for the Wnt inhibition assays) post-transfection, cells were dissociated from the growth surface using 0.05 g/l trypsin (Life Technologies) and plated in triplicate onto a solid white tissue-culture treated 96-well plate (Costar®, CLS3917). To avoid any position bias error of the luminometer, sample order was randomized for each independent experimental repeat. The remaining cells were re-plated for SDS-PAGE and western blot analysis and lysed at the time of the reporter assay. Either 16 (for ZIC trans-activation and competition assays) or 19.5 hours (for Wnt inhibition assays) after re-plating, cells in each well were lysed by
incubation with 100 μl of a 1:1 dilution of luciferase substrate (ONE-
Glo™ Luciferase Assay System, Promega) with DMEM and the
luminescence from each well measured in a GloMax®-96 Microplate
Luminometer (Promega). The luciferase activity was normalized
to the pcDNA3.1/nV5-DEST™ negative control and the average
value and standard deviation calculated from the three internal
repeats. At least three independent experiments were performed
for each assay, with one representative experiment shown. For
statistical analysis, GenStat was used to perform ANOVA with
Fischer's unprotected post hoc test.

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The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS
J.N.A. and R.G.A. developed the concepts and approach, performed experiments,
analysed data and prepared the manuscript. N.W., H.M.W., H.M.B., K.S.B. and A.J.T.
analysed data and prepared the manuscript. N.W., H.M.W., H.M.B., K.S.B. and A.J.T.
performed experiments and analysed data. R.G.A. oversaw the project, developed
the concepts and approach, analysed data and prepared the manuscript.

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