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Molecular Signatures of Major Depression

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Molecular Signatures of Major Depression

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Adverse life experiences, particularly in early life, can cause illness. Clues to the responsible mechanisms may lie with the discovery of molecular signatures of stress, some of which include alterations to an individual’s somatic genome. Here, using genome sequences from 11,670 women, we observed a highly significant association between a stress-related disease, major depression, and the amount of mtDNA ($p = 9.00 \times 10^{-42}$, odds ratio $1.33$ [95% confidence interval [CI] = 1.29–1.37]) and telomere length ($p = 2.84 \times 10^{-14}$, odds ratio $0.85$ [95% CI = 0.81–0.89]). While both telomere length and mtDNA amount were associated with adverse life events, conditional regression analyses showed the molecular changes were contingent on the depressed state. We tested this hypothesis with experiments in mice, demonstrating that stress causes both molecular changes, which are partly reversible and can be elicited by the administration of corticosterone. Together, these results demonstrate that changes in the amount of mtDNA and telomere length are consequences of stress and entering a depressed state. These findings identify increased amounts of mtDNA as a molecular marker of MD and have important implications for understanding how stress causes the disease.

**INTRODUCTION**

Adverse life experiences, particularly those in childhood, contribute to disease morbidity and mortality [1–7]. There is considerable interest in understanding the mechanisms through which they do so, as it remains unclear how illness becomes apparent decades after the presumed initiating event. Long-standing hypotheses include chronic activation of the hypothalamic-pituitary-adrenal axis [8–10] and alterations of neuroimmune function [11]. Molecular signatures of stressful life experiences and their relation to disease are therefore of special interest to clarify the causal relationship between signature, disease, and stress.

Causal associations between stressful life events and early adversities such as childhood sexual abuse and major depression (MD) are well documented [12–14], suggesting that molecular signatures of stress may be enriched in sufferers of MD. The disease, and stress.

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**RESULTS**

Shortened Telomeres and Increased mtDNA Are Associated with Adversity

We first examined the relationship between mean telomere length and amount of mtDNA with MD. We assessed the mean length of telomeres (across all chromosomes) from low-coverage whole-genome sequencing (mean coverage of 1.7X) of saliva DNA samples of 11,670 subjects (Experimental Procedures). MD was associated with shorter mean telomere length: in a logistic regression model, the odds ratio for the contribution of normalized measure of mean telomere to the risk of MD is 0.85 (95% CI = 0.81–0.89, $p = 2.84 \times 10^{-14}$).

Abnormal mitochondrial morphology and altered metabolic activity has been reported in mood disorders [28]. Our aim was to establish whether telomere length and the amount of mtDNA represent markers of stress-related illness and to explore how such molecular signatures might arise.

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We replicated the association between MD and increased amounts of mtDNA in a European case-control study [29, 30]. In contrast to the CONVERGE sample, the DNA was extracted from blood, and samples were of both sexes. We obtained quantitative PCR (qPCR) measures of mtDNA from 216 individuals (108 cases and 108 controls, 123 women and 93 men). In a logistic model, the odds ratio for the normalized measure of mtDNA’s contribution to the risk of MD was 1.35 (95% CI = 1.11–2.10, $p = 8.3 \times 10^{-18}$; Figure 1C).

We next explored the association in the CONVERGE data between stressful life events and both mean telomere length and amount of mtDNA. Telomere length was significantly shorter in those who had experienced more stressful life events ($p = 0.0018$, by linear regression) and in those reporting childhood sexual abuse ($p = 0.043$, by linear regression) (Table 1). The amount of mtDNA was significantly correlated with both the total number of stressful life events (linear regression $p = 4.83 \times 10^{-4}$) and childhood sexual abuse (linear regression $p = 3.65 \times 10^{-3}$). The association of both molecular markers with childhood sexual abuse was stronger with increasingly severe abuse (Table 1).

**Molecular Changes Are Not Due to Technical or Biological Artifacts**

We explored a number of explanations for the association between molecular markers and MD (Figures 1, S1, and S2; Tables 1 and S1; Supplemental Experimental Procedures). First, we considered artifacts arising from incorrectly mapped reads. We found that none of the reads used for assessing the amount of mtDNA mapped to a set of all bacterial and plasmid genomes, and none mapped to nuclear copies of mtDNA.

Second, we considered whether the molecular changes might be due to medication. We could not explain the telomere length or mtDNA changes as a result of cases taking antidepressant medication: among the MD cases, 975 reported never having taken any antidepressants. Neither the amount of mtDNA nor telomeric length in these subjects differed significantly from that assayed in the 4,861 individuals reporting taking antidepressants ($t$ test $p = 0.96$ and $p = 0.88$, respectively).

Third, we considered whether the effects might be explained by alterations in the cellular composition of the saliva between cases and controls (see Supplemental Experimental Procedures). Methylation of cytosine residues at cytosine-guanine ( CpG ) dinucleotides differs between cell types [31–35] and thus contains information about the cellular composition of the tissue from which it was extracted [36–38]. We assessed methylation in 156 individuals (78 cases and 78 controls), selected from the extremes of the distribution of amount of mtDNA, and matched for age and other potential confounds. The sites assayed are shown in Table S1, and the percentage of methylation at each CpG site is shown in Figure S1. MD case-control status remained highly significantly associated with the amount of mtDNA ($t$ test $p$ value $= 5.14 \times 10^{-18}$) and telomere length ($p = 6.83 \times 10^{-5}$) after accounting for the degree of methylation at each of the sites (Figure S2). Expressed as a change in effect size using Nagelkerke’s $R^2$ measure, there is a 6% reduction in the $R^2$ in a model including methylation and the amount of mtDNA to predict MD and a 9% reduction for telomere length. From this analysis, we concluded that the cellular composition of saliva collected from cases differed slightly from that of controls and explained less than 10% of the differences in the amount of mtDNA and telomere length between cases and controls.

**Molecular Changes Are Contingent on the Depressed State**

To investigate a causal relationship between stressful life events, MD, amount of mtDNA, and telomere length, we performed a...
Table 1. Relationship between Childhood Sexual Abuse, Telomere Length, and the Amount of Mitochondrial DNA

<table>
<thead>
<tr>
<th>CSA Type</th>
<th>Excess Telomeric DNA</th>
<th>t Value</th>
<th>p Value</th>
<th>Excess mtDNA</th>
<th>t Value</th>
<th>p Value</th>
<th>Number Cases</th>
<th>Number Controls</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-genital CSA</td>
<td>0.02</td>
<td>0.35</td>
<td>0.73</td>
<td>0.08</td>
<td>1.37</td>
<td>0.169</td>
<td>186</td>
<td>81</td>
<td>267</td>
</tr>
<tr>
<td>Genital CSA</td>
<td>−0.08</td>
<td>−1.27</td>
<td>0.20</td>
<td>0.11</td>
<td>2.02</td>
<td>0.045</td>
<td>240</td>
<td>47</td>
<td>287</td>
</tr>
<tr>
<td>Intercourse CSA</td>
<td>−0.20</td>
<td>−2.45</td>
<td>0.01</td>
<td>0.38</td>
<td>4.67</td>
<td>3.05 x 10^{-6}</td>
<td>159</td>
<td>17</td>
<td>176</td>
</tr>
</tbody>
</table>

Results for analysis of variance in which different forms of childhood sexual abuse (CSA) predict telomere length and the amount of mtDNA. Non-genital CSA refers to sexual invitation, sexual kissing, and exposing; genital CSA refers to fondling and sexual touching; and intercourse CSA refers to attempted or completed intercourse.

- Estimated excess of telomeric or mtDNA over mean telomeric DNA or mtDNA in individuals with no CSA.
- t statistic of tests of hypotheses that underlying excess is zero.
- p value of tests of hypotheses that underlying excess is zero.
- Number of MD cases.
- Number of controls.
- Number of total individuals.

Table 2 shows the counts of individuals categorized by MD disease status and number of stressful life events, with the means and SEs for the amount of mtDNA (Table 2) and telomere length (Table 2) within each category.

If stressful life events have independent causal effects on MD and the molecular measures, then the latter should become independent of MD after conditioning on the number of stressful events. Table 2 shows this is not the case because the mean differences in amount of mtDNA and telomere length between cases and controls, when stratified for the number of stressful life events, remained highly significant (t tests in third column of Table 2; mtDNA p values range from 1.25 x 10^{-10} to 0.37; telomere length p values range from 4.23 x 10^{-5} to 0.0083).

We next asked whether the effect of stressful life events on MD is entirely indirect, acting via changes in the amount of mtDNA or telomere length. We rejected this explanation because the association between MD and stressful life events remains highly significant after conditioning on either amount of mtDNA (p = 5.6 x 10^{-99}; see Table S2, i) or telomere length (p = 2.10^{-100}; Table S3, i) in a logistic regression model. In contrast, the association between stress and amount of mtDNA or telomere length disappeared when conditioned on MD (p = 0.11 Table S4, i, and p = 0.11 Table S5, i, respectively). In other words, the predictive power of stress on amount of mtDNA and telomere length is mediated through a history of MD.

These conclusions also hold when the number of stressful life events is replaced by a history of childhood sexual abuse. In particular, there was no significant difference in the amount of mtDNA or telomere length when comparing controls who reported a history of childhood sexual abuse with those who did not. Mean values of normalized mtDNA for controls who reported any form of childhood sexual abuse was −0.136 (SE = 0.007) and −0.095 (SE = 0.001) for no such history; t test p value = 0.66. Comparable values for telomere length were 0.168 (SE = 0.0125) and 0.072 (SE = 0.001); t test p value = 0.27.

These analyses indicate that the molecular markers represent the current state of illness, regardless of the path by which it is reached, and predict that the most pronounced changes would be found in subjects currently reporting a severe mood disorder. Our analyses up to this point used subjects for whom we did not have a current state measure of mood. We therefore measured the amount of mtDNA in a separate Chinese case-control cohort of MD [39] where a state measure of mood was available (the Hamilton rating scale [40]). We selected 29 cases with scores greater than 25 (very severe) and 25 controls with scores of less than 5. Despite using such a small sample, we observed a highly significant difference (t test p value = 0.0008) and an odds ratio of 2.94 (95% CI 1.26–6.02), more than twice the odds ratio seen in the CONVERGE sample (odds ratio = 1.33).

Stress Increases the Amount of mtDNA and Shortens Telomeres

To gain a mechanistic understanding of the relationship between stress, amount of mtDNA, and telomere length, we undertook a mouse experiment. Sixteen C57BL/6J mice (eight males and eight females) were stressed for 4 weeks (for 5 days, a different stressor was administered: tail suspension, force-swim, foot shock, restraint, and sleep deprivation, followed by 2 days rest). After 0, 2, and 4 weeks of stress, amount of mtDNA and telomere length were assessed by qPCR and compared to age-matched non-stressed controls (eight males and eight females).

Consistent with our findings in humans, in mice, stress significantly increased the amount of mtDNA and decreased telomere length in saliva and in blood (Figure 2). After 4 weeks of stress, there was a mean increase in the amount of mtDNA of 210% compared to the unstressed animals in saliva (t test p = 0.0036) and 240% in blood (t test p = 6.1 x 10^{-5}). At the same time, the length of telomeric DNA was reduced 28% in saliva (t test p = 0.0001) and 30% in blood (t test p = 0.0017) in stressed mice as compared to non-stressed. There were no significant differences in the white cell parameters between stressed and non-stressed animals (all p values > 0.05), indicating that this result is unlikely to be due to differences in the blood cellular composition.

After 4 weeks of stress, half of the animals (eight stressed mice and eight controls) were kept in home cages without any intervention to model a recovery period of no stress. Molecular markers were again tested in blood and saliva, and results
For each category of stressful life event (#SLE, ranging from none [0] to more than four [4+] reported events), Table 2 reports the means and SEs of the normalized mtDNA levels (top section of the table) and normalized telomere length measures (bottom section of the table), followed by the numbers of individuals, for MD cases and controls. The last column gives the t statistic and p value for the difference between cases and controls.

### Mitochondrial Function Is Altered in Tissues with Increased mtDNA

Altering the amount of mtDNA presumably reflects functional changes in mitochondria, a hypothesis we tested by measuring and comparing the oxidative phosphorylation (OXPHOS) capacity of mitochondria-enriched fractions from the liver of stressed and non-stressed mice. Figure 4A shows the mean values for the change in oxygen concentration over time for liver mitochondrial preparations taken from eight stressed and eight control (not stressed) animals. Addition of an equal amount of ADP (driving force for the electron transport chain after depletion of residual driving force in the fraction with excess glutamate and malate) induced a greater increase in oxygen consumption in mitochondria from the non-stressed animals than stressed ones (p = 0.038, from a linear model; Figure 4B). The complete quenching of OXPHOS upon addition of electron transport chain inhibitor potassium cyanide in both stressed and non-stressed mice showed oxygen consumption during the experiment was solely due to OXPHOS. Results of this experiment showed that OXPHOS efficiency was reduced in the liver tissue of mice whose amount of mtDNA had increased in response to stress, suggesting either an adaptive switch to glycolysis or a compromise in mitochondrial function.

### Glucocorticoid Administration Reproduces the Effects of Stress

What might be inducing the molecular changes? We considered one mechanism: activation of the hypothalamic pituitary adrenal (HPA) axis [41–48]. We administered corticosterone to eight C57BL/6J female mice over 4 weeks and oil vehicles of the same volume to eight control mice of the same strain. Figures 5 shows that after 4 weeks, there was significantly more mtDNA in the saliva (p = 0.011) and in the blood (p = 0.0013) of treated mice compared to controls and that telomere length had significantly reduced in both tissues (in saliva: p = 0.0023; in blood: p = 0.0016; all p values from t tests).

### DISCUSSION

We report here two important observations on the relationship between MD and two molecular signatures of adversity, the amount of mtDNA and mean telomere length. First, the changes in amount of mtDNA and telomere length are contingent on the presence of MD. We found no significant molecular changes in those who reported stressful life events, including childhood sexual abuse, but had never been depressed. Second, in a mouse model, while stress over a period of weeks did increase the amount of mtDNA and shorten telomere length, both changes were at least partly reversible. While early environmental adversity may result in permanent changes in physiology and risk of disease [49], our results indicate that it is important to recognize two trajectories, one leading to molecular signatures of stress and one to illness.

For the first trajectory leading from adversity to molecular changes, one possible pathway is through the endocrine
system, particularly the activation of the hypothalamic pituitary axis, since changes in both molecular markers could be reproduced in mice by administration of corticosterone. Release of glucocorticoids is known to increase in response to stress. Severe stressors, such as childhood sexual abuse [42, 50], alter pituitary-adrenal and autonomic reactivity. In some circumstances, the consequences may be deleterious rather than adaptive: glucocorticoids have been implicated in the pathophysiology of posttraumatic stress disorder [51, 52], and it has been known for many years that some patients with MD exhibit hypersecretion of cortisol [41, 53, 54], in part due to corticotrophin releasing factor (CRF) hypersecretion [55].

For the second trajectory leading to illness, we hypothesize that while adversity may on its own have an effect on both the amount of mtDNA and mean telomere length, the extent and persistence of these molecular changes depend on an individual’s susceptibility to MD, either from genetic or additional environmental predisposing factors. In many individuals, the molecular signatures will be small and transitory, but in those with MD, the effects may be larger or last for a longer period of time. Subjects who have never been diagnosed with MD, yet suffered severe adversity, may have had detectable alterations in mtDNA levels and mean telomere length in particular tissues at the time they experienced stressful life events, but these changes would have reversed and no longer be detectable by the time they were interviewed. We emphasize that the molecular changes we observe are neither risk factors nor causes of MD. The correlation between stress, mtDNA, and telomere length is contingent upon MD; we could find no evidence that stressful life events act via changes in mtDNA or telomere length to increase the risk of MD. Thus, our data provide no support for a role of changes in the amount of mitochondrial DNA or length of telomeres in regulating mood.

The disease-state dependence of the measures is important when considering the potential use of the changes as biomarkers. It is noteworthy in this regard that in a sample when we assayed amount of mtDNA in currently severely ill subjects, a robust difference was detected in a comparison of just 29 cases and 25 controls. This suggests that, despite the relatively small effects and large variances seen in the saliva sample, there may be circumstances where the amount of mtDNA could serve as a useful biomarker. The relatively larger increases seen in the mouse experiment (up to 4-fold) suggest that controlling for inter-individual variation would improve the chances of the biomarkers having a clinical application.

Changes in mean telomere length and levels of mtDNA presumably reflect altered metabolic strategies in times of perceived or expected stress. Experiments assessing OXPHOS efficiency in mice showed a decrease in OXPHOS energy production in stressed mice with elevated mtDNA levels. The tissue-specific effects of stress on amount of mtDNA and mean telomere suggest different, or possibly sequential, pathways governing tissue-specific change. It is possible that these changes might in part explain changes in appetite and sleep occurring during the state of depression.
mtDNA in multiple tissues

Telomere length in multiple tissues

Figure 3. Alterations of mtDNA in Different Tissues after 4 Weeks of Stress

Top: Assessment of mtDNA in four tissues. Bottom: Assessment of telomere length in four tissues. The vertical axis shows the amount of mtDNA or telomere length assessed by qPCR, relative to the mean of the values obtained for control animals (no stress). The horizontal axis gives the names of the tissues for the two conditions: stress (red) and no stress (blue).

EXPERIMENTAL PROCEDURES

The CONVERGE Study, Samples, DNA Preparation, and Sequencing

All 11,670 samples are drawn from the CONVERGE study of MD. The study protocol was approved centrally by the Ethical Review Board of Oxford University (Oxford Tropical Research Ethics Committee) and the ethics committees responsible for each hospital in China. The study posed minimal risk to the subjects (an interview and saliva sample). Stressful life events and childhood sexual abuse were assessed retrospectively. The stressful life events section of the CONVERGE interview was developed for the Virginia Adult Twin Study of Psychiatric and Use Disorders (VATSPUD) [17]. It assesses 16 traumatic life events and the age at their occurrence. The childhood sexual abuse was a shortened version of the detailed module used in the VATSPSUD study, which was in turn based on the instrument developed by Martin et al. [56]. DNA was extracted from saliva samples using the Oragene protocol.

Sequencing libraries were constructed from DNA fragmented using the Covaris Adaptive Focused Acoustics (AFA) technology. QIAquick Gel Extraction kit was used to purify the DNA fragments. Each DNA sample was uniquely tagged for the purpose of mapping. The reference genome NC_012920.1 was extracted from the Genome Reference Consortium Human Build 37 patch release 5 (GRCh37.p5) with Stampy (v.1.0.17) [57] and stored in BAM format [58]. The estimated genome coverage and the GC content of DNA; it was then regressed with mean telomere length output from Telseq was already corrected for whole-genome coverage and the GC content of DNA; it was then regressed using the linear function in the R statistical software language [59].

Estimation of mtDNA Copy Number

Average read depth per 100 bp is calculated for the mtDNA reads mapped to NC_012920.1 both before and after filtering out poorly mapped reads including those potentially from bacterial genomes using SAMTOOLs (v.0.1.18) [56]. There are regions in the mitochondrial genome replicated in the nuclear genome commonly known as nuclear copies of mitochondrial DNA (NUMTs), which would most likely be present as secondary alignments. We calculated average read depth per 100 bp in the mtDNA alignments between before and after filtering and compared the two sets of read depths. To reduce errors in estimation of coverage due to NUMTs, segments with big differences in read depth (>5% of the filtered read depth) between the filtered and unfiltered alignments that are more likely to span NUMTs were excluded from our calculation of mtDNA copy number. We arrived at a measure of mtDNA copy number by taking the mean read depth in the filtered alignments across all remaining 100 bp segments, then regressing it with sequencing batch, sample age, and average filtered read depth on chromosome 20, then transforming the residuals to normality using a quantile normal transformation of the MT-CYB gene on the mitochondrial chromosome in duplex with the TaqMan RNaseP Copy Number Reference Assay (Life Technologies, part number 4403326) as an internal control.

Estimation of Mean Telomere Length

Mean telomere length was quantified from low-coverage whole-genome sequencing data mapped to Genome Reference Consortium Human Build 37 patch release 5 (GRCh37.p5) with Telseq v.0.0.1 [60]. The estimated mean telomere length output from Telseq was already corrected for whole-genome coverage and the GC content of DNA; it was then regressed with...
under the following conditions: denaturation 95°C for 10 min followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. An estimate of the mtDNA copy number was calculated using the mean of Gapdh as a control [63].

All samples were duplicated at each time point. PCR efficiencies were between 90%–110% (average coefficient variance: 0.806). PCR runs were discarded if they failed to meet the following criteria: no template control (NTC) with a quantitation cycle (Cq) < 38 cycles; sample with a Cq > 30 cycles; PCR efficiency > 90% and < 110.0%; standard curve R² < 0.980; replicate group Cq SD greater than 0.20. qPCRs were carried out at the end of each experiment and all time points were analyzed on a single plate, thus excluding batch effects.

**Quantification of Telomere Length by Monochrome Multiplex qPCR**

Average telomere length was measured from mouse DNA using a previously described monochrome multiplex qPCR (MMqPCR) method [64].

**Mitochondrial Oxygen Consumption**

We measured oxygen consumption from mouse liver mitochondrial preparations over time using a Clark electrode. After the addition of respiratory substrates (glutamate and malate), oxygen consumption was monitored for 100 s, after which ADP was added and oxygen consumption measured for a further 100 s. Potassium cyanide (KCN) was added 100 s later to inhibit all mitochondrial oxygen consumption.

**Animal Experiments**

All experiments were carried out in strict accordance with the recommendations in the Guide for Laboratory Animals Facilities and Care as promulgated by the Council of Agriculture, Executive Yuan, ROC, Taiwan. The protocol was approved by the Institutional Animal Care and Use Committee of Chang Gung University (permit number: CGU13-067). Animals were housed and randomly assigned to stress or non-stress experiments.

Mouse stress experiment: mice (strain C57BL/6J, female n = 8, male n = 8, aged 12 months) were stressed over 5 days followed by 2 days rest, repeated for 4 weeks. On the first day, animals were suspended from their tails for 10 min. This was repeated three times, with 5 min rest between tail suspensions. On the second day, animals were placed in a cylinder of deep water from which there was no escape for 10 min. The forced swim was repeated...
twice with a 10 min rest. On the third day, a foot shock was administered three times (0.75 mA for 10 s with 10 s rest). On the fourth day, animals were restrained in a cylindrical tube (12 cm in length and 3 cm in diameter) for 3 hr. On the fifth day, animals were sleep deprived for 24 hr (mice were put in water tank, containing multiple and visible platforms [4.5 cm in height and diameter] surrounded by water for 24 hr). For the glucocorticoid experiment, mice (strain C57BL/6J, female n = 8, aged 12 months) underwent daily subcutaneous injection of 30 mg/kg corticosterone (Sigma) or vehicle (oil) for 28 days. Association between mtDNA, telomere length, and stress was performed in a linear mixed model using the lme4 package in the statistical software language R [59]. The null model included only weight. Variation in the amount of mtDNA between different tissues was assessed by a t test, comparing values between controls and experimental animals.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.03.008.

AUTHOR CONTRIBUTIONS

N.C., Yihan Li, S.C., K.K., R.M., and J.F. prepared the manuscript. Y.C., Yihan Li, H.D., B.D., Keqing Li, W.S., J.G., J.F., Jian Hu, C.H., G.H., G.J., Youhui Li, Kan Li, Yi Li, G.L., L. Liu, T.J., Y. Lu, X.Z., Y.Y., Yingrui Li, H.Y., Jian Wang, X.G., R.M., J.M., J.F., Jun Wang, and X.X. carried out genome sequencing and analysis. N.C., Yihan Li, J.F., and R.M. carried out genetic analysis. N.C. identified the shortened telomeres and examined the relationship between both molecular markers with stress and depression. S.C. performed all animal experiments and analyzed the qPCR and OXPHOS output data. Yihan Li identified the excess of mtDNA in low-coverage sequencing data in cases of MD as compared to controls.

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