Drosophila Nnf1 paralogs are partially redundant for somatic and germ line kinetochore function

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Abstract Kinetochores allow attachment of chromosomes to spindle microtubules. Moreover, they host proteins that permit correction of erroneous attachments and prevent premature anaphase onset before bi-orientation of all chromosomes in metaphase has been achieved. Kinetochores are assembled from subcomplexes. Kinetochore proteins as well as the underlying centromere proteins and the centromeric DNA sequences evolve rapidly despite their fundamental importance for faithful chromosome segregation during mitotic and meiotic divisions. During evolution of Drosophila melanogaster, several centromere proteins were lost and a recent gene duplication has resulted in two Nnf1 paralogs, Nnf1a and Nnf1b, which code for alternative forms of a Mis12 kinetochore complex component. The rapid evolutionary divergence of centromere/kinetochore constituents in animals and plants has been proposed to be driven by an intragenome conflict resulting from centromere drive during female meiosis. Thus, a female meiosis-specific paralog might be expected to evolve rapidly under positive selection. While our characterization of the D. melanogaster Nnf1 paralogs hints at some partial functional specialization of Nnf1b for meiosis, we have failed to detect evidence for positive selection in our analysis of Nnf1 sequence evolution in the Drosophilid lineage. Neither paralog is essential, even though we find some clear differences in subcellular localization and expression during development. Loss of both paralogs results in developmental lethality. We therefore conclude that the two paralogs are still in early stages of differentiation.

Keywords Meiosis · Centromere drive · Mis12 complex · Gene duplication · Kinetochore

Introduction

Centromeres are essential for faithful chromosome segregation during mitotic and meiotic divisions. Nevertheless, centromeres evolve rapidly. Rapid divergence occurs in centromeric DNA sequences and also in the associated centromere and kinetochore proteins (Fukagawa and Earnshaw 2014; Henikoff et al. 2001). Centromeric DNA sequences can be short as in Saccharomyces cerevisiae, where they conform to a 125-bp consensus sequence that is sufficient to specify centromere identity. In contrast, animal and plant species have centromeric DNA extending over many kilobases, either dispersed along holocentric chromosomes as in Caenorhabditis elegans or focused into regional centromeres as for example in Drosophila melanogaster and Homo sapiens. Moreover, centromeric DNA in animals and plants is usually highly repetitive. These rapidly evolving tandem repeat sequences are of little immediate importance for centromere function, as clearly demonstrated by the identification and characterization of human neocentromeres (Marshall et al. 2008; Voullaire et al. 1993). Some of the neocentromeres that were identified in...
human patients appear to be fully functional even though they are located in regions devoid of any alpha-satellite repeats, which are characteristically present within the normal centromeres of all human chromosomes. Epigenetic rather than DNA sequence-based centromere specification has been demonstrated experimentally in several organisms (Fukagawa and Earnshaw 2014; Karpen and Allshire 1997).

The fact that centromere-specific chromatin rather than a specific DNA sequence motif marks centromere identity in most eukaryotes explains the divergence of centromeric DNA sequences to some extent. However, it does not clarify immediately why centromere proteins are also strongly diverged (Meraldi et al. 2006; Schleiffer et al. 2012). For example, a most important centromere protein is the centromere-specific histone H3 variant named Cenp-A in humans. While orthologs can be identified throughout the eukaryotic domain of life, their sequences are far less conserved than those of the canonical histone H3 proteins (Henikoff et al. 2000; Talbert et al. 2004). Moreover, several species including some kinetoplastids (Akiyoshi and Gull 2014; Berriiman et al. 2005) and holocentric insects (Drinnenberg et al. 2014) have lost the centromere-specific histone H3 gene. This loss is even more surprising, given that Cenp-A and the orthologous proteins of other species are assembled into centromere-specific nucleosomes that appear to function as epigenetic centromere mark (Fachinetti et al. 2013; Guse et al. 2011; Hori et al. 2013; Mendiburo et al. 2011; Westhorpe and Straight 2015). In addition, Cenp-A starts a recruitment cascade that allows assembly of kinetochore proteins at the start of M phase. As in the case of Cenp-A, the additional centromere and kinetochore proteins in general are characterized by limited sequence conservation and some lineage-specific gene losses. 

D. melanogaster provides a well-studied example illustrating the evolutionary plasticity of centromere and kinetochore organization. All 16 centromere proteins of the so-called constitutive centromere-associated network (CCAN) with the exception of Cenp-C are absent in the fly (Heeger et al. 2005; Przewloka et al. 2007; Schittenhelm et al. 2007; Westermann and Schleiffer 2013). Cenp-C, which binds directly to Cenp-A/Cid, recruits the kinetochore proteins of the KMN network composed of Knl1/Spc105 and the heterotetrameric Mis12 and Ndc80 complexes. Several features of the KMN network are distinct in Drosophila. Drosophila Spc105 has divergent MELT repeats even though canonical repeats have been shown to be essential for the binding of the spindle assembly checkpoint proteins in yeast and mammals (London et al. 2012; Primorac et al. 2013; Schittenhelm et al. 2009; Shepperd et al. 2012). Kmn2, the putative Drosophila Spc24 homolog (Schittenhelm et al. 2007), lacks the characteristic coiled-coil region present in other organisms, where it is crucial for integration into the Ndc80 complex. Moreover, the Drosophila Mis12 complex lacks the Dsn1 subunit present in yeast and vertebrates (Przewloka and Glover 2009; Przewloka et al. 2007; Schittenhelm et al. 2007). Overall, in comparison to mammals, Drosophila has evolved a far simpler centromere and kinetochore structure. The main microtubule binding activity at the kinetochore, the Ndc80/Nuf2 heterodimer, is linked to centromeric Cenp-A/Cid nucleosomes uniquely via Cenp-C and a variant KMN network, while other eukaryotes use CCAN components as an additional platform for KMN recruitment (Gascoigne et al. 2011; Kim and Yu 2015; Klare et al. 2015; Malvezzi et al. 2013; Nishino et al. 2013; Rago et al. 2015; Schleiffer et al. 2012).

The rapid divergence of both centromeric DNA sequences and associated proteins has been proposed to involve an evolutionary conflict in animals and plants (Henikoff et al. 2001; Malik and Henikoff 2009). The proposed intragenome conflict appears to arise in these species because the female sex transmits only one of the four meiotic haploid products to the next generation. Centromere sequence variants that induce their preferential meiotic segregation into the female pronucleus are predicted to increase their abundance in the population very rapidly. Selfish centromere variants might result from expansion of existing or new centromeric satellite arrays enabling enhanced recruitment of centromere proteins or spindle microtubules. Although centromeric DNA repeats are not absolutely essential for centromere function, it seems very likely that they exert some influence, given their pervasive presence in established euchromatic centromeres (Fukagawa and Earnshaw 2014). Spindle assembly during female meiosis is often acentrosomal, as also in Drosophila, where microtubules accumulate around meiotic chromosomes, thereby providing opportunities for selfish centromeres to bias their orientation within the asymmetric spindles via associated proteins that affect microtubule behavior. As the expansion of a selfish centromere within a population is likely to have negative effects, including retention of linked deleterious mutations, positive selection is predicted to favor centromere/kinetochore protein variants that specifically suppress the strength of an expanding selfish centromere (Henikoff et al. 2001; Malik and Henikoff 2009). Intriguingly, the centromere-specific histone H3 variants of Drosophila, primates, fish, and plants, but not of budding yeast where meiosis is always symmetric, appear to have evolved under positive selection. Additional centromere proteins have been found to evolve under positive selection (Abbey and Kral 2015; Axellsson et al. 2010; Beck and Llopart 2015; Cooper and Henikoff 2004; Finseth et al. 2015; Malik and Henikoff 2001; Schueler et al. 2010; Talbert et al. 2002, 2004; Yuan et al. 2015). Moreover, although still few, cases of centromere drive have been clearly documented in the plant Mimulus (Finseth et al. 2015; Fishman and Saunders 2008) and in the mouse (Chmatal et al. 2014).

Centromere drive might also be relevant for the retention of duplicated Nnf1 gene copies within the D. melanogaster subgroup (Schittenhelm et al. 2007). Nnf1 (necessary for nuclear function 1) was originally identified in budding yeast and subsequently shown to be a component of the Mis12 complex
(Shan et al. 1997; Westermann et al. 2003). The Mis12 complex subunits Nsl1 and Dsn1 form direct contacts with Knl-1/ Spec105 and the Spec24/25 subunits of the Ndc80 complex (Malvezzi et al. 2013; Petrovic et al. 2010, 2014). By binding to Cenp-C, the Mis12 complex recruits the KMN network to the centromere (Przewloka et al. 2011; Scegman et al. 2011). Drosophila Nnf1 binds directly to Cenp-C in vitro (Przewloka et al. 2011). Cross-linking analyses in yeast have revealed additional interactions of Mis12 complex subunits and Cenp-C (Hornung et al. 2014). An initial comparison of the expression pattern of the two paralogous Nnf1 genes in D. melanogaster (Schittenhelm et al. 2007) suggested that Nnf1b might be germ line-specific, raising the possibility that it provides a meiosis-specific function. Accordingly, this but not the other paralog might evolve under positive selection when engaged in suppression of a hypothetical centromere drive during female meiosis. To address these possibilities, we have further characterized the two Drosophila Nnf1 paralogs. Beyond evolutionary sequence analyses, we have generated null mutations and report single- and double-mutant phenotypes. Our analyses reveal a partial functional specialization of the Nnf1 paralogs without evidence for strong positive selection.

**Materials and methods**

**Drosophila genetics**

Df(2R)Exel6070 and Df(2R)Exel7164 (Parks et al. 2004) were obtained from the Bloomington Drosophila Stock Center. PCR assays using the primer pair AB3 (5′-TCGCAACCACAAAGTCAAC-3′) and AB4 (5′-AACGGCTTTTGGCCATAGC-3′) were used for confirmation that Df(2R)Exel7164 deletes Nnf1a. These primers anneal to regions flanking the deficiency break point and amplify a 2-kb fragment only when the deficiency is present in genomic template DNA.

PBac{SAstopDsRed}4L02791 (Schuldiner et al. 2008) was obtained from the Drosophila Genetic Resource Center (DGRC, Kyoto Institute of Technology). Information on the transposon insertion site provided by FlyBase and DGRC were not fully consistent. Our analyses by PCR and sequencing revealed an insertion site in the second TTAA motif within intron 1 of Nnf1a.

Two deficiencies that delete Nnf1b were isolated. Df(2L)MK01 was generated as described (Parks et al. 2004) by FLP-mediated recombination between the two FRT transposon insertions P{XP}d00346 and PBac{WH}f01300 (Thibault et al. 2004), which were obtained from the Exelixis Collection at the Harvard Medical School. For the isolation of Df(2L)NK01, we mobilized the transposon insertion P{SUPor-P}KG07276 (Bloomington Drosophila Stock Center, no. 14327; Bellen et al. 2004), which is marked with a mini-w+ gene, by crossing with y′ w∗; CyO, H{w+mC ΔPDelta2-3}HoP2.1Bc (Bloomington Drosophila Stock Center, no. 2078). Single white-eyed progeny males obtained from a cross of w∗; P{SUPor-P}KG07276/CyO, H{w+mC ΔPDelta2-3}HoP2.1Bc with w+; If/CyO females were used to establish 250 lines after backcrossing to w∗; If/CyO females. None of the w+ revertant second chromosomes present in these lines was free of recessive lethal mutations, suggesting the presence of such second site mutations on the starting P{SUPor-P}KG07276 chromosome. Genomic DNA isolated from balanced w+ revertant flies was analyzed with a multiplex PCR using the three primers OZH47 (5′-TCGCAACCACAAAGTCAAC-3′), OZH48 (5′-CCTCCAGAAGAAGACGA-3′), and CL18 (5′-CGCAGGTACCTTTAGTTATTCATATTG-3′) for the identification of imprecise excision events. These primers amplify three fragments of different length from P{SUPor-P}KG07276/CyO, one from the balancer and two from the transposon chromosome which include the regions flanking the transposon insertion on the left and right sides, respectively. Revertant lines, where specifically the flanking fragment on the Nnf1b side could no longer be amplified, were further characterized. In case of Df(2L)NK01, additional characterization by PCR and sequencing revealed the presence of a deletion with break points within the P{SUPor-P} transposon just upstream of Nnf1b and within the second exon of the Dbp21E2 gene just downstream of Nnf1b. To confirm the absence of Nnf1b gene in w+; Df(2L)MK01, gDSP II.2/Df(2L)NK01, gDSP II.2 flies, a multiplex PCR with the Nnf1b-specific primers NT60 (5′-CTGCAACCACAAAGTCAAC-3′) and NT53 (5′-TTAAATGCTTTTGCCCATAGC-3′) in combination with the primers AB1 (5′-TTCGACCTGCAACCACAAAGTCAAC3′) and NT58 (5′-CTGCAACCACAAAGTCAAC-3′) to amplify a control fragment from Nnf1a, were used. In combination with wild-type genomic DNA, this multiplex PCR results in the amplification of a 826-bp Nnf1a and a 388-bp Nnf1b fragment.

For Nnf1a Nnf1b double-mutant analysis, we generated stocks with second chromosomes obtained by standard meiotic recombination with w+; Df(2L)MK01, gDSP II.2, PBac{SAstopDsRed}4L02791/CyO and w+; Df(2L)NK01, gDSP II.2, Df(2R)Exel7164/CyO. Twenty-five percent of the zygotes generated by a cross between these two stocks have the genotype w+; Df(2L)MK01, gDSP II.2, PBac{SAstopDsRed}4L02791/Df(2L)NK01, gDSP II.2, Df(2R)Exel7164 and therefore neither zygotic Nnf1a nor Nnf1b function. Stocks with a CyO version carrying P{Dfd-GMR-nYFP} (Le et al. 2006) were used for the identification of double-mutant embryos and larvae based on absence of EYFP fluorescence in the head region.

Lines carrying the gDSP, g-Nnf1a, and g-Nnf1b transgenes were generated by standard germ line transformation using the...
pCaSpeR-4 constructs described below. Lines carrying the transgenes g-EGFP-Nnf1a, g-Nnf1a-EGFP, g-EGFP-Nnf1b, and g-Nnf1b-EGFP were obtained by integrating the attB constructs described below into the attP landing site PBac[y']-attP-9A/VK00020 (Venken et al. 2006). Lines carrying the transgenes ga-EGFP-Nnf1a and ga-EGFP-Nnf1b were made by integrating the pattB constructs described below into the attP landing site P[Car]pattP2 (Groth et al. 2004).

Crosses for phenotypic analyses were performed at 25 °C. w1 was used as wild-type control. To assess male fertility, single males of a given genotype were crossed to three w1 virgin females. For examination of female fertility, three virgins of a genotype were pooled and crossed to three w1 males. In both cases, ten replicate crosses were set up. Flies were allowed to mate for 2 days, then transferred to a fresh vial, and discarded after two more days. The eclosing adult progeny from the second vial was counted for 8 days. For X chromosome nondisjunction (X-ND) tests, we used the stock C(1;Y)1, y v B: y+/C(1)RM, y2 su(w1) w (Bloomington Drosophila Stock Center, no. 700). Males with a compound XY chromosome were crossed to Nnf1bnull females. Regular gametes produced by these females develop into either XXY females expressing Bar eyes or X0 males with normal eyes after fertilization with XY or 0 sperm, respectively. Irregular nullo-X gametes produced by females after X-ND develop into XY males with Bar eyes after fertilization with XY sperm. Moreover, irregular diplo-X gametes resulting from X-ND in females develop into adult females with normal eyes after fertilization with 0 sperm. In contrast, XXXY gametes resulting from X-ND in females develop into either XXY females expressing Bar eyes and males with normal eyes after fertilization with XY or 0 sperm, respectively. Irregular nullo-X gametes produced by females after X-ND develop into XY males with Bar eyes after fertilization with XY sperm. Therefore, to determine the rate of X-ND, we multiplied the number of irregular adult progeny (males with Bar eyes and females with normal eyes) by two before dividing by the total number of adult progeny.

Plasmids

A pCaSpeR-4 construct was made for the generation of gDSP transgenic flies. The pCaSpeR-4-dDSP construct contains a 6.3-kb genomic fragment with the genes Dhp21E2, Saq6, and Pex12 that are deleted in Df(2L)MK01 apart from Nnf1b. The first part of this genomic region was amplified using the primers OZH-20 (5'-ATATGGTACCCTTC GATTGGGTTAGCTATGGCC-3') and OZH-21 (5'- ATATCCGGGTTAGCTATGGCCTTG-3') from BAC16101 (Hoskins et al. 2000), which contains an insert fragment of the corresponding Drosophila genome region. After digestion with Acc65I and Smal, the fragment was inserted into the corresponding sites of pSLfa1180fa (Horn et al. 2000). The resulting first cloning intermediate was then digested with PacI and Smal for insertion of the second part of the genomic region which was isolated from the same BAC with primers OZH-22 (5'-CTACTGCTTACACTGATA TACC-3') and OZH-23 (5'-ATATCCGGGTTAGCTTG CAGTTTTTGCGCTG-3') and digested with the same restriction enzymes. The complete 6.3-kb genomic region was released with Acc65I and Smal from the second cloning intermediate and inserted into the Acc65I and Hpal sites of pCaSpeR-4.

We also generated pCaSpeR-4 constructs for the production of g-Nnf1b and g-Nnf1a transgenic flies. Primers AB26 (5'-TTTTGGCGCCGCAATCGATGCTGGAAGGCTTG-3') and AB31 (5'-CAATTCTAGACATTGCGCAGGAGGCTTG-3') were used for the enzymatic amplification of the Nnf1b genomic region from pFlyFos030346 (Ejsmont et al. 2009). After digestion with BamHI and Xbal, the fragment was inserted into the corresponding sites of pCaSpeR-4. A 1.8-kb fragment with Nnf1a was amplified from w1 genomic DNA using the primers CL126 (5'-TTTTGGCGCCGCAATCGATGCTGGAAGGCTTG-3') and CL127 (5'-GACAATG GTACCAGTTTAAAATAATTGTAATGTC-3'). After digestion with NotI and Acc65I, the fragment was inserted into the corresponding sites of pCaSpeR-4. Characterization of construct and w1 genomic DNA by sequencing revealed some genetic polymorphisms, where the w1 genome sequence differs from the FlyBase genome sequence.

For the generation of attB transgene constructs allowing expressions of Nnf1a and Nnf1b with N- or C-terminal EGFP extension, we first derived the vectors pattB-ORF-EGFP and pattB-EGFP-ORF from pattB (Bischof et al. 2013). In case of pattB-ORF-EGFP, the primers AB17 (5'-GACTCGCCCGACATGGGAGCAAGGCTTG-3') and AB19 (5'-GACTTCTAGACATTGCGCAG GCTAGCTGCTG-3') were used for the enzymatic amplification of the EGFP coding region (with stop codon), followed by digestion with NotI and Xbal and insertion into the corresponding sites in pattB. In case of pattB-EGFP-ORF, the primers AB17 and AB18 (5'-GACTCTAGACATTGCGCAG GCTAGCTGCTG-3') were used for the enzymatic amplification of the EGFP coding region (without stop codon), followed by digestion with NotI and Xbal and insertion into the corresponding sites in pattB. For the construction of pattB-g-Nnf1a-EGFP, the 3' flanking region of Nnf1a was first amplified with primers AB24 (5'-GAAGTCAAGGACTTTTACCTTACAAACTT AATGTTGCTACCACTTG-3') and AB25 (5'-GAAGTCAAGGACTTTTACCTTACAAACTT AATGTTGCTACCACTTG-3') from pCaSpeR-4-g-Nnf1a and cloned into pattB-ORF-EGFP using Xhol and Xbal. The Nnf1a gene and upstream sequences were then inserted using BamHI and NotI after enzymatic amplification with the primers AB20 (5'-GAAGTCAAGGACTTTTACCTTACAAACTT AATGTTGCTACCACTTG-3') and AB23 (5'-TTAAAGGCGCGCGA GTACGTTCAATGCTCGG-3').
For the construction of pattB-g-EGFP-Nnf1a, the Nnf1a gene and 3′ flanking sequences were amplified from pCasPeR-4-g-Nnf1a with the primers AB22 (5′-AATTTCCAGGATTGAGATCGGCAAGC-3′) and AB25 (5′-GACTCTGAAATTTAATTGTGTAATGTCAATG-3′) and cloned into the corresponding sites of pattB-EGFP-ORF using Xhol and Xbal. The Nnf1a upstream sequences were then inserted using BamHI and NotI. EGFP-Nnf1b is fused to Nnf1a 3′ region from pattB-g-EGFP-Nnf1a using NotI and XbaI. The Nnf1a 3′ primers AB17 and AB90, which is 199 bp long and introduces enzymatic amplification from pattB-g-EGFP-Nnf1b using the primers AB26 (5′-CAATTCTAGACATTTGCCGGCAAGT-3′) and AB27 (5′-GAATTCTCGAGATGGAGGATTCGGAAGC-3′) were amplified with AB26 and AB27 (5′-TAAGCGGCCGACCAATTTGCTCTCAG-3′) and inserted using BamHI and NotI.

For the construction of pattB-g-Nnf1b-EGFP, the Nnf1b 3′ flanking region was amplified from psFlyFos030346 with AB28 (5′-TGGTTGTCGACATTGAATATTTGCA-3′) and AB31 (5′-CAATTCTAGACATTTGCCGGCAAGT-3′) and cloned into pattB-ORF-EGFP using Xhol and Xbal. The Nnf1b gene and 5′ upstream sequences were then amplified with the primers AB26 (5′-TTAGCGGCCGCAACCATTTCGCAACATG-3′) and AB29 (5′-TTAGCGGCCGCAACCATTTCGCAACATG-3′) and inserted using BamHI and NotI.

For the construction of pattB-g-EGFP-Nnf1b, the Nnf1b gene and 3′ flanking sequences were amplified from psFlyFos030346 with AB28 (5′-TGGTTGTCGACATTGAATATTTGCA-3′) and AB31 (5′-CAATTCTAGACATTTGCCGGCAAGT-3′) and cloned into pattB-ORF-EGFP using Xhol and Xbal. The Nnf1b gene and 5′ upstream sequences were then amplified with AB26 and AB27 (5′-TAATACGGCGCCGATTTCCATTTCTCAG-3′) and inserted using BamHI and NotI.

For the construction of pattB-ga-EGFP-Nnf1b, we first removed the region coding for EGFP-Nnf1a and the 3′ flanking region from pattB-g-EGFP-Nnf1a using NotI and Xbal. The deleted region was then replaced with a fragment where EGFP-Nnf1b is fused to Nnf1a 3′ sequences obtained by enzymatic amplification from pattB-g-EGFP-Nnf1b using the primers AB17 and AB90, which is 199 bp long and introduces the Nnf1a 3′ sequences (UTR and flanking region).

The correctness of the transcribed region in all the final attB constructs was confirmed by sequencing.

qRT-PCR

Total RNA was isolated from 0–2-h embryos collected from either PBac{SstopDsRed}L02797, PBac{SstopDsRed}L02797/CyO, w*; Df(2L)MK01/Df(2L)NK01; gDSP III.2/+, or w+ control flies. Of the embryos for each genotype, 300–1500 were used for RNA isolation with TRIzol (Invitrogen) followed by DNase digestion (DNA-Free Kit, Ambion). Complementary DNA (cDNA) synthesis was performed with 500 ng RNA per reaction using a Transcriptor High-Fidelity cDNA Synthesis Kit (Roche). Real-time quantitative PCR was performed using SYBR Green and an Applied Biosystems 7900HT using the recommended two-step cycling protocol with the following primer pairs: AB1 (5′-TTCAATGAGGATTGGAAGC-3′) and NT46 (5′-TCAATGCGCAAGATTTGGTT-3′), NT47 (5′-TAAACAATTTTGCGACCTTG-3′) and NT48 (5′-TGAGGTTAATTTGCGCTATC-3′), NT47 and AB2 (5′-TCTACGATGCTTTCCGTT-3′), NT50 (5′-CATGCCGATTTGTGACTCT-3′) and NT51 (5′-GGCATTGGGACATTGCTCTT-3′), and NT57 (5′-GAGCCTGGGACACACGTGCACACT-3′) and NT61 (5′-TCCTCGAGGGAGACAGATT-3′). For normalization, three primer pairs (sequences provided upon request) were used for amplification of transcripts from the genes act5C, alphaTub84B, and Tbp. At least three replicates were performed.

Immunofluorescence and immunoblotting

Eggs were collected from crosses that generate Nnf1a Nnf1b double mutants on apple agar plates at 25 °C for 3 h, followed by ageing for 13 h before fixation with 4 % formaldehyde in phosphate-buffered saline (PBS) and devitellinization. For analyses of embryos during the syncytial blastoderm stages, we collected eggs for 1 h following by ageing for an additional hour before methanol fixation and devitellinization. Embryos were stained with a rabbit anti-serum against Drosophila cyclin B (1:2000; Jacobs et al. 1998) and a DNA stain (Hoechst 33258, 1 μg/ml) according to standard procedures. Ovaries and testes were dissected in testis buffer (183 mM KCl, 47 mM NaCl, and 10 mM Tris–HCl pH 6.8) and fixed in 4 % formaldehyde in PBS and 0.1 % Triton X-100 for 10 min. DNA was stained with Hoechst 33258 (1 μg/ml) in PBS and 0.1 % Triton X-100 for 10 min. Samples were washed twice with PBS and mounted in 70 % glycerol, 50 mM Tris pH 8.5, 10 mg/ml propyl gallate, and 0.5 mg/ml phenylenediamine. Testis squash preparations were prepared essentially as described previously (White-Cooper 2004). For quantification of the kinetochore signals in synecytial embryos, image stacks of 15 focal planes with 300-nm spacing that contained the nuclear layer were acquired with a 100×/1.4 objective and a Cell Observer HS (Zeiss) microscope. For quantification of the kinetochore signals in prometaphase I cells in testes, focal planes with 250-nm spacing were acquired with a 63×/1.4 objective. After maximum projection, kinetochore signals were quantified as described previously (Schipper et al. 2010). Maximum intensity projections are displayed in the figures except where stated differently. Immunoblotting was performed using anti-EGFP (Schittenhelm et al. 2007) at 1:4000 and anti-PSTAIR (Sigma, P7962) at 1:4000. Peroxidase-coupled goat antibodies (Jackson Immuno Research) against mouse or rabbit IgG were used as secondary antibodies at 1:2000 and detected
using Amersham ECL (GE Healthcare). Quantification of band intensities was performed as previously described (Radermacher et al. 2014).

Phylogenetic reconstruction

We obtained the coding DNA sequences of Nnf1 from 19 different Drosophila species using BLAST searches of their annotated genomes. Nine species predate the duplication of Nnf1 (D. ananassae, D. pseudoobscura, D. persimilis, D. willistoni, D. mojavensis, D. virilis, D. grimshawi, D. kikkawai, and D. pectinata). In the other ten species, Nnf1 has been duplicated and their genomes harbor two copies of the gene (D. simulans, D. sechellia, D. melanogaster, D. yakuba, D. erecta, D. eugracilis, D. biarmipes, D. takahashii, D. elegans, and D. rhopaloa). We aligned the 29 Nnf1 coding sequences using the PRANK algorithm (Löytynoja and Goldman 2008) and employed a recent whole-genome phylogeny as a guide tree for the aligner (Seetharam and Stuart 2013). We removed all nucleotide sites with gaps from the resulting codon-based nucleotide alignment using trimAl v1.2 (Capella-Gutierrez et al. 2009), which resulted in an alignment with 507 aligned nucleotides (or 169 aligned codons; Online Resource 1, Supplementary Fig. 1). We then used MEGA v6.06 to calculate the mean pairwise nucleotide and amino acid distance between the paralogs Nnf1a and Nnf1b (Tamura et al. 2013). To compute nucleotide distances, we used the maximum likelihood composite model (Tamura et al. 2004), while for amino acid distances, we used a Poisson’s correction (Zuckerkandl and Pauling 1965). We then used PhyML v3 (Guindon et al. 2010) with 1000 bootstrap replicates to build a gene tree through phylogenetic inference by maximum likelihood. Following the Akaike information criterion in jModelTest v2.1.7, we chose the nucleotide substitution model TIM1 (“transition model”) + I + Γ (Posada 2008). We rooted the tree according to the known phylogeny for Drosophila species (Seetharam and Stuart 2013). All downstream analyses of selection are based on the gene tree resulting from this procedure.

Analyses of selection

To explore the selective pressures in the different lineages of the Nnf1 tree, we estimated the ratio ω of the nonsynonymous and synonymous substitution rates (ω = dN/dS). To perform these analyses, we employed three classes of codon substitution models, the branch models, the site models, and the branch-site models that we implemented using the codeml program from the package PAML v4.7 (Yang 2007). For all three models, we used likelihood ratio tests (LRTs) to compare a complex model against a simpler nested model (Huelsenbeck and Crandall 1997; Yang 1998). We computed twice the difference in the log-likelihoods (2Δℓ) of the two nested models and compared its value against a χ² distribution, with the degrees of freedom being the difference in the number of estimated parameters between the two models. In all these analyses, we estimated the codon frequencies empirically, using the codon model F3x4. Also, we estimated all branch lengths of the gene tree from the initial values obtained with the one-ratio M0 model (Goldman and Yang 1994). This simple model assumes a single ratio ω for all tree branches and all codon sites in the sequence alignment.

In our phylogenetic analysis, we first used branch models (Yang 1998), which allow us to estimate different ω ratios for different branches in the phylogeny. However, for a given branch, these models do not allow variation in ω among different codon sites. Second, we used site models (Nielsen and Yang 1998; Wong et al. 2004; Yang et al. 2000, 2005), which do allow ω to vary among sites but not among branches. We used these models to test if specific sites were affected by positive selection during the evolution of Nnf1. Among these kinds of models, the neutral model M1a specifies the following two classes of codon sites: conserved sites under purifying selection (ω < 0) and neutral sites (ω = 0). We compared this model against the M2a model, which allows an additional class of codon sites that are under positive selection (ω > 1) (Nielsen and Yang 1998; Wong et al. 2004; Yang et al. 2000). We also compared the neutral model M7, where ω varies among codons according to a beta distribution, to the model M8, which includes an additional codon class subjected to positive selection (Yang et al. 2000). Finally, we also used branch-site models (Yang and Nielsen 2002; Zhang et al. 2005), which allowed us to ask whether particular sites evolve under positive selection for specific branches (foreground branches, while the others are named background branches). For the branch and branch-site models, we divided the gene tree of Nnf1 into pre-duplication branches (assumed to evolve at a rate ω₀), branches immediately following the duplication event (ω₁), and branches of the clades Nnf1a (ω₂) and Nnf1b (ω₃).

Results

Nnf1b is not essential

The two Nnf1 paralogs of D. melanogaster appear to have arisen after duplication of a primordial Nnf1 gene during evolution of the melanogaster species group presumably about 10 million years ago (Schittenhelm et al. 2007; see also below). The genomic location of the Nnf1a paralog on chromosome 2R corresponds to that of the primordial Nnf1 gene according to gene synten analysis, while Nnf1b is in a distinct region on chromosome 2L. The exon-intron structure of the two Nnf1 paralogs is identical, indicating that the duplication event did not involve an mRNA intermediate. Published data
Concerning the expression pattern of Nnf1b during Drosophila development indicate maximal transcript levels in ovaries of adult females and at the earliest embryonic stages (Chintapalli et al. 2007; Graveley et al. 2011; Schittenhelm et al. 2007). In contrast, Nnf1a transcript levels are minimal at the earliest embryonic stages, but they increase later during embryogenesis when Nnf1b transcripts rapidly vanish. Therefore, Nnf1b appears to be the predominant form present during female meiosis, and the fixation of the Nnf1b paralog during evolution might reflect functional specialization for support of meiosis-specific kinetochore behavior. The success of meiosis relies on specific control of sister kinetochore autonomy. At the onset of the first meiotic division, the two sister kinetochores behave as a functional unit. Their co-orientation in the meiosis I spindle allows for reductional segregation of homologs to opposite poles. In contrast, during all other divisions (meiosis II and mitosis), sister kinetochores behave as autonomous units, allowing their bi-orientation within the spindle. The control of sister kinetochore behavior during meiosis is poorly understood, and a functional specialization of Nnf1b in this context would be of great interest. To address Nnf1b function, we generated zygotes lacking Nnf1b for phenotypic analyses.

For elimination of Nnf1b, we first generated a small deficiency, Df(2L)MK01, using FLP/FRT-mediated recombination between flanking transposon insertions carrying FRT sites (Parks et al. 2004). Beyond Nnf1b, Df(2L)MK01 deletes the three downstream genes Dhp21E2, Saf6, and Pex12 (Fig. 1a). To restore these three genes, we generated a transgene (gDSP) carrying an appropriate genomic fragment (Fig. 1a).

![Diagram](image)

**Fig. 1** Nnf1b is not essential for viability and fertility. a Scheme of the Nnf1b region in the Drosophila melanogaster genome. Nnf1b and the three downstream genes Dhp21E2, Saf6, and Pex12 are shown (open boxes untranslated regions and filled boxes coding regions), as well as insertion positions of transposons (triangles) used for the generation of the deficiencies Df(2L)MK01 and Df(2L)NK01 (black bars). The genomic region present in the gDSP transgene is indicated as well (open bar). b A multiplex PCR assay confirmed absence of Nnf1b in Nnf1bnull-2 mutant flies. Products amplified from w control (+) or Nnf1bnull-2 (null-2) genomic DNA or in the absence of template DNA (H2O) were analyzed by agarose gel electrophoresis. A molecular weight marker (M) was used for size comparison of the fragments amplified from Nnf1a and Nnf1b (arrowheads). e Loss of Nnf1b does not interfere with development to the adult stage. Homozygous Df(2L)MK01, gDSP II.2 females were crossed with males that were either Df(2L)NK01/CyO (null-1) or Df(2L)NK01, gDSP II.2/CyO (null-2), and the number of adult progeny with (+) and without (−) CyO was determined and is displayed (percent of total adult progeny). d Fertility in the absence of Nnf1b is normal. The fertility of males and females with the genotypes w for control (+), Nnf1bnull-1 (null-1), or Nnf1bnull-2 (null-2) was determined. Fertility of controls was set to 100 %. Relative average (bars) and standard deviation (whiskers) are displayed (n=10). e Loss of Nnf1b does not increase the rate of X chromosome nondisjunction (X-ND) during female meiosis. The rate of X-ND (in percent of total meioses) in females with the genotypes w for control (+), Nnf1bnull-1 (null-1), or Nnf1bnull-2 (null-2) was determined (see “Materials and methods” section). Based on distinct adult visible phenotypes, males (m regular) and females (f regular) resulting from euploid oocytes as well as adult progeny (irregular) indicating X-ND were scored and counted.
addition, we isolated Df(2L)NK01, an even smaller deficiency, as imprecise excision after mobilization of a P element insertion upstream of Nnf1b (Fig. 1a). This second deficiency deletes Nnf1b completely and Dhp21E2 partially (Fig. 1a). To assess the loss-of-Nnf1b phenotype, we arranged a cross generating the genotype Df(2L)MK01, gDSP II.2/

**Fig. 2** Nnf1a is not essential for viability and fertility. **a** Scheme illustrating the structure of the Nnf1a gene (open boxes untranslated regions and filled boxes coding regions) as well as the insertion position of the PBacLL02791 transposon (triangle), which includes a splice acceptor site (SA) followed by stop codons (stop) in all three reading frames. Primer pairs used for the analysis of the effects of the transposon insertion on the Nnf1a transcript splice pattern are indicated by colored arrows. **b** The PBacLL02791 insertion prevents production of normally spliced Nnf1a transcripts. RNA isolated from embryos during the syncytial stages was analyzed by qRT-PCR with the primer pairs illustrated in **a** and an additional Nnf1b-specific primer pair (magenta). The isolated RNA is of maternal origin. The mothers had the genotype w for control (+), PBacLL02791/CyO (PBac/CyO), PBacLL02791 (PBac), or Df(2L)MK01/Df(2L)NK01; gDSP III.2/+ (Nnf1b null). Product amount detected in PBac/CyO with a given primer pair was set to 100 %. Relative average (bars) and standard deviation (whiskers) are displayed (n ≥ 3). nd not determined. e Loss of Nnf1a does not interfere with development to the adult stage. PBacLL02791/CyO virgin females were crossed with males that were either PBacLL02791/CyO (PBac), Df(2R)Exel6070/CyO (Df6070), or Df(2R)Exel7164/CyO (Df7164), and the number of adult progeny with (+) and without (−) CyO was determined and is displayed (percent of total adult progeny). **d** Fertility is not affected severely by absence of Nnf1a. The fertility of males and females with the genotypes w for control (+), PBacLL02791 (PBac), PBacLL02791/Df(2R)Exel6070 (Df6070), or PBacLL02791/Df(2R)Exel7164 (Df7164) was determined. Fertility of controls was set to 100 %. Relative average (bars) and standard deviation (whiskers) are displayed (n = 10)
Nnf1a is not essential

The apparent absence of abnormalities resulting from complete elimination of Nnf1b gene function might reflect redundancy. To address potential functional overlap with Nnf1a, we determined whether the transposon insertion PBac[SSTOPDSRed]^{LL02791} eliminates Nnf1a gene function (Fig. 2a). The transposon was found to be integrated within the first intron. As the transposon includes a splice acceptor site followed by stop codons in all three frames (Schuldiner et al. 2008), it is expected to cause premature termination after translation of only the N-terminal 17% of the normal full-length protein. To evaluate whether the splice acceptor site within the transposon is used indeed as expected, we performed quantitative RT-PCR experiments (Fig. 2b). As expected, in transposon heterozygotes, we detected wild-type transcripts as well as transcripts containing the transposon-derived exon. In contrast, in transposon homozygotes, we detected exclusively the latter transcripts and in wild-type controls, only the former transcripts. We conclude that the transposon insertion precludes expression of normal transcripts, and therefore, it appears to be a null allele.

To assess phenotypic consequences resulting from elimination of Nnf1a, we crossed balanced females heterozygous for PBac[SSTOPDSRed]^{LL02791} with balanced males of the same genotype, as well as with balanced males heterozygous for Df(2R)Exel6070 or Df(2R)Exel7164 which both delete Nnf1a. Adult progeny homozygous or hemizygous for PBac[SSTOPDSRed]^{LL02791} were obtained with frequencies very close to their Mendelian ratio (Fig. 2c), indicating that their viability is similar to that of the balanced siblings. Compared to hemizygotes, the homozygous flies were weaker and had a shorter life span, arguing for some second site effects in PBac[SSTOPDSRed]^{LL02791} homozygotes (Online Resource 1, Supplementary Fig. 2). However, also hemizygous flies had a life span that was only about half of that of control flies. To evaluate the importance of Nnf1a for fertility, we crossed homozygous and hemizygous PBac[SSTOPDSRed]^{LL02791} flies with w\textsuperscript{1} control flies. Based on progeny counts, hemizygous males lacking Nnf1a were fully fertile. Female fertility appeared to be at most slightly reduced in PBac[SSTOPDSRed]^{LL02791}/Df(2R)Exel7164 flies, as the observed reduction was not statistically significant. The somewhat stronger effects observed in case of PBac[SSTOPDSRed]^{LL02791}/Df(2R)Exel6070 and even more in case of PBac[SSTOPDSRed]^{LL02791} homozygous females (0.3% in w control, 0.3% in PBac[SSTOPDSRed]^{LL02791}/Df(2R)Exel6070, 0.5% in PBac[SSTOPDSRed]^{LL02791}/Df(2R)Exel7164, and 2.2% in PBac[SSTOPDSRed]^{LL02791}; n > 600). We conclude that Nnf1a is not absolutely essential for development into fertile adults, but vitality and perhaps to a minor extent also female fertility are no longer fully normal.

Nnf1a and Nnf1b have overlapping functions

The development of fertile adults in the absence of either Nnf1a or Nnf1b function might be explained by functional overlap among the two paralogs. To address redundancy, we analyzed double mutants (Df(2L)MK01, gDSP II.2, PBac[SSTOPDSRed]^{LL02791}/Df(2L)NK01, gDSP II.2, Df(2R)Exel7164). Double mutants did not develop to the adult stage. The large majority of the double mutants (between 80 and 97%, n > 50) still completed embryogenesis and reached the early larval stages presumably by exploiting maternally provided Nnf1. Analysis of double-mutant embryos revealed that they were no longer fully normal at late embryonic stages, during which abnormalities were clearly detectable within the central nervous system (CNS; Fig. 3). During wild-type embryogenesis, mitotic cell proliferation lasts longer in the CNS than in most other embryonic tissues. The maternal Nnf1 contribution therefore seems to run out before completion of the late CNS proliferation. The abnormalities observed in the double-mutant CNS after labeling with a DNA stain and anti-cyclin B were highly reminiscent of those observed in mutant embryos lacking zygotic function of other KMN...
components (Schittenhelm et al. 2007, 2009). Some cells were larger and displayed increased cyclin B labeling, presumably reflecting cell cycle defects triggered by partially functional kinetochores and hyperploidy resulting from chromosome missegregation. These developmental abnormalities might perhaps also explain why double-mutant larvae failed to grow and reach the third instar-wandering stage. In conclusion, the complete developmental lethality of double mutants in contrast to the viability and fertility of single mutants indicates that the two Nnf1 paralogs have partially overlapping functions.

To establish that the developmental lethality of double mutants results from a loss of Nnf1 function and not from genetic background effects, we performed rescue experiments with transgenes expressing Nnf1a or Nnf1b with N- or C-terminal EGFP extensions. The regulatory regions controlling expression of these transgenes were those present at the endogenous loci. All transgenes were inserted into the same chromosomal attP landing site.

When double mutants inherited either g-EGFP-Nnf1a or g-Nnf1a-EGFP from the father, they developed into fertile adults. These observations demonstrate that the lethality of double mutants reflects a loss of Nnf1 function. Moreover, EGFP-Nnf1a and Nnf1a-EGFP are functional proteins.

In contrast to the Nnf1a transgenes, g-EGFP-Nnf1b and g-Nnf1b-EGFP did not prevent the lethality of double mutants. However, interpretation of these rescue failures was complicated by our subsequent finding that a single endogenous Nnf1b+ copy does not support the development of Nnf1a mutants to the adult stage and that homozygosity of the transgenes g-EGFP-Nnf1b and g-Nnf1b-EGFP resulted in lethality even in an otherwise wild-type background. Moreover, an additional, P element-based transgene (g-Nnf1b) with the same regulatory region driving expression of untagged Nnf1b failed to promote double-mutant development to the adult stage even when present in two copies. Both g-Nnf1b insertions used in these experiments were homozygous viable and fertile in the wild-type background. We assume therefore that the Nnf1b regulatory regions present in our transgenes are not fully functional. Some regulatory elements might be outside of the 1.2-kb upstream region used in our transgenes, perhaps within the additional 20 kb of intergenic region upstream of Nnf1b.

Fig. 3 Simultaneous loss of Nnf1a and Nnf1b results in embryonic abnormalities. Embryos were collected from a cross of Df(2L)MK01, gDSP II.2, PBacLL02791/CyO, Dfd-EYFP females with Df(2L)NK01, gDSP II.2, Df(2R)Exel7164/CyO, Dfd-EYFP males. Embryos were fixed and stained with anti-cyclin B (CycB) and a DNA stain (DNA). Nnf1a Nnf1b double mutants (Nnf1a− Nnf1b−), which did not express Dfd-EYFP in the head region unlike balanced sibling embryos (Nnf1a+ Nnf1b+; arrowhead), contained some cells in the CNS that were enlarged and/or expressed cyclin B at higher levels. Bottom row shows single sections from CNS regions indicated in the top row (dashed rectangles) at higher magnification. Scale bars correspond to 100 μm (top) and 20 μm (bottom), respectively.
Nnf1b accumulates to higher levels than Nnf1a at kinetochores of syncytial embryos. 

a During mitosis but not during interphase, EGFP-Nnf1a and EGFP-Nnf1b both localize to kinetochores in syncytial blastoderm embryos generated by Nnf1a Nnf1b double-mutant females carrying two transgene copies of either ga-EGFP-Nnf1a (upper part) or ga-EGFP-Nnf1b (lower part). The metaphase plates indicated by the arrowheads are shown at higher magnification in the insets. Scale bar 10 μm. 

b The intensity of EGFP signals at kinetochores in syncytial blastoderm embryos during prometaphase and metaphase were quantified. The genotypes of the mothers are indicated below the bars. Two copies of ga-EGFP-Nnf1a (ga-E-Nnf1a) or ga-EGFP-Nnf1b (ga-E-Nnf1b) were present in either a Nnf1a Nnf1b double-mutant background (null) or in a background with Nnf1a and Nnf1b function (+). In the latter background, we also analyzed single copies of g-EGFP-Nnf1a (g-E-Nnf1a), g-Nnf1a-EGFP (g-Nnf1a-E), g-EGFP-Nnf1b (g-E-Nnf1b), or g-Nnf1b-EGFP (g-Nnf1b-E). 

c EGFP-Nnf1b levels are higher than those of EGFP-Nnf1a also when expressed under control of the same cis-regulatory sequences. Total extracts from syncytial blastoderm embryos were analyzed by immunoblotting with anti-EGFP and anti-PSTAIR used as loading control. The embryos were collected from mothers carrying either ga-EGFP-Nnf1a (ga-E-Nnf1a), ga-EGFP-Nnf1b (ga-E-Nnf1b), g-EGFP-Nnf1a (g-E-Nnf1a), or g-EGFP-Nnf1b (g-E-Nnf1b) or no transgene (+). All transgenes were homozygous except g-EGFP-Nnf1b which was present in one copy. For accurate quantification, a dilution series of the ga-EGFP-Nnf1b extract was loaded in the first three lanes. Bar diagram represent expression levels (mean and SD) resulting from immunoblot quantification (n = 3), where levels of the different EGFP fusion proteins were quantified relative to those obtained with ga-EGFP-Nnf1b. EGFP-Nnf1b levels were set to 100 in each blot.
Stage-specific differences in Nnf1a and Nnf1b subcellular localization during the cell cycle

For a comparison of potential functional specialization of the paralogous Nnf1 proteins, we generated two additional transgenic lines (ga-EGFP-Nnf1a and ga-EGFP-Nnf1b). Both transgenes were inserted into the same chromosomal attP landing site and contained the same Nnf1a 5′ and 3′ regulatory region. However, they coded for either EGFP-Nnf1a or EGFP-Nnf1b. When present in one copy, both transgenes supported the development of double mutants into fertile adults that eclosed with the expected Mendelian frequency (67% expected and 65% observed), n > 200. Moreover, rescued females had at most slightly elevated X-ND (w control 0.3% and 0.4% and 0.5% for double mutants rescued with ga-EGFP-Nnf1a and ga-EGFP-Nnf1b, respectively; n > 1100). Both EGFP-Nnf1a and EGFP-Nnf1b are therefore functional proteins.

To compare the subcellular localization of EGFP-Nnf1a and EGFP-Nnf1b during the cell cycle, we analyzed syncytial blastoderm embryos which express maternally contributed EGFP fusion proteins. Because these embryos were derived from Nnf1a Nnf1b double-mutant mothers rescued by either ga-EGFP-Nnf1a or ga-EGFP-Nnf1b, they did not express any untagged wild-type Nnf1 protein. With both paralogs, EGFP signals were clearly detectable at kinetochores throughout mitosis (Fig. 4a). In contrast, during interphase, we did not observe localized EGFP signals. The same localization behavior was also observed after cellularization during progression through the embryonic cell division cycles (data not shown). While subcellular localization of EGFP-Nnf1a and EGFP-Nnf1b during the embryonic cell cycles was indistinguishable, signal intensities were different (Fig. 4b). Kinetochore signals during mitosis in case of EGFP-Nnf1b were considerably stronger than those of EGFP-Nnf1a. Independent quantifications of embryos expressing ga-EGFP-Nnf1a or ga-EGFP-Nnf1b in the presence of competing wild-type Nnf1 confirmed this difference (Fig. 4b). Immunoblotting also suggested the presence of slightly elevated levels of EGFP-Nnf1b compared to EGFP-Nnf1a (Fig. 4c), also when both were expressed from transgenes with identical regulatory sequences and chromosomal integration position. Hence, Nnf1b translation efficiency and/or stability appears to be slightly higher compared to Nnf1a. In combination with the differential activity of the endogenous cis-regulatory sequences that direct production of the maternal contribution, a clear quantitative dominance of Nnf1b over Nnf1a is resulting at kinetochores during the syncytial mitoses in early embryos (Fig. 4b, c).

To evaluate the presence of the different Nnf1 proteins on meiotic kinetochores, we analyzed the EGFP-tagged proteins expressed from our transgenes (g-EGFP-Nnf1a, g-Nnf1a-EGFP, g-EGFP-Nnf1b, and g-Nnf1b-EGFP) in testes and ovaries. In testes, EGFP signals resulting from the Nnf1b transgenes were readily detected at kinetochores during both meiotic divisions (Fig. 5b; data not shown). As in syncytial embryos, no localized EGFP signals were detected during interphase in spermatocytes before the onset of the meiotic divisions (Fig. 5a) and also not in spermatids after completion of the meiotic divisions (data not shown). Analogously, during progression through the gonial cell division cycles preceding meiosis, kinetochore signals were only detected during mitosis. Interestingly, a different behavior was observed for Nnf1a. In this case, EGFP dots were not only present during M phase (Fig. 5b) but also during interphase (Fig. 5a). Signals at centromeres during interphase were observed in spermogonial cells (data not shown), as well as in spermatocytes before meiosis (Fig. 5a), but not in the terminally differentiated mitotically quiescent epithelial cells of the testis sheath and seminal vesicle (data not shown). During the meiotic divisions, Nnf1a was observed to be at kinetochores at lower levels than Nnf1b (Fig. 5c).

The differential localization of Nnf1a and Nnf1b during interphase was also observed in our analyses in ovaries (Fig. 5d, e). EGFP-tagged Nnf1a was present at interphase centromeres during the gonial division cycles, in nurse cell and oocyte nuclei throughout oogenesis, and in the somatic follicle cells during the early stages of oogenesis as long as progression through mitotic division cycles occurs. In contrast, in case of Nnf1b, we never observed centromeric EGFP dots during interphase. Centromeric signals during interphase were also not observed when EGFP-Nnf1b expression was directed by the Nnf1a cis-regulatory sequences (data not shown), indicating that differences in protein properties are responsible for the distinct interphase localization. However, during mitotic divisions, as well as in mature stage 14 oocytes that are arrested in metaphase of the first meiotic division, Nnf1b was present at kinetochores and also Nnf1a (Fig. 5d, e insets). We conclude that consistent with the observed genetic redundancy, Nnf1a and Nnf1b are both present at meiotic kinetochores. However, these two proteins have acquired distinct properties, at least with regard to localization during interphase in proliferating nonembryonic cells where Nnf1a is centromeric but not Nnf1b.

No evidence for functional divergence of Nnf1a and Nnf1b by positive selection

The partially redundant D. melanogaster Nnf1 paralogs are distinct with regard to localization properties and expression levels during development. Nnf1b is particularly abundant during female meiosis. In the context of the centromere drive model, it is conceivable therefore that Nnf1b might evolve under more prominent positive selection than Nnf1a. To address Nnf1 sequence evolution, we compared the coding regions of the Nnf1 homologs present in Drosophilid species of which whole-genome sequences are known. A single homolog was found in species outside the melanogaster subgroup (Fig. 6; Schittenhelm et al. 2007). In contrast, two paralogs, Nnf1a and Nnf1b, were
present in most of the melanogaster subgroup species (Fig. 6) (Schittenhelm et al. 2007). Comparison of the Nnf1 gene phylogeny with whole-genome species trees (Chen et al. 2014; Seetharam and Stuart 2013) indicates that occurrence and fixation of the Nnf1 gene duplication happened at the base of the branch leading to all the sequenced species with an evolutionary origin younger than D. kikkawai. The paralogs Nnf1a and Nnf1b are highly diverged. The mean nucleotide distance between the two clades is 0.443 and the mean amino acid distance is 0.568. To explore if this divergence was driven by positive selection, we studied the ratio $\omega$ of nonsynonymous substitutions $d_N$ to synonymous substitutions $d_S$ ($\omega = d_N/d_S$) during the evolution of Nnf1, as reflected in the gene tree of Fig. 6. Values of this ratio above one reflect the action of positive selection. More specifically, to find out whether Nnf1a and Nnf1b are evolving under different selective pressures over the entire gene length, we first employed a class of codon substitution models known as branch models (Yang 1998). These models allow the estimation of different $\omega$ ratios for different branches in a phylogenetic tree. For this analysis, we partitioned the Nnf1 phylogeny (Fig. 6) into pre-duplication branches ($\omega_0$), branches immediately following the duplications ($\omega_1 = \omega_2 = \omega_3$), and branches of the clade Nnf1a ($\omega_2$), and branches of the clade Nnf1b ($\omega_3$).

We first considered the one-ratio model M0, where $\omega$ is identical across all the branches of the Nnf1 phylogeny (Yang 2007). The estimate of $\omega$ under this null model was 0.199, indicating that the evolution of Nnf1 was dominated by purifying selection (Table 1). A more complex model M2, where two different $\omega$ ratios are estimated for pre-duplication ($\omega_0$) and post-duplication branches ($\omega_1 = \omega_2 = \omega_3$), yielded a significantly better fit than the
simpler model M0 ($2\Delta\ell = 14.340, df = 1, P = 1.53 \times 10^{-4}$; Table 2). This suggests that after the duplication of Nnf1, the duplicates experienced relaxed selection that translated into an increase in the rate of evolution by a factor of 1.6 ($\omega = 0.145$ to $\omega = 0.231$; Table 1). However, there was no evidence of positive selection acting after the duplication event, because the ratio $\omega$ in the post-duplication branches was still below one. To explore if the rate of evolution of Nnf1 was significantly higher immediately following the gene duplication ($\omega_1$) than the average rate for the other post-duplication branches ($\omega_2 = \omega_3$), and the rate for the pre-duplication branches ($\omega_0$), we compared a three-ratio M2 model against the two-ratio M2 model (Table 2). The estimated $\omega$ for the $\omega_1$ branches was 0.318 and thus higher than for the other branches (Table 1), but this more complex model did not yield a significantly better fit ($2\Delta\ell = 0.958, df = 1, P = 0.358$; Tables 1 and 2). Lastly, to find out if Nnf1a and Nnf1b experienced different selection pressures after the gene duplication, we compared a four-ratio M2 model against the simpler two-ratio M2 model, but the differences were not significant ($2\Delta\ell = 3.040, df = 2, P = 0.149$; Table 2). Together, these findings suggest that after the duplication event, the rate of evolution increased in both Nnf1a and Nnf1b, but the rates were not significantly different ($\omega_0$).

Although our analysis thus far had found no evidence for functional divergence of Nnf1a and Nnf1b through positive selection, the tests we had employed would not be able to detect signals of positive selection that occur only at one or few amino acid. To find out whether such localized signatures of selection exist, we next employed site models (Nielsen and Yang 1998; Wong et al. 2004; Yang et al. 2000, 2005). These models allow $\omega$ ratios to change among different codon sites, although not among different lineages. None of the models allowing sites to

### Table 1

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<th>Model Type</th>
<th>Estimated Parameters</th>
<th>Log-likelihood ($\ell$)</th>
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<tr>
<td>M0: one-ratio</td>
<td>$\omega = 0.19873$</td>
<td>$-8056.613025$</td>
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<td>M2: two-ratio</td>
<td>$\omega = 0.14473, \omega_1 = 0.23133$</td>
<td>$-8049.443005$</td>
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<td>M2: three-ratio</td>
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<td>M2: four-ratio</td>
<td>$\omega_0 = 0.14461, \omega_1 = 0.32065, \omega_2 = 0.25121, \omega_3 = 0.20458$</td>
<td>$-8047.922804$</td>
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*a Models from Yang (1998)*

*b See branch-specific $\omega$ ratios in Fig. 6*
but only when two functional Nnf1a protein, development into fertile adults is still possible embryonic cell proliferation in somatic cell lineages. Without degrees of freedom $df$ evolve under positive selection ($\omega > 1$) showed a significantly better fit than nearly neutral models, where sites can only evolve neutrally ($\omega = 0$) or experience purifying selection ($\omega < 1$; Table 3). In sum, when all the branches in the Nnf1 phylogeny are analyzed together, there is no evidence of adaptive evolution at any particular site of this gene.

In a final analysis, we also employed branch-site models where $\omega$ ratios can change both among codon sites and lineages (Yang and Nielsen 2002; Zhang et al. 2005). They allowed us to ask if positive selection could have acted on specific sites along specific branches of the Nnf1 phylogeny (foreground branches). This analysis did not yield evidence for positive selection in any of the four classes of branches defined in Fig. 6 and Table 4.

In conclusion, taking together all these results, the evolution of Nnf1 does not seem to be driven by positive selection. Yet, after the duplication event, both paralogs Nnf1α and Nnf1β accelerated their rates of evolution but not significantly different.

Discussion

Our results demonstrate that Nnf1α and Nnf1β are not functionally differentiated to the point where either gene can no longer provide all functions essential for development into fertile adults under laboratory conditions. However, the cis-regulatory regions of these paralogs are functionally distinct to a readily detectable degree. Expression of Nnf1β is maximal during oogenesis and early embryogenesis when that of Nnf1α is minimal. In contrast, Nnf1α expression is dominant after cellularization during the embryonic cell proliferation in somatic cell lineages. Without Nnf1α protein, development into fertile adults is still possible but only when two functional Nnf1β gene copies are present.

Table 2  Likelihood ratio tests of branch models

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<th>Model comparisons $^a$</th>
<th>$2\Delta \ell$</th>
<th>$df$</th>
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<td>M0 (one-ratio) vs. M2 (two-ratio)</td>
<td>14.340 $^*$</td>
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</tr>
<tr>
<td>M2 (two-ratio) vs. M2 (three-ratio)</td>
<td>3.040</td>
<td>1</td>
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<td>M2 (two-ratio) vs. M2 (four-ratio)</td>
<td>2.081</td>
<td>2</td>
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$df$ degrees of freedom

$^a$ Models from Yang (1998)

$^*$ $P < 0.001$

Such Nnf1α mutant adults with two functional Nnf1β gene copies are weak. They have also a reduced life span. These disadvantageous phenotypic consequences of a loss of Nnf1α function are likely sufficient to explain why Nnf1α is maintained in current D. melanogaster populations. In contrast, our phenotypic analyses cannot explain the maintenance of Nnf1β. Loss of Nnf1β function does not have a readily detectable effect on viability and fertility in the laboratory. Moreover, we have failed to detect an increase in chromosome nondisjunction when analyzing X chromosome segregation during female meiosis in Nnf1β mutants, even though Nnf1β appears to be predominant at kinetochores during the meiotic divisions. We emphasize that X-ND tests are far more sensitive than fertility tests. In principle, readily detectable highly significant increases in X-ND can occur in the absence of noticeable effects on fertility.

Apart from the cis-regulatory regions, the paralogous coding sequences are different as well with consequences for intracellular localization. We find that only Nnf1α but not Nnf1β is centromeric during interphase in gonads during production of gametes in both sexes. However, both proteins are at kinetochores during M phase. This difference between the Nnf1 paralogs has also been observed previously after transfection of Drosophila cells (Przewloka et al. 2007; Schittenhelm et al. 2007). Interestingly, we find that this intracellular localization difference during interphase is developmentally controlled. During the early embryonic division cycles, Nnf1α and Nnf1β are still localized indistinguishably also during interphase where neither is centromeric.

The striking difference in the ability of Nnf1α and Nnf1β to associate with centromeres already in interphase in certain cell types might be relevant for the previously observed developmental control of Drosophila Mis12 localization. In embryos, Mis12 is not or at most weakly detectable at interphase centromeres, whereas it is clearly centromeric in gonads and cultured cells already during interphase (Schittenhelm et al. 2007; Venkei et al. 2012) (unpublished data). In cultured cells, centromeric localization of Mis12 and of an unidentified Nnf1 form was shown to be co-dependent (Venkei et al. 2012). Our findings implicate Nnf1α in this co-dependent localization at interphase centromeres.

Table 3  Likelihood ratio tests of site models

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<thead>
<tr>
<th>Model comparisons $^a$</th>
<th>$2\Delta \ell$ $^b$</th>
<th>$df$</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1a vs. M2a</td>
<td>$-3.38 \times 10^{-3}$</td>
<td>2</td>
</tr>
<tr>
<td>M7 vs. M8</td>
<td>$-6.60 \times 10^{-5}$</td>
<td>2</td>
</tr>
</tbody>
</table>

$df$ degrees of freedom

$^a$ See “Materials and methods” for details

$^b$ None of these model comparisons indicated a significant difference

$\ell$ degrees of freedom

Table 4  Likelihood ratio tests of branch-site models

<table>
<thead>
<tr>
<th>Foreground branch $^a$</th>
<th>$2\Delta \ell$ (A vs. Anull) $^{b,c}$</th>
<th>$df$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\omega_0$</td>
<td>2.723</td>
<td>1</td>
</tr>
<tr>
<td>$\omega_1$</td>
<td>0.000</td>
<td>1</td>
</tr>
<tr>
<td>$\omega_2$</td>
<td>0.000</td>
<td>1</td>
</tr>
<tr>
<td>$\omega_3$</td>
<td>0.000</td>
<td>1</td>
</tr>
</tbody>
</table>

$df$ degrees of freedom

$^a$ See branch names in Fig. 6

$^b$ Models from Zhang et al. (2005)

$^c$ None of these model comparisons indicated a significant difference

$\Delta \ell$ likelihood ratio

$\Delta \ell = \ell_{M2} - \ell_{M0}$
Mis12 is present as well but not Nnf1b. In contrast, in early embryos where Nnf1a is low and unable to associate with interphase centromeres, Mis12 is also absent.

Although Nnf1a and Nnf1b are distinct with regard to centromere localization during interphase, this obvious functional difference does not appear to be of crucial physiological significance since single mutants were definitely not affected severely. Moreover, the weakness and reduced life span associated with loss of Nnf1a reflect reduced Nnf1 levels rather than absence of interphase centromere localization, as these traits were not observed in double mutants rescued by a single transgene copy expressing either Nnf1a or Nnf1b under Nnf1a control.

Localization of the Mis12 complex to interphase centromeres has also been observed in mammalian cells (Kline et al. 2006; McAinsh et al. 2006). While centromere recruitment of the Mis12 complex during M phase is clearly essential for kinetochore function, the role of centromeric Mis12 complex components during interphase remains to be clarified. In Drosophila, the mechanisms for centromere recruitment during interphase and mitosis have been demonstrated to be distinct (Venkei et al. 2012). Centromeric Mis12 complex components might act in a functionally redundant manner in heterochromatin regulation, since the heterochromatin protein HP1 has been demonstrated to recruit the Mis12 complex to interphase centromeres in mammalian cells (Kiyomitsu et al. 2010; Obuse et al. 2004). Accordingly, the absence of heterochromatin during the early syncytial cycles of Drosophila embryogenesis (Rudolph et al. 2007), when extremely rapid DNA replication in the absence of widespread transcription prevails, might explain the absence of Nnf1a from interphase centromeres specifically during the initial embryonic stages.

Our characterization of the Nnf1 paralogs provides a basis for future analyses addressing the evolutionary significance of gene duplications which have attracted increasing interest ever since Ohno’s seminal publication (Ohno 1970). Our comparison of Drosophilid sequences suggests that the Nnf1 duplication was followed by accelerated sequence divergence. However, we have been unable to detect positive selection. Positive selection in particular in the Nnf1b branch would have been of great interest in the context of the centromere drive hypothesis (Henikoff et al. 2001). Nnf1b has properties that appear to make it a good target for mutations beneficial for suppression of preferential meiotic segregation of centromere variants into the female pronucleus. Nnf1b predominates in kinetochores during female meiosis where it forms a bridge (Przewloka et al. 2011) between Cenp-C, an inner centromere protein with noticeable direct DNA-binding activity (Hori et al. 2008; Politi et al. 2002; Sugimoto et al. 1994; Yang et al. 1996), and the rest of the KMN network, the main microtubule-binding activity that might have a prominent role in the assembly and positioning of the acentrosomal spindles in oocytes (Radford et al. 2015). Clearly, the absence of evident positive selection in the Nnf1b branch does not argue against centromere drive as an important evolutionary force within the D. melanogaster lineage, where positive selection in the cid/Cenp-A gene has actually been reported in support of the centromere drive hypothesis (Cooper and Henikoff 2004; Malik and Henikoff 2001). Interestingly, a CENH3/Cenp-A duplication has been recently described in Minimus, where centromere drive is clearly ongoing, and differential evolution of the paralogs has been detected in this case (Finseth et al. 2015). Transient positive selection in the context of centromere drive early after Nnf1 duplication cannot be ruled out by our data and might therefore still have been important for the retention of the duplicated Nnf1 genes. Apart from centromere drive, an adaptive conflict caused by distinct selective pressures on Nnf1 function during meiosis and mitosis, for example, might have favored retention of the duplicated copies by permitting functional optimization of Nnf1b for meiosis. Nnf1b specialization for the extremely rapid mitoses of early embryogenesis might be another conceivable alternative. If still present in D. melanogaster, such Nnf1b specialization would have to be subtle, as we have not detected obvious effects after loss of Nnf1b. However, since Nnf1b is predominant during both female and male meiosis, we favor meiotic specialization apart from the evident partial subfunctionalization of the cis-regulatory regions as probable explanations for the maintenance of the functionally overlapping Nnf1 paralogs.

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Compliance with ethical standards This article does not contain any studies performed by any of the authors with human participants or animals (except for the invertebrate Drosophila melanogaster which is not subject to animal research legislation).

Conflict of interest The authors declare that they have no competing interests.

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Chen ZX et al. (2014) Comparative validation of the D. melanogaster modENCODE transcriptome annotation. Genome Res 24:1209–1223. doi:10.1101/gr.159384.113


