Original Article

Longevity GWAS Using the Drosophila Genetic Reference Panel

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Abstract

We used 197 Drosophila melanogaster Genetic Reference Panel (DGRP) lines to perform a genome-wide association analysis for virgin female lifespan, using ~2M common single nucleotide polymorphisms (SNPs). We found considerable genetic variation in lifespan in the DGRP, with a broad-sense heritability of 0.413. There was little power to detect signals at a genome-wide level in single-SNP and gene-based analyses. Polygenic score analysis revealed that a small proportion of the variation in lifespan (~4.7%) was explicable in terms of additive effects of common SNPs (≥2% minor allele frequency). However, several of the top associated genes are involved in the processes previously shown to impact ageing (eg, carbohydrate-related metabolism, regulation of cell death, proteolysis). Other top-ranked genes are of unknown function and provide promising candidates for experimental examination. Genes in the target of rapamycin pathway (TOR; Chrb, slif, mipp2, dredd, RpS9, dm) contributed to the significant enrichment of this pathway among the top-ranked 100 genes ($p = 4.79 \times 10^{-06}$). Gene Ontology analysis suggested that genes involved in carbohydrate metabolism are important for lifespan; including the InterPro term DUF227, which has been previously associated with lifespan determination. This analysis suggests that our understanding of the genetic basis of natural variation in lifespan from induced mutations is incomplete.

Key Words: Ageing—Gene-based analysis—Gene ontology—Polygenic score analysis—Target of rapamycin—Insulin signaling pathway.

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to be segregating in natural populations (including human populations). It is well established that there is a genetic component for survival into old age in humans. Estimates of heritability from twin-registry and population-based studies range from ~20% to ~30% (6–8), and can increase with age (7). However, the paucity of gene variants found by genome-wide association studies (GWAS) of lifespan in humans suggests that effects of individual genetic variants are small. Thus far, the most robustly replicated finding is the importance of the APOE gene (6,9–11), where the ε4 isoform is repeatedly associated with increased mortality in carriers (12). Therefore, APOE is considered to be a “frailty gene,” rather than a “longevity gene” (6,12). GWAS have also implicated several other genes associated with longevity, although none reach genome-wide significance (reviewed in 6). In addition to GWAS, association analyses of human orthologs of candidate longevity genes found in model organisms have been performed (13–15). These studies have identified genes in the IIS signaling pathway as being associated with human longevity. Indeed, the human forkhead box O3 transcription factor (FOXO3) in this pathway is often described as the second gene most commonly associated with longevity (15–17), although these findings have not been replicated in other studies (9,10,18,19).

Thus, studying natural variation in longevity in animal models could bridge the gap between mutations with a large effect, found in model organisms, and natural variants in humans. A substantial difference between studies in humans and those in animal models is the controlled environment in which the latter are living. This reduces the impact of environmental factors, allowing a larger proportion of differences in the phenotypic variation to be attributed to genetic variation.

Genome-wide association studies of ageing in model organisms have rarely been performed. The Drosophila melanogaster Genetic Reference Panel (DGRP) of ~200 inbred, sequenced lines is a community resource for analysis of population genomics and genome-wide association mapping of quantitative traits (20,21). Here, we performed a GWAS of virgin female lifespan using 197 DGRP lines (for a list of the lines used and their mean lifespan see Supplementary Table 1). Our aim was to measure the influence of naturally occurring genetic variants on lifespan to discover genes and mechanisms associated with natural variation in lifespan. We utilized an unbiased, multilayer approach building on a single-nucleotide polymorphism (SNP) GWAS as a primary unit, and tested whether individual genes, including regulatory regions, are associated with lifespan. We also explored whether enrichment of particular domains and/or biological pathways contribute to an increased or reduced mean lifespan. In addition, we investigated how much of the phenotypic variance in lifespan is explicable in terms of the additive effects of common genetic variants.

Materials and Methods

Lines Used
The Drosophila Genetic Reference Panel, Freeze 2.0 (20,21), comprises 205 D. melanogaster lines derived by 20 generations of full-sib mating from inseminated wild-type caught females from Raleigh, North Carolina. Whole-genome sequencing data, along with genotype calls, are available for all 205 lines (http://dgrp2.gnets.ncsu.edu). Lifespan data were obtained for virgin females for 197 DGRP lines, with a sample size of N = 25 females per line (five females in each of five replicate vials). A subset of the lifespan data has been published previously (22). The Wolbachia infection status and karyotype for major inversions for each line were downloaded from the DGRP website.

Quality Control
The original number of DGRP SNPs, after removing nonpolymorphic and SNPs with ≥ 3 alleles, was 3,963,420. We further filtered these data. All SNPs from the different chromosomes (ie, 2L/2R, 3L/3R, 4, and X) were combined before performing these filters. We set the SNP call rate cut-off at 0.9 (Supplementary Figure 1), that is, 90% of the lines were required to have a genotype call for a particular SNP to be included for further analysis. The minor allele frequency (MAF) cut-off was set at 0.02 (Supplementary Figure 2), that is, SNPs that had MAF < 2% were excluded from further analysis. Finally, at least 80% of the SNPs were present in all individuals (Supplementary Figures 3 and 4), thus no individual lines were removed. A total of 2,193,745 SNPs remained for 197 DGRP lines after applying these filters (summary statistics are presented in Supplementary Table 1).

Single-SNP Association (GWAS) Analysis
In order to identify SNPs associated with lifespan in the DGRP inbred lines, linear regression under an additive model was used, with a covariate included for the presence of Wolbachia pipiensis and the first four principal components [using Eigenstrat software (23)]. For the calculations, we used PLINK v. 1.07 (24). All single SNP association results are available upon request.

Gene-Based Association Analysis
We assigned the 2,193,745 SNPs to genes (including intronic regions) (Ensembl; http://www.ensembl.org/biomart/martview; BDGP5; as defined by the “Gene start (bp)” and “Gene end (bp)” in biomart). In some cases, SNPs were assigned to multiple genes, that is, when genes overlapped. We used a previously described, set-based method (25), whereby p values from all SNPs within a gene from the single-SNP association, corrected for Wolbachia status and the first four principal components, were combined using Fisher’s T-statistic:

\[
T = -2 \sum_{i=1}^{N} \log_{e} p_i
\]

where N is the number of SNPs (tests) and \( p_i \) (i = 1, ..., N) is the corresponding p value. For each gene, this was termed original statistic. Only genes with at least two SNPs were used in the calculations of the gene-based analysis. To calculate empirical gene-wide significance for each gene, we performed 10,000 genome-wide permutations (with replacement) for each set of SNPs within a gene. For each gene in each permutation, we calculated a permuted Fisher’s T-statistic and compared it with the original statistic. The empirical p value obtained for each gene was the proportion of permuted Fisher’s T-statistic that was found equal or larger to the original statistic. For the top 40 genes we performed 1,000,000 permutations, in order to get a more accurate estimate of the empirical gene-significance. All gene-based results are available upon request. We also performed similar gene-based analysis for SNPs within ± 5 kb (kilobases) of each gene.

Polygenic Score Analysis
Testing the polygenic score association (as described in the International Schizophrenia Consortium (ISC) study, 26) with
lifespan is based on the notion that a phenotype, under the polygenic model, is affected/influenced by the combined effect from numerous variants, each with a small effect. Such variants would not generally be significant in a single-SNP analysis. Nevertheless, such truly associated variants would be clustered in the lower percentile of the distribution of the association of SNPs. Hence, polygenic scores, created by using weighted allele dosage of SNPs passing a certain permissive p value threshold, could explain a relatively large proportion of the phenotypic variance. An independent set of lines with recorded lifespan was not available in order to calculate polygenic scores on the DGRP sample (discovery set) and test in a test sample set (target set), as done in the ISC. Instead, we performed a cross-validation analysis using permutations (with replacement). The DGRP sample set was randomly split in two sets (discovery and target sample sets) with roughly equal number of lines (99 vs 98). As in the ISC study, we pruned (maximum linkage-disequilibrium: r^2 = 0.25) the SNPs present in each of the discovery sample sets (99 lines) within a sliding window of 250 kb and kept the highest associated SNP (using clump function in PLINK v 1.07). We utilized 14 separate p value thresholds (p ≤ 0.1, p ≤ 0.09, p ≤ 0.08, p ≤ 0.07, p ≤ 0.06, p ≤ 0.05, p ≤ 0.04, p ≤ 0.03, p ≤ 0.02, p ≤ 0.1, p ≤ 0.05, p ≤ 0.01, p ≤ 0.005, p ≤ 0.001, p ≤ 0.0005, p ≤ 0.0001) for selection of SNPs in the discovery sample set for the calculation of the polygenic scores. The polygenic scores were calculated by using the score function in PLINK, based on the number of alleles from the discovery set that passed a certain p value threshold and weighted by the ß-coefficients from the linear regression in the discovery set (including Wolbachia status and the first four principal components as covariates). The final variance explained in terms of R^2 was calculated by means of linear regression of lifespan on polygenic scores. This procedure was repeated 100 times and means and standard deviations for each of the sets were calculated.

Gene Ontology and InterPro

Based on the ranking of the gene-based association analysis, we performed a Gene Ontology (GO, http://geneontology.org) and InterPro (http://www.ebi.ac.uk/interpro/ (27)) over-representation/enrichment analysis. Conversion from FlyBase gene identifiers to GO and InterPro was achieved by using FlyBase version FB2014_04. The ranked gene list (14,146 genes for SNPs within genes and 15,145 InterPro categories) was achieved by using Catmap (28). Catmap uses a Wilcoxon rank-sum test to assign significance of GO and/or InterPro categories among ranked list of genes.

Results

Phenotypic Variation in Lifespan in the DGRP

The overall mean lifespan for the 197 lines used was 55.28 days (Figure 1). A list of the lines used, along with lifespan data and Wolbachia status, can be found in Supplementary Table 1. We found considerable genetic variation in lifespan, with a broad-sense heritability of H^2 = 0.413 (Figure 1, Supplementary Tables 1 and 2).

There is considerable lifespan variability between the fly lines (Figure 1). The maximal mean lifespan in the DGRP (80.29 days) is similar to the maximal mean lifespan achieved in mutants (long-lived isogenic fly line 1L18; mean lifespan 82.1 (29) and median lifespan 82.5 days for fly strain wDah; dilp2-3,5 (30)). In addition, the lifespan variability within the fly lines is not that different from a survival analysis of large number of Drosophila controls (31). The mean lifespan variability of Drosophila strains wDah and w1118 is ~10.37 (data not shown), similar to the variability within the DGRP fly lines (μ = 10.6; Supplementary Figure 5).

Analysis of Single-SNP Association with Lifespan

Several large polymorphic inversions segregate in the DGRP, and about 50% of the lines are infected with the endosymbiotic bacterium Wolbachia pipientis (21). Since both inversion karyotypes and Wolbachia infection can cause cryptic relatedness and bias GWAS, we performed a principal component analysis of the DGRP genotypes. We found that In(3R)Mo and In(2Lt) are associated with genetic variation and relatedness within the 197 lines (Supplementary Figure 6). We therefore adjusted the phenotypic data to account for the first four principal components and Wolbachia status, although we did not observe a significant effect of Wolbachia status on lifespan, and performed a single-variant GWAS for lifespan using the 2,193,745 SNPs with MAF ≥ 0.02. None of the SNPs tested reached the Bonferroni threshold for genome-wide significance (p = 2.28×10^{-8}; Figure 2 and Supplementary Figure 7). The top 50 SNPs and nearby genes are listed in Supplementary Table 3.

In order to assess whether there was sufficient power to detect a relatively large effect on lifespan, we performed power calculations (Supplementary Figure 8). The power to detect a relatively large effect (10 days difference in lifespan between homozygous genotypes) at α = 2.28×10^{-8} ranged from 15% to 68%, corresponding to MAF of 0.1–0.2 (Supplementary Figure 8). The power increased to >91% power for MAF ≥ 0.3.

Thus, there is enough power to detect common SNPs with large effects on lifespan, indicating that there are no common alleles with large effects segregating in the DGRP. There was also adequate power to detect at least some lower frequency SNPs with large effects, so perhaps these do not segregate in the population either. We therefore infer that the individual variants contributing to the heritability are likely to have effects that are too small to be reliably detected in a population of 197 inbred lines.
Several of the genes tagged by top SNPs have been shown previously to affect lifespan (brummer, Rpd3, Thor, hairy, sima, and RFeSP). Lifespan extension by dietary restriction (DR) is mediated by an increased activity of Thor (32), although this may depend on the type of DR, as flies null for Thor respond normally to DR (33). brummer controls organismal fat storage in Drosophila and flies lacking brummer under DR live ~56% longer than controls (34). Females heterozygous for a Rpd3 hypomorphic allele have a lifespan extension of 52% and experimental data suggest that Rpd3 is likely to be within a pathway related to DR (35).

Both hairy and sima are involved in cellular responses to hypoxia (36,37), although the relationship between tolerance to hypoxia and lifespan is not entirely clear. That is, flies selected for hypoxia tolerance do not have altered lifespan (36), direct knockdown of genes leading to increased hypoxia resistance in worms also do not affect lifespan (38), although hypoxia significantly induces reactive oxygen species (ROS) within mice adipocytes and tissue hypoxia increases with age (39). Thus, it is unclear if hypoxia tolerance will result in fewer ROS and potentially increased lifespan. Nevertheless, deletions of the C. elegans ortholog of sima, the hypoxia-inducible factor HIF-1, modify longevity in a temperature-dependent manner (40). Additionally, the worm ortholog of Drosophila RFeSP, isp-1, extends lifespan (41).

Gene-Based Analysis

As noted above, power calculations suggest that single-SNP association analysis is underpowered for SNPs with MAF < 0.3. Gene-based GWAS on the other hand can increase the power to detect the combined effects of multiple variants within a gene (25,42). We therefore assessed the significance of the combined Fisher’s T-statistic for all common SNPs (MAF ≥ 2%; see Materials and Methods for definition of gene positions) in each annotated Drosophila gene. Of the 15,683 genes in the fly genome, 14,146 had at least two SNPs (MAF≥2%). None of the gene-based association tests exceeded the Bonferroni-corrected significance threshold ($p = 3.53 \times 10^{-06}$, Figure 3 and Supplementary Figure 9).

The top 30 genes are listed in Supplementary Table 4. In order to allow for SNPs in regulatory regions, we also performed a gene-based analysis with gene positions extended by 5 kb from the 5′ and 3′ ends. None of the 15,145 such tests of gene regions with
at least two SNPs were significant at a stringent Bonferroni threshold ($p = 3.30 \times 10^{-06}$ Supplementary Figures 10, 11; Supplementary Table 5).

Several of the top-ranking genes from the gene-based associations are plausible candidate genes affecting lifespan determination. CG11523 is predicted to have a GSK3β interaction domain (GSKIP; InterPro term: IPR023231). The human ortholog (GSKIP) protein interacts in vitro and in vivo with GSK3β and inhibits the GSK3β activity (43). Mouse GSK3β is a critical regulator of mTORC1, autophagy, and ageing (44); In addition, GSK3β controls the activity of S6 kinase 1, a crucial component of the TOR pathway, in human cell lines (45). CG6030 is ranked 10th in our gene-based analysis. CG6030 encodes the ATP synthase subunit delta, part of the complex V of the mitochondrial electron transport chain, which is the main energy-generating complex (46). Its role in ageing/lifespan is currently unknown, although the inhibition of a different subunit from the same complex (subunit β) has been shown to extend lifespan in worms (47).

Neo1 (Nep1) was the top-ranked gene-based association when genes were extended to include 5 kb from the 5′ and 3′ ends. Thus, it is possible that several SNPs within the regulatory regions of Nep1 could contribute to determination of female lifespan in these lines. Nep1 is integral for female fertility (48) and likely to be essential for female reproductive fitness. Fertility has been previously shown to be associated with ageing, that is, the “trade-off” theory of ageing, although the relationship is unclear and the effect of fertility can be separated from lifespan (49). Furthermore, Nep1 is the major β-amyloid degrading enzyme in mice and humans, and is associated with progressive Nep1 protein level decline with age in mice (50,51).

Many of the top genes from the gene-based analyses are computationally predicted and have no defined biological processes or molecular functions. The proportion of predicted genes was 36.7% (11 genes) and 53% (16 genes) among the top 30 genes from the strict and extended (genes ± 5 kb) gene-based analyses, respectively.

**IIS/TOR Pathway Enrichment**

Mutations in several genes in the IIS and TOR pathways affect lifespan (1,3), and there is a considerable literature on their effects and interactions. We previously used this knowledge to build a comprehensive, manually curated signaling network model of the TOR and IIS pathways (2,52). We assessed whether the top-ranked genes in our gene-based analyses were significantly enriched for genes in the IIS and TOR pathways (Figure 4; Supplementary Table 6).

Chrb, slf, Mipp2, dm, and dredd are in the TOR pathway, and ranked 33rd, 36th, 38th, 71st, and 93rd, respectively, in the extended gene-based analysis. Diminutive is downstream of the TOR pathway (2,52) and heterozygous mutants have an increased lifespan (53). Similarly, Mipp2 is a crucial component of the TORC1 complex (http://flybase.org (54)), part of the TOR pathway and slf is an amino acid transporter that activates TOR signaling in the fat body (55). Further, charybde is upstream of the TSC1 complex and inhibits growth, possibly via down-regulating S6K activity (56).

**Figure 4.** Overview of the insulin/insulin-like growth factor signaling (IIS) and target of rapamycin pathway (TOR) pathways. Rectangles represent genes; diamonds: molecules; triangles: environmental factors; trapezoids: other than IIS or TOR pathways; octagons: transcription factors; arrow lines represent activation; red t-shaped lines represent inhibition; brown boxes starting with c_ represent complexes; yellow boxes represent putative genes part of the IIS (CG11523) or TOR (RpS9) pathways; genes circled in red represent genes found in the top-ranked genes in the gene-based analysis; black horizontal line represents a symbolic separation between the TOR and IIS pathways.
**Polygenic Score Analysis**

We performed a polygenic score analysis (26) to assess the extent to which the additive effect of common genetic variants predict lifespan. Although the broad-sense heritability of line means is $H^2 = 0.413$, we were only able to achieve a maximum mean $R^2 = 0.047 ± 0.0047$ (α = 0.05; for SNPs with $p < 0.005$; Figure 5). Thus, only 4.7% of the phenotypic variation in lifespan is explained by the additive effect of common variants.

Estimates of $R^2$ using polygenic score analysis can be inflated if the sample size is small (64). We performed 100 permutations using the same procedure for calculating the polygenic scores and $R^2$, but randomly permuting the lifespan phenotypes, to estimate $R^2$ when there is no association between the phenotype and genotype. The results from these random permutations suggest that lifespan variation could be explained by no more than 1.99% ($p \leq 0.0005$; $R^2 = 0.019 ± 0.0019$), when the phenotype is not associated with the genetic variants (Supplementary Figure 14). Furthermore, for all $p$ value thresholds the mean variation explained was in all cases significantly higher than random permutations of lifespan ($p \leq 4 \times 10^{-6}$ for all $p$ value thresholds; Supplementary Table 9). Therefore, the $R^2$ estimates from the polygenic score analysis are unlikely to be due to chance. We can conclude that a relatively small proportion (~5%) of variation in lifespan in the DGRP is likely to be due to the additive effect of common genetic variants. The phenotypic variance of lifespan explained by common variants in the polygenic score analysis is similar to that obtained for starvation resistance (median $R^2 = 0.074$ and startle response (median $R^2 = 0.08$) with genotypic data for the same fly lines (65).

**Discussion**

We performed a GWAS of virgin female lifespan using the DGRP (20,21). The primary single-SNP GWAS did not reveal any variants that passed the genome-wide Bonferroni significance threshold. Power calculations suggest that 350–400 lines would be needed to detect single variants with a small to modest effects (<10 days) with > 80% power for MAF < 0.3. This suggests that common alleles (MAF ≥ 2%) with relatively large effects on lifespan are not segregating in the DGRP. Several of the top SNPs are within genes that have been previously shown to impact longevity. The remaining genes tagged by the top SNPs are largely uncharacterized and are novel candidates, likely affecting lifespan determination and aging.

**Gene-Based Association Analysis**

We showed that TOR-related genes are significantly enriched among the top-ranked genes in our gene-based association analysis, corroborating that genes shown to be important in lifespan determination and/or aging are found among the top-ranked genes in our analysis. Thus, the other top-ranked genes are likely to be promising longevity candidates as well. The finding that alleles within TOR-related genes are enriched and found associated with lifespan within these fly lines does not necessarily mean that TOR-related genes have the highest effect on lifespan. Since gene mutants within the TOR pathway have been known for some time to have a significant impact on longevity and we have a better understanding of their effect and relationship, we are more likely to detect their effect. This scenario does not negate the significance/importance and potential role of these genes in lifespan determination in the DGRP. Furthermore, a large proportion of the genes ranked at the top of the gene-based analysis are currently without a known function. It is nearly impossible to determine...
the biological function of these genes or assess their significance with respect to longevity without detailed lab-based experiments. Similar observations, that is, large number of uncharacterized genes, were also made in two separate genome screens (P-element insertions (66) and sequencing of flies divergent for late-age fertility and lifespan (67)). Given the highly ranked genes and genes ±5 kb with previously known association with lifespan and ageing, as well as the enrichment in TOR-related genes, the highly ranked uncharacterized genes present good candidates for further investigation with respect to lifespan.

We also draw conclusions based on the gene-based analysis of SNPs within genes and genes ±5 kb almost interchangeably. Without additional transcriptome-based data and additional analysis (eg, eQTLs) we are unable to determine if SNPs in gene regulatory regions (eg, promoter regions) are likely or not to affect transcription of these genes and subsequently affect lifespan. Additional transcriptome-based data or experimental assays could shed light on the effect of such regulatory elements on the expression of such genes and their effect on lifespan.

**Gene Ontology and InterPro Analysis**

Around half of the top 20 GO categories in the gene-based analysis are related to carbohydrate metabolism. This certainly implies that genes up or downstream of nutrient-sensing pathways, such as IIS or TOR, are likely to play a crucial role in lifespan determination in the DGRP. Apart from several statistically enriched InterPro terms, the rest of the top InterPro/GO terms and associated genes have thus far not been shown to be involved in lifespan determination. These could potentially be involved in biological mechanisms of ageing that are novel and could shed some light on biological pathways previously not implied in ageing. In addition, a large proportion of the top genes in our gene-based analysis are currently without a clearly defined function, hence we cannot utilize these in any meaningful way. Clearly, the GO and InterPro terms rely on previously defined biological and molecular function of such genes, thus these genes will be missed in any high-level analysis.

**Genetic Contribution to Lifespan**

We also explored how much of the phenotypic variance of lifespan, is potentially explicable in terms of common genetic variants. We showed that common genetic variants significantly contribute to lifespan determination, as compared to a randomly permuted lifespan data, whereby the relationship between the genotypes and phenotype is interrupted. Nevertheless, the additive effects of common genetic variants only explain ~5% of the phenotypic variance, in agreement with similar analyses of other phenotypes in the DGRP (65). The low phenotypic variance explained could be due to an increased variability in the lifespan determination of each individual line and of course all pairwise and higher order epistatic interaction, which are not interrogated using the additive, infinitesimal model.

Rare genetic variants are also likely to play a significant role in lifespan determination within these fly lines. These variants were excluded from analysis in this study, due to the minimal power to detect an association with such variants.

![Figure 5. Polygenic score and lifespan. Each box represents the interquartile range (IQR) with the median as a black horizontal line; the whiskers represent values 1.5*IQR; outliers are represented as separate points. The continuous black horizontal line connects the means within each p value threshold.](http://journals.gerontology.org/)

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*Figure 5. Polygenic score and lifespan. Each box represents the interquartile range (IQR) with the median as a black horizontal line; the whiskers represent values 1.5*IQR; outliers are represented as separate points. The continuous black horizontal line connects the means within each p value threshold.*
Conclusions
We utilized a multifaceted, hypothesis-free, and unbiased genome-wide approach in order to identify genes and pathways are associated with variation in lifespan. The analysis was based on whole-genome sequencing data, part of the valuable community resource: DGRP. Even though our results suggest that there is little power to detect single-SNP associations with small effects on lifespan, a gene-based analysis revealed additional information about genes and pathways that are likely to have an impact on lifespan, including several genes in the TOR pathway that have been previously shown to have an impact on lifespan. Experiments are now needed to refute or refine these associations.

Supplementary Material
Supplementary material can be found at: http://biomedgerontology.oxfordjournals.org/

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