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**The orphan nuclear receptor TLX regulates hippocampal transcriptome
changes induced by IL-1 β**

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22 Abbreviations: TLX, Orphan nuclear receptor tailless homolog; IL-1 β , interleukin-1 beta;
23 TNF, tumor necrosis factor; NPCs, neural progenitor cells; DG, dentate gyrus; LTP, long
24 term potentiation; P, post-natal day; KO, knockout; WT, wildtype; PBS, phosphate buffered
25 saline; GO, gene ontology; FDR, false discovery rate; RNASeq, RNA sequencing; DAVID,
26 database for annotation visualisation and integrated discovery; STRING, search tool for the
27 retrieval of interacting genes/proteins.

28 **Abstract**

29 TLX is an orphan nuclear receptor highly expressed within neural progenitor cells (NPCs) in
30 the hippocampus where it regulates proliferation. Inflammation has been shown to have
31 negative effects on hippocampal function as well as on NPC proliferation. Specifically, the
32 pro-inflammatory cytokine IL-1 β has been shown to suppress NPC proliferation as well as
33 TLX expression in the hippocampus. However, it is unknown whether TLX itself is involved
34 in regulating the inflammatory response in the hippocampus. To explore the role of TLX in
35 inflammation, we assessed changes in the transcriptional landscape of the hippocampus of
36 TLX knockout mice (TLX^{-/-}) compared to wildtype (WT) littermate controls with and
37 without intrahippocampal injection of IL-1 β using a whole transcriptome RNA sequencing
38 approach. We demonstrated that there is an increase in the transcription of genes involved in
39 the promotion of inflammation and regulation of cell chemotaxis (*Tnf*, *Il1b*, *Cxcr1*, *Cxcr2*,
40 *Tlr4*) and a decrease in the expression of genes relating to synaptic signalling (*Lypd1*, *Syt4*,
41 *Cplx2*) in cannulated TLX^{-/-} mice compared to WT controls. We demonstrate that mice
42 lacking in TLX share a similar increase in 176 genes involved in regulating inflammation
43 (e.g. *Cxcl1*, *Tnf*, *Il1b*) as WT mice injected with IL-1 β into the hippocampus. Moreover,
44 TLX^{-/-} mice injected with IL-1 β display a blunted transcriptional profile compared to WT
45 mice injected with IL-1 β . Thus, TLX^{-/-} mice, which already have an exaggerated
46 inflammatory profile after cannulation surgery, are primed to respond differently to an
47 inflammatory stimulus such as IL-1 β . Together, these results demonstrate that TLX regulates
48 hippocampal inflammatory transcriptome response to brain injury (in this case cannulation
49 surgery) and cytokine stimulation.

50

51 Keywords: RNA sequencing, TLX, interleukin-1 beta, hippocampus, neuroinflammation,
52 nuclear receptors,

53 1: Introduction

54 The hippocampus is one of two regions of the adult brain that neural progenitor cells (NPCs)
55 reside throughout the lifespan (Gage, 2000, Zhao et al., 2008). Specifically within the dentate
56 gyrus (DG) of the hippocampus, these NPCs are core components of neurogenesis (i.e. the
57 birth of new neurons) and this process is thought to contribute to hippocampal cognitive
58 functions such as spatial memory (Kempermann et al., 2004, Shors et al., 2002) as well as
59 playing a role in the regulation of emotion (O'Leary and Cryan, 2014). For hippocampal
60 neurogenesis to occur, NPCs must successfully progress from a proliferative state to fully
61 mature integrated neurons (Deng et al., 2010). This progression is under strict regulation by a
62 host of intrinsic and extrinsic factors (Suh et al., 2009). One of these regulators is the orphan
63 nuclear receptor subfamily 2 group E member 1 (Nr2e1 or TLX) (Niu et al., 2011, Li et al.,
64 2012, Shi et al., 2004, Zhang et al., 2008). TLX is required to maintain NPCs in a
65 proliferative state and to prevent ectopic neural differentiation (Shi et al., 2004, Zhao et al.,
66 2010). Its expression in the adult mouse brain is localised to the neurogenic niches and
67 specifically within the NPCs of these niches (Monaghan et al., 1995). Mice lacking TLX
68 display reduced hippocampal volume as well as impaired neurogenesis due to the fact that
69 NPCs fail to proliferate (Shi et al., 2004). TLX deficient mice also have impaired long-term
70 potentiation (LTP) in the DG and display deficits in hippocampal neurogenesis-associated
71 spatial memory cognition (O'Leary et al., 2016b, Christie et al., 2006, Roy et al., 2002,
72 O'Leary et al., 2016a). Although there are profound behavioural and cellular defects observed
73 in mice lacking the TLX, to date it has not been determined how the whole hippocampal
74 transcriptome may be affected by the loss of this NPC-specific transcription factor. Moreover,
75 whether intrinsic regulators of neurogenesis, such as TLX, can regulate wider hippocampal
76 transcriptional responses to negative stimuli such as inflammation is not fully known.

77

78 It has been established that hippocampal inflammation can induce deficits in hippocampal
79 neurogenesis and associated cognitive function (O'Leime et al., 2017a, Kohman and Rhodes,
80 2013, Yirmiya and Goshen, 2011). Interestingly, it has been demonstrated that NPCs
81 themselves can regulate the inflammatory environment when transplanted into a region of
82 tissue damage (Pluchino et al., 2005, Martino and Pluchino, 2006). Interleukin-1 β (IL-1 β) is a
83 pro-inflammatory cytokine whose receptor, IL-1 receptor type 1 (IL-1R1) is expressed in
84 proportionately higher levels in the hippocampus compared to other brain regions (Parnet et
85 al., 1994, Farrar et al., 1987). IL-1R1 is also expressed on NPCs in the hippocampus (Green
86 et al., 2012; Ryan et al., 2013). IL-1 β is predominantly responsible for the negative effects of
87 neuroinflammation on hippocampal neurogenesis and hippocampal-related impairments due
88 to its receptor expression profile (Ryan et al., 2013, Green and Nolan, 2012, Kelly et al.,
89 2003, Koo and Duman, 2008, Hein et al., 2010). It is noteworthy that IL-1 β has been
90 demonstrated to inhibit the expression of TLX in both embryonic and adult hippocampal
91 NPCs (Green and Nolan, 2012, Ryan et al., 2013). However, it is unknown whether TLX
92 itself can regulate the IL-1 β -induced inflammatory response within the hippocampus. To
93 address this, we compared the transcriptome changes in the hippocampus of TLX knockout
94 (TLX^{-/-}) mice to wild type (WT) mice following an inflammatory stimulus with IL-1 β .

95

96

97 **2: Methods**

98 *2.1: Animals*

99 Postnatal day (P) 56 male TLX^{-/-} and wildtype (WT) littermate control mice (on a cross
100 BL6/129S1 background) were used in this study (n=4-8). Breeding pairs were kindly
101 provided by Prof. Elizabeth Simpson, University of British Columbia and were generated as
102 previously described (Wong et al., 2010). Briefly, male TLX heterozygous mice (TLX^{+/-}) on
103 a 129S1 background were crossbred with female BL6 TLX^{+/-} mice to generate TLX wildtype
104 controls (TLX^{+/+}), TLX^{+/-}, and TLX knockout (TLX^{-/-}) pups with offspring genotypes
105 followed Mendelian inheritance (See figure 1 for breeding summary and numbers of mice
106 used per group). All pups were weaned at P21 and tailsnips were taken for genotype analysis
107 which was carried out using an Extract N' Amp kit (Sigma-Aldrich) as per the
108 manufacturer's instructions. After genotyping, the animals were single housed under standard
109 housing conditions (temperature 21°C and relative humidity 55%), with food and water
110 available *ad libitum*. All experiments were conducted in accordance with the European
111 Directive 2010/63/EU, and under an authorization issued by the Health Products Regulatory
112 Authority Ireland and approved by the Animal Ethics Committee of University College Cork.
113

114 *2.2: Experimental design*

115 For RNA sequencing analysis, TLX^{-/-} and WT mice were injected with either IL-1 β or
116 vehicle (phosphate buffered saline (PBS)) via cannulation, thus there were four experimental
117 groups for the RNA sequencing section of this study (Figure 1, Table 1). Hippocampal tissue
118 from an additional group of non-cannulated TLX^{-/-} and WT mice was taken for PCR analysis
119 to assess the effect of cannulation on cytokine (IL-1 β and TNF α) expression.
120

121 *2.3: Stereotaxic surgery for hippocampal cannulation*

122 At P52 (4 days prior to IL-1 β or PBS microinjection), TLX^{-/-} and WT mice were
123 anaesthetised using a mixture of ketamine (0.25ml), xylazine (0.2ml) and sterile 0.9% NaCl
124 (2.05ml) at a dose of 0.1mls/10g (i.p.) and placed in a Kopf stereotaxic frame. A guide
125 cannula (*Plastics1*, Gauge: 22, Pedestal Length: 4mm, Projection: 1.5mm) was implanted
126 unilaterally into the dorsal hippocampus at the following co-ordinates: AP -1.7, ML -1.2 and
127 DV -1.5 relative to bregma (Haettig et al., 2013). Animals were randomly implanted on the
128 left or right hemisphere. The cannula was secured in place using superglue as a base layer
129 followed by dental cement. After the dental cement had hardened sufficiently, animals were
130 administered Carprofen (0.1ml/30g, i.p.) and 0.5ml of 5% Glucose (5g/100ml; i.p.), and
131 returned to their home cage.

132

133 *2.4: Intrahippocampal microinjections*

134 At P56 mice were unilaterally injected with 1 μ l of either recombinant mouse IL-1 β (10ng/ μ l;
135 R&D systems) or PBS (filtered using a 0.2 μ m sterile filter) and infused at a rate of 0.5 μ l/min
136 using an automated 'Pico Plus' microinjector (Harvard Apparatus, Kent, UK). The injection
137 cannula projected a further 0.5mm past the guide cannula to give a total depth of 2.0mm and
138 was left in place for an additional 2 min for diffusion before the needle was withdrawn. Mice
139 were sacrificed by decapitation one hour after injection. This timepoint was chosen as we
140 have previously demonstrated that IL-1 β can induce a significant reduction in TLX gene
141 expression in hippocampal NPCs at this timepoint (O'Leime et al., 2017b). Moreover, we
142 aimed to assess the immediate gene expression changes induced by IL-1 β rather than
143 secondary gene expression changes induced by the release of other regulators of gene
144 expression as a result of IL-1 β injection. The injection needle was attached to the guide
145 cannula and mice were allowed to move freely during the injection. After one hour, left or
146 right hippocampi were removed, flash frozen on dry ice and stored at -80°C.

147

148 *2.5: Hippocampal RNA Extraction*

149 Total RNA was extracted and DNase treated from hippocampal tissue from both WT and
150 TLX^{-/-} animals using the mirVanaTM total RNA extraction kit (Ambion/Life Technologies,
151 Dublin, Ireland) and Turbo DNA-free kit (Ambion/life technologies) as per the
152 manufacturer's instructions. The total concentration of extracted RNA was quantified using a
153 Nanodrop 2000 (Thermo Scientific, UK) and was stored at -80°C until sent for sequencing.

154

155 *2.6: mRNA sequencing*

156 Equal volumes of total hippocampal RNA from each animal was sent for sequencing by
157 Exiqon (Vedbaek, Denmark) and conducted on an Illumina NextSeq500 sequencer with an
158 average of 30 million reads with a 50 basepair paired-end read length. Annotation of the
159 obtained sequences was performed using the reference genome annotation: Mus musculus
160 (organism), GRCm38 (reference genome), Ensembl_70 (annotation reference).

161

162 *2.7: Differential gene expression and functional enrichment analysis*

163 Data analysis was conducted by Exiqon using XploreRNA automated analysis software. The
164 data analysis pipeline used in this software is based on the Tuxedo software package which is
165 a combination of open-source software and makes use of peer-reviewed statistical methods.
166 Additionally, Exiqon employs specialised software developed at Exiqon to interpret and
167 enhance the readability of the finalised results. The components of the data analysis pipeline
168 used at Exiqon for RNA sequencing include Bowtie2 (v. 2.2.2.), Tophat (v. 2.0.11.), and
169 Cufflinks (v. 2.2.1.). Briefly, Bowtie2 is a sequence aligner used by Tophat to align the
170 sequencing reads to the reference genome (GRCm38, UCSC Genome browser and
171 Ensembl_70 (annotation reference)). Cufflinks uses the alignment results from Tophat and

172 assembles the aligned sequences into transcripts to construct a map of the transcriptome.
173 Cufflinks assembles the aligned reads into transcript isoforms based on exon usage and also
174 determines transcriptional start sites. Additionally, Exiqon performs fragment bias correction
175 which corrects for sequence bias during library preparation. Cuffdiff is used when comparing
176 groups to calculate the number of fragments per kilobase of transcript per million mapped
177 fragments (FPKM) and to determine differential gene expression and regulation based on the
178 assembled transcripts from the submitted samples using the Cufflinks output. An adjusted p-
179 value of ≤ 0.05 was considered significantly differentially regulated. Differentially regulated
180 genes were analysed for enrichment of Gene Ontology (GO) terms and KEGG pathways
181 using the DAVID Bioinformatics Resources (v6.8) (Huang da et al., 2009).

182

183 2.8 Quantitative real-time PCR (qRT-PCR)

184 Validation of RNA sequencing was performed using specific PrimerTime[®] qPCT assays
185 sourced from IDT (Integrated DNA Technologies) (Table 2). cDNA was reversed transcribed
186 using the high capacity cDNA reverse transcription kit (Applied Biosystems) using the
187 SureCycler[®] 8800 (Agilent Technologies) and diluted to a final concentration of 10ng/ μ l. All
188 qRT-PCR was performed in 3 technical replicates for each biological sample on a
189 LightCycler[®] 480 Instrument II (Roche). Quantification of gene expression was analysed
190 using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). RNA sequencing data was validated
191 for four genes that contributed to the enrichment for TNF signalling in our KEGG pathway
192 analysis using the following primers from Integrated DNA Technologies (IDT): SDHA
193 (Mm.Pt.56a.12170577), IL-1 β (Mm.PT.58.41616450), IL-6 (Mm.Pt.58.10005566), TNF
194 (Mm.PT.58.12575861).

195

196 2.9 STRING analysis

197 The STRING database, which stems from computational predictions was used to investigate
198 direct (physical) and indirect (functional) associations between differentially regulated genes.
199 An interaction network was created using a list of differentially regulated genes between WT
200 and TLX^{-/-} and genes that were commonly up-regulated in WT vs. TLX^{-/-} and WT vs. WT +
201 IL1B (172 genes). The string network was visualized using the following parameters:
202 excluded any genes that there was no evidence of association of connectivity, minimum
203 required interaction score was set at high confidence (0.007), and meaning of network edges
204 was set to line thickness indicating strength of data support. KEGG pathway analysis was
205 also conducted in STRING which indicated among the 172 up-regulated genes that TNF
206 signalling pathway was the most significantly enriched pathway $p < 0.001$ (red nodes).

207

208 *2.10 Statistics*

209 An adjusted p value (q value, Benjamini-Hochberg method) of ≤ 0.05 for RNA sequencing
210 data was considered to indicate significantly differentially regulated gene expression. For
211 Gene Ontology (GO) and KEGG analysis a cut off of 0.05 FDR p value was used to
212 determine significance. For RT-PCR validations, one-way ANOVA followed by Tukey's
213 *post-hoc* analysis or student's t -test was used where appropriate to determine statistical
214 significance.

215

216 **3: Results**217 *3.1: TLX regulates inflammatory gene expression in the hippocampus of mice.*

218 By performing pairwise analysis of whole transcriptome RNA sequencing data, we compared
219 differentially expressed genes in the hippocampus between vehicle injected cannulated WT
220 and TLX^{-/-} mice. We identified a large number of differentially expressed genes in the
221 hippocampus of TLX^{-/-} mice compared to WT controls (1542 genes) (Figure 2A, B). Of these
222 1542 genes, the majority were up-regulated (1272 genes) with a smaller subset down-
223 regulated (274 genes) in TLX^{-/-} mice (Figure 2A, B, C). Next we assessed the biological
224 functions of these up- and down-regulated genes in TLX^{-/-} mice. We observed a significant
225 enrichment in GO terms such as regulation of inflammatory response, cytokine production,
226 and cellular responses to cytokine stimulus (e.g. *Tnf*, *Il1b*, *IL6*, *Tlr2*, *Nfkbia* and *Tlr4*) (Figure
227 2D). GO analysis on the list of down-regulated genes observed in TLX^{-/-} mice revealed an
228 enrichment in genes involved in synaptic signalling such as *Syt4*, *Syt17*, and *Lypd1* compared
229 to WT control (Figure 2E).

230

231 *3.2: TLX^{-/-} mice display similar transcriptional profile to that of WT mice injected*
232 *intrahippocampally with IL-1 β .*

233 We found a large number of genes to be similarly up-regulated in TLX^{-/-} and WT + IL-1 β
234 mice compared to WT mice (Figure 3A, B). Of the 1272 genes whose expression increased in
235 TLX^{-/-} mice compared to WT mice, 176 or 13.8% these genes are also increased in WT + IL-
236 1 β . We assessed the biological function of these genes and observed an enriched
237 enrichment in GO terms for cellular response to cytokines (e.g. *Cxcl1*, *Il1b*, *Tnf*, *Ccl2*),
238 regulation of inflammatory response (e.g. *Tlr2*, *Ccl4*, *Ccl7*, *Ier3*), and apoptotic processes
239 (e.g. *Ier3*, *Cyr61*, *Lgals3*, *Perp*) (Figure 3C, E). KEGG pathway analysis revealed a
240 significant enrichment for genes involved in inflammatory pathway signalling such as TNF

241 signalling (*Tnf*, *Fos*, *Jun*, *Il1b*), cytokine-receptor interaction (*Csf3*, *Il6*, *Ccl2*, *Il1b*, *Il1a*), NF-
242 κ B signalling (*Nfkb1a*, *Lbp*, *Ptgs2*), and TLR signalling (*Tlr2*, *Il1b*, *Nfkb1a*, *Lbp*) (Figure 3D,
243 E). This indicated that in TLX^{-/-} mice, there is a similar dysregulation of inflammatory gene
244 expression to that induced by IL-1 β within the hippocampus of WT mice. Additionally,
245 KEGG pathway analysis using two web based algorithms (DAVID and STRING) indicated
246 that TNF signalling was the most significant and prominent pathway to be enriched in this
247 subset of differentially up-regulated genes (172) (Figure 4A, B).

248

249 *3.3: Cannulated TLX^{-/-} mice have a significantly different cytokine response to IL-1 β*

250 WT + IL-1 β mice show a down-regulation in 7 genes and an up-regulation in 221 genes

251 within the hippocampus compared to cannulated WT mice (Figure 5A, B). TLX^{-/-} + IL-1 β

252 mice display a much reduced transcriptional response as they display a differential

253 transcriptional response in 38 genes and only 5 of those were up-regulated compared to TLX^{-/-}

254 ^{-/-} mice (Figure 5A, B). GO enrichment analysis only showed significant enrichment among

255 down-regulated genes (33 genes) between TLX^{-/-} and TLX^{-/-} + IL-1 β groups of mice (Figure

256 5A). Specifically, we observed enrichment in GO terms such as cell (including leukocytes

257 and neutrophils) chemotaxis, (*Cxcl13*, *Ccr1*, *Trem1*), defence to bacterium (*Lyz2*, *Lyz1*),

258 response to LPS (*Lcn2*, *Cxcl13*), and acute inflammatory response (*S100a8*, *Saa3*) (Figure

259 5C).

260

261 *3.4: TLX potentially regulates TNF signalling indirectly via two separate gene interactions*

262 *and cannulation induces a significant increase in IL-1 β and TNF α expression in TLX^{-/-} mice*

263 As we identified TNF signalling as a possible mechanism through which TLX regulates

264 inflammation, we again used STRING analysis to identify the link between TLX (*Nr2e1*) and

265 TNF signalling. We identified *Top2a* and *Bcl6b* as two genes from all differentially regulated

266 genes (1542 genes) in TLX^{-/-} mice compared to WT mice that may interact with TLX and
267 potentially mediate the regulatory effects of TLX on TNF signalling (Figure 6A). We
268 confirmed our sequencing results using qRT-PCR that TNF α and IL-1 β , which have been
269 shown from our KEGG analysis to be highly involved in regulating TNF signalling, have
270 elevated expression levels in cannulated TLX^{-/-} mice (Figure 6B, C). We also assessed the
271 expression of these genes in non-cannulated mice. Non-cannulated TLX^{-/-} mice have similar
272 hippocampal expression of IL-1 β and TNF α to that of non-cannulated WT mice (Figure 6B,
273 C). However, after hippocampal cannulation surgery, there is a significant increase in IL-1 β
274 (F(3,24)=12.34, $P=0.0001$, ***= $P<0.01$) and TNF α (F(3,24)=10.12, $P=0.0003$;
275 ***= $P<0.01$) expression in TLX^{-/-} mice only (Figure 6B, C). There is a non-significant trend
276 towards an increase in these cytokines in WT mice after hippocampal cannulation surgery
277 (IL-1 β : t-test comparison between WT/Naive and WT/PBS indicates $P=0.0862$, TNF α : t-test
278 comparison between WT/Naive and WT/PBS indicates $P=0.0588$) which may be due to
279 limited samples numbers or to significant variations among individual mice. Likewise, PCR
280 analysis revealed that there was no statistically significant difference in IL-6
281 mRNA expression between cannulated and non-cannulated mice for both WT and KO strains
282 (Figure 6D).

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290 **Discussion**

291 In the present study, we demonstrate that TLX acts to regulate the transcription of genes
292 involved in maintaining a normal inflammatory profile after cannulation surgery and in
293 response to an IL-1 β stimulus in the hippocampus of mice. Firstly, we demonstrated an
294 orchestrated up-regulation in genes involved in inflammatory processes in the hippocampus
295 of TLX^{-/-} mice when compared to WT mice. WT mice injected with IL-1 β into the
296 hippocampus display a significant up-regulation in genes involved in cellular responses to
297 cytokines and inflammatory signalling pathways, and these genes overlap with the genes
298 increased in TLX^{-/-} mice. We showed that TLX^{-/-} mice have a blunted transcriptional response
299 to IL-1 β after cannulation surgery compared to cannulated WT mice and we highlight that
300 TLX may act to regulate inflammatory responses via TNF signalling. Finally, we outline that
301 there was no difference in the expression levels of IL-1 β and TNF α between TLX^{-/-} and WT
302 naïve mice but that these cytokines were significantly increased in TLX^{-/-} mice after surgery.
303 Taken together, these data indicate that the absence of TLX results in disruption of the
304 hippocampal inflammatory transcriptome response.

305

306 There was a large dysregulation in the transcriptional landscape of the hippocampus of TLX^{-/-}
307 mice evident with 1542 genes differentially expressed compared to WT mice. The majority of
308 these genes (1272 in total) were up-regulated, which is in keeping with the current literature
309 describing TLX as a transcriptional repressor (Sun et al., 2007, Islam and Zhang, 2014).

310 Similarly, whole transcriptome analysis of another strain of TLX knockout mice revealed a
311 large number of differentially regulated genes (1721) in NPCs from the subventricular zone
312 of the lateral ventricles (Niu et al., 2011). Most likely these genes are not all targets of TLX
313 but rather are up-regulated as a knock-on effect due to a lack of regulation of TLX target
314 genes. These genes showed enrichment for GO terms such as the regulation of inflammatory

315 processes and included genes such as *Tnf*, *Tlr2*, *Tlr4*, and *Il1b*. We have recently
316 demonstrated that there is a significant increase in microglial cell density and activation
317 (increased somal area) in the DG of TLX^{-/-} mice compared to WT counterparts under baseline
318 conditions, while there is no change in the number of apoptotic cells in the DG of these mice
319 (Kozareva et al., 2017a, Kozareva et al., 2017b). Thus, there is evidence to suggest that TLX
320 is intrinsically linked with inflammatory processes in the hippocampus as the absence of TLX
321 leads to increased microglial activation at baseline and a dysregulated inflammatory gene
322 expression profile.

323

324 As proliferating NPCs are the predominant source of TLX within the hippocampus (Shi et al.,
325 2004), it could be suggested that NPCs may function to normalise inflammatory gene
326 expression. Moreover, it has been demonstrated that TLX is not expressed, or expressed at a
327 very low level within cerebral cortex microglia (EMBL gene expression atlas
328 (<http://www.ebi.ac.uk/gxa/genes>)), and thus it is likely that the regulatory role of TLX on
329 inflammation within the hippocampus is mediated by its functions within NPCs (Zhang et al.,
330 2014, Li et al., 2012). To this end, data from transplantation studies have demonstrated that
331 NPCs themselves have indirect beneficial effects on the surrounding tissue by modulating the
332 inflammatory environment (Pluchino et al., 2005, Martino and Pluchino, 2006).

333

334 Among the genes that were down-regulated in TLX^{-/-} mice compared to WT mice, a
335 significant enrichment for genes involved in synaptic signalling such as *Syt4* and *Syt17*, was
336 observed. This supports a previous report, also using mice with a spontaneous deletion of
337 TLX, which demonstrates an impairment in LTP formation in the DG in TLX^{-/-} mice
338 compared to WT mice (Christie et al., 2006). Interestingly, that study demonstrated that LTP
339 was specifically impaired in TLX^{-/-} mice in the DG and not the CA1 region of the

340 hippocampus. As the DG is the primary area where NPCs are found, and TLX is primarily
341 expressed in proliferating NPCs, it can be suggested that impairments in synaptic plasticity
342 resulting from TLX deletion occurs as a results of its disfunction or absence in NPCs. The
343 dysregulation in synaptic gene expression and impairment in LTP may also underlie the
344 hippocampal-dependent behavioural deficits that we and others have previously observed in
345 these TLX^{-/-} mice (O'Leary et al., 2016a, Christie et al., 2006, Young et al., 2002, O'Leary et
346 al., 2016b).

347

348 Apart from genes involved in the regulation of inflammation, there are a large number of
349 genes involved in other processes such as intracellular signal transduction, cell chemotaxis,
350 and organ development that are differentially expressed in TLX^{-/-} mice. These mice have a
351 spontaneous deletion and are thus devoid of TLX from embryonic development through to
352 postnatal development and adulthood (Young et al., 2002). Therefore, the wide ranging
353 biological processes dysregulated in these mice are possibly due to the absence of TLX
354 during critical moments of various organ development rather than a specific lack of TLX in
355 adulthood (Monaghan et al., 1997, Roy et al., 2002). Further studies should assess the
356 inflammatory profile of mice with a conditional knockout of TLX in adulthood to address the
357 role that loss of TLX during embryonic development has on the development of the immune
358 system and its function in adulthood.

359

360 As we observed a significant dysregulation in inflammatory gene expression in TLX^{-/-} mice
361 and because we have previously reported enhanced microglial activation in the DG of TLX^{-/-}
362 mice, we aimed to assess whether an inflammatory cytokine stimulus could induce similar
363 transcriptome changes in WT mice. The pro-inflammatory cytokine IL-1 β , whose receptor is
364 expressed on NPCs in the hippocampus (Green et al., 2012), has previously been shown to

365 downregulate TLX expression in NPCs *in vitro* (Green and Nolan, 2012, Ryan et al., 2013,
366 Farrar et al., 1987). In response to hippocampal IL-1 β injection in WT mice, we observed a
367 significant increase in inflammation and chemotaxis related genes such as *Cxcl1*, *Il1b*, *Tnf*,
368 and *Tlr2*. This is supported by reports that increased cytokine expression or administration of
369 exogenous cytokines to the hippocampus can induce further endogenous cytokine expression
370 (Anisman et al., 2008, Moore et al., 2009, Shaftef et al., 2007, Skelly et al., 2013, Balschun et
371 al., 2004, del Rey et al., 2013). We did not observe a significant decrease in TLX expression,
372 However, this may be due to the fact that TLX is only expressed in a subset of cells within
373 the hippocampus and as we extracted RNA from the whole hippocampus, any changes in
374 specific TLX-expressing cells may be not be detected.


375

376 With regard to the similarity in transcriptome changes in TLX^{-/-} mice and IL-1 β -injected WT
377 mice compared to WT mice, there were 176 genes (of the 1272 genes up-regulated in TLX^{-/-}
378 mice) also up-regulated in WT mice in response to IL-1 β . It should be noted that RNA
379 sequencing analysis only highlighted similarly upregulated genes however, and did not
380 compare the magnitude of gene expression change between the two groups. Notwithstanding,
381 these data suggest that the absence of TLX expression leads to an inflammatory state in the
382 hippocampus similar to that induced by IL-1 β in WT mice. KEGG pathway analysis of the
383 overlapping genes shared between TLX^{-/-} and WT mice injected with IL-1 β compared to WT
384 mice reveal that TNF signalling is a common pathway. This then suggests that TLX may act
385 to repress TNF signalling, which in turn is increased by knocking down TLX expression or
386 by increasing IL-1 β expression. It has been established that IL-1 β can induce the expression
387 of other cytokines such as TNF α , and that TNF α can itself suppress proliferation of TLX
388 expressing NPCs and subsequent neuronal differentiation (Monje et al., 2003, Ben-Hur et al.,
389 2003, Keohane et al., 2010). Likewise, TNF α can induce the expression of other cytokines


390 such as IL-6 and IL-10 (del Rey et al., 2013, Skelly et al., 2013). Therefore, for future studies
391 on the mechanism of the regulatory role of TLX in inflammation, it will be important to
392 assess the effects of TLX on various cytokines and their respective signalling pathways. From
393 the current study it appears that TLX maintains normal inflammatory status in the
394 hippocampus and that the TNF signalling pathway may be a major pathway through which
395 TLX carries out this function.

396

397 We conducted STRING analysis on the up-regulated genes in TLX^{-/-} compared to WT mice
398 enriched for TNF signalling. This analysis predicts potential protein-protein interactions
399 based on evidence from the literature and from online protein databases. The thickness of the
400 interconnecting lines depicted in Figure 6 is indicative of the amount of evidence supporting
401 the interaction between any two proteins. We observed that TLX does not appear to directly
402 interact with TNF protein. Rather, TLX may interact with TNF signalling proteins via its
403 potential interactions with Top2a and Bcl6b proteins. Both of these genes are up-regulated in
404 our sequencing data suggesting that they may be repressed by TLX signalling. However, the
405 evidence for an interaction between TLX and Top2a and Bcl6b proteins is not strong, as
406 indicated by the thickness of the connecting lines in Figure 6A. Specifically, STRING
407 analysis highlighted these two proteins due to the fact that structural homologs of the proteins
408 (TLX, Top2a, and Bcl6b) have been shown to interact in human tissue and there is greater
409 evidence for the interactions between protein homologs of TLX and Bcl6b (Ku et al., 2009,
410 Diner et al., 2015). Nuclear transrepression is also another potential mechanism by which
411 TLX regulates inflammation (Glass and Saijo, 2010). Nuclear transrepression involves the
412 ‘tethering’ of nuclear receptor to other signalling factors in order to prevent their signalling.
413 This is a common mechanism through which glucocorticoid receptors and PPARs act to
414 suppress inflammatory-related NF- κ B signalling (Glass and Ogawa, 2006, Glass and Saijo,

415 2010, De Bosscher et al., 2003, Delerive et al., 1999). It may be possible that TLX regulates
416 inflammation via transrepression of TNF signalling or other inflammation-related pathways.
417 However, to date no such transrepression mechanism has been described for TLX and any
418 component of TNF signalling. 

419

420 Somewhat surprisingly, we observed a drastically different alteration in the transcriptional
421 landscape of TLX^{-/-} mice in response to IL-1 β compared to WT mice injected with IL-1 β .
422 TLX^{-/-} mice have a blunted response with only 38 differentially expressed genes compared to
423 228 genes differentially regulated in the WT mice after IL-1 β injection. Moreover,
424 enrichment could only be achieved for the down-regulated genes in the TLX^{-/-} mice in
425 response to IL-1 β , and this revealed an enrichment in GO terms such as cell chemotaxis
426 (*Cxcl13* and *Ccr1*). These genes are important for NPC-neuron-microglia communication
427 (Cartier et al., 2005, Li, 2013). A possible explanation for this blunted response is that the
428 TLX^{-/-} mice already have an elevated inflammatory response to the cannulation surgery.
429 Specifically, we observed a significant increase in the expression of IL-1 β and TNF α in TLX^{-/-}
430 mice only after surgery. Thus, it could be suggested that these mice have reached an
431 inflammatory plateau after cannulation surgery and are resistant to further cytokine stimulus.
432 Additionally, these data indicate that TLX^{-/-} mice may be more sensitive to brain injury
433 compared to WT mice. Despite the fact that we did not see any differences in IL-1 β or TNF α
434 expression levels between TLX^{-/-} and WT mice at baseline, we have previously reported 
435 increased microglial activation in TLX^{-/-} mice which indicates a heightened inflammatory
436 state in these mice at baseline (Kozareva et al., 2017a). With regards the role of TNF
437 signalling and its regulatory role on inflammation, it should also be noted that IL-1R1 is
438 expressed on endothelial cells throughout the brain (Matsuwaki et al., 2014). These cells also
439 express genes involved in the TNF signalling pathway and are thus critical in the regulation

440 of neuroinflammation (Liu et al., 2015). Thus mechanistically, it is possible that the absence
441 of TLX can disrupt both NPC-endothelial and NPC-microglia communication which
442 subsequently results in blunted responses to stimulation by inflammatory cytokines as well as
443 an enhanced inflammatory response to cannulation surgery. We assessed IL-6 mRNA
444 expression via PCR and observed no significant differences between cannulated and non-
445 cannulated mice. While IL-6 is a key marker of brain injury (Woiciechowsky et al., 2002,
446 Shohami et al., 1994), it may be the case that the TLX^{-/-} mice used in the current study are
447 more susceptible to changes in IL-1 β and TNF α than to IL-6 as much greater differences in
448 the mRNA expression of IL-1 β and TNF α are observed. However, it should be noted that
449 there was a large degree a variability in the PCR results for IL-6 which may be due to the
450 relatively limited number of samples available. This result highlights the facts that compared
451 to RNASeq, PCR analysis requires highly specific primer sequences and possibly larger
452 sample sizes to accurately highlight smaller gene expression differences (Griffith et al.,
453 2010).

454

455 Disruption of TLX as a result of spontaneous deletion results in abnormalities in motor,
456 cognitive and anxiety-related behaviours. The most striking behavioural phenotype is
457 increased aggression (Young et al., 2002; O'Leary et al., 2016 review). The serotonin_{2A/C}
458 receptor has been shown to mediate the aggressive phenotype of TLX^{-/-} mice (Juarez et al.,
459 2013) and interestingly, from our GO enrichment of genes for biological processes we
460 observed an increase in expression in genes associated with serotonin transport and release
461 (*Cd300a*, *Syk*, *Fcerig*, *Lgals3* and *Fcgr3*) in TLX^{-/-} compared to WT mice. Hyperactivity has
462 also been consistently observed in TLX^{-/-} mice, and we have recently reported a progressive
463 decline in motor performance on the accelerating rotarod (O'Leary et al., 2016) which
464 implicate corticostriatal pathways. With regard to hippocampal-associated behaviours, TLX^{-/-}

465 mice display poor performance in working memory as assessed by spontaneous alternations
466 in the Y-maze, as well as and contextual fear conditioning (O'Leary et al., 2016a). Extensive
467 evidence in the literature shows that hippocampal administration of IL-1 β results in
468 impairments in hippocampal-associated spatial learning and memory tasks and in LTP
469 (Moore et al., 2009, Kohman and Rhodes, 2013, Vereker et al., 2000). Although we did not
470 assess behavioural outcomes of IL-1 β administration to TLX^{-/-} mice in the current study, it is
471 possible that IL-1 β may not exacerbate the already robust deficits in cognitive behaviours due
472 to the significant dysregulated and indeed pro-inflammatory transcriptional profile of the
473 TLX^{-/-} mice in this study.

474

475 In conclusion, this study provides valuable insights into the role of TLX as a regulator of
476 inflammation and lays the ground work for future studies assessing the effects of TLX on
477 inflammatory and cognitive processes. Moreover, as TLX is a nuclear receptor and thus has
478 the potential to be targeted therapeutically (Benod et al., 2014), future studies should aim to
479 identify selective ligands for TLX and determine the ability of modulating TLX activity to
480 mitigate the effects of extensive inflammation on hippocampal-associated cognition.

481

ACCE

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488

ACCEPTED MANUSCRIPT

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669 81.

670

671

672 **Table 1:** Meaningful pairwise comparisons for gene expression changes conducted using
 673 RNA sequencing (4 unique comparisons).

Experimental group	Abbreviation	n	Pairwise comparison
Wild type + PBS	WT	6	WT vs. WT + IL-1 β
TLX knockout + PBS	TLX ^{-/-}	4	WT vs. TLX ^{-/-}
Wild type + IL-1 β	WT + IL-1 β	7	WT + IL-1 β vs. TLX ^{-/-} + IL-1 β
TLX knockout + IL-1 β	TLX ^{-/-} + IL-1 β	6	TLX ^{-/-} + IL-1 β vs. TLX ^{-/-}

674

675

676 **Figure Legends**

677

678

679 **Figure 1:** Schematic of experimental design and timeline. WT, wildtype; TLX^{-/-}, TLX
 680 knockout; PBS, Phosphate buffered saline; IL-1 β , Interleukin-1 beta; WT + PBS, Wildtype
 681 PBS injected mice; WT + IL-1 β , Wildtype IL-1 β injected mice; TLX^{-/-} + PBS, TLX knockout
 682 PBS injected mice; TLX^{-/-} + IL-1 β , TLX knockout IL-1 β injected mice.

683

684 **Figure 2:** TLX^{-/-} mice display elevated transcription of inflammatory genes in the
 685 hippocampus compared to WT controls. **A)** The number of differentially expressed genes
 686 (DEGs) between all pairwise comparisons across the groups. **B)** A volcano plot depicting the
 687 number of differentially regulated genes between WT and TLX^{-/-} mice. Each gene is graphed
 688 as the fold change (log 2) versus q value (p adjusted value) for multiple comparisons. **C)**
 689 Venn diagram outlining the number of DEGs either up-regulated (green circles) or down-
 690 regulated (blue circles) across all groups. **D)** GO enrichment in biological processes for up-
 691 regulated genes comparing TLX^{-/-} to WT mice. Dotted line represents significance level, with

692 values above this line deemed significant. **E)** GO enrichment in biological processes for
693 down-regulated genes comparing TLX^{-/-} to WT mice. Dotted line represents significance
694 level, with values above this line deemed significant.

695

696 **Figure 3:** IL-1 β induces the transcription of inflammatory-related genes in WT mice similar
697 to that in TLX^{-/-} mice. **A)** Volcano plot of all DEGs when comparing WT + IL-1 β and WT
698 mice vs. WT and TLX^{-/-} mice. **B)** Venn diagram of all DEGs either up-regulated (green
699 circles) or downregulated (blue circles) and the overlap of these genes when comparing TLX^{-/-}
700 and WT mice. **C)** GO enrichment in KEGG pathway for up-regulated genes comparing the
701 overlap of WT vs. WT + IL-1 β and WT vs. TLX^{-/-} mice. The dotted line represents
702 significance level, with values above this line deemed significant. **D)** GO enrichment in
703 biological processes for up-regulated genes comparing the overlap of WT vs. WT + IL-1 β
704 and WT vs. TLX^{-/-} mice. The dotted line represents significance level, with values above this
705 line deemed significant. **E)** Venn diagram comparing the overlap in gene transcriptional
706 changes between WT vs. WT + IL-1 β and WT vs. TLX^{-/-} mice.

707

708 **Figure 4:** String analysis showing interactions between genes similarly increased in TLX^{-/-}
709 mice and WT mice injected with IL-1 β compared to WT mice that are involved in TNF
710 signalling. Line thickness between nodes indicates the strength of the evidence to support the
711 interaction.

712

713 **Figure 5:** TLX^{-/-} mice have a blunted transcriptional response to IL-1 β compared to WT mice
714 and an increased inflammatory response to cannulation surgery. **A)** Venn diagram showing
715 the total number of DEGs down-regulated when comparing WT + IL-1 β to TLX^{-/-} + IL-1 β .
716 **B)** Venn diagram showing the total number of DEGs up-regulated when comparing WT + IL-

717 1 β to TLX^{-/-} + IL-1 β mice. **C)** GO enrichment in biological processes for down-regulated
718 genes comparing the overlap of TLX^{-/-} to TLX^{-/-} + IL-1 β -treated mice. The dotted line
719 represents significant level with values above this line deemed significant.

720

721 **Figure 6: A)** STRING analysis showing interactions between genes increased in TLX^{-/-} mice
722 compared to WT mice that are involved in TNF signalling, and how TLX interacts with TNF
723 signalling. Thicker lines indicate stronger network connections between genes. Line thickness
724 between nodes indicates the strength of the evidence to support the integration. **B), C)** and **D)**
725 TNF α , IL-1 β and IL-6 mRNA expression in non-cannulated and cannulated TLX^{-/-} and WT
726 mice, n.s = non-significant (data expressed as mean \pm SEM, ***= P <0.01 ANOVA and
727 Tukey's *post-hoc* test). All data are presented as mean \pm SEM.

728

729

Ó Léime et al. 2017: Highlights

730

- TLX regulates inflammatory transcriptional profile within the hippocampus of mice.

731

- IL-1 β induces inflammatory transcriptional changes in wildtype mice.

732

- TLX knockout mice have a blunted hippocampal transcriptional response to IL-1 β .

733

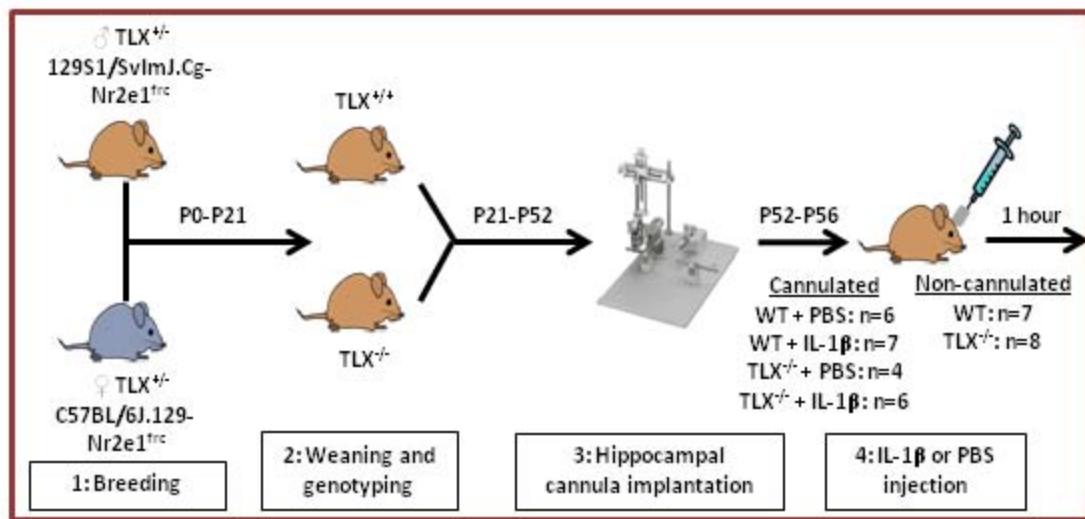
- TLX may interact with TNF signalling to regulate hippocampal inflammation.

734

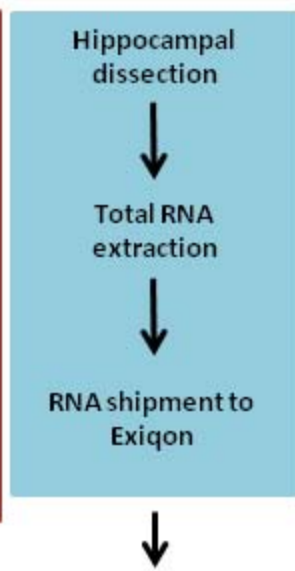
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ACCEPTED MANUSCRIPT

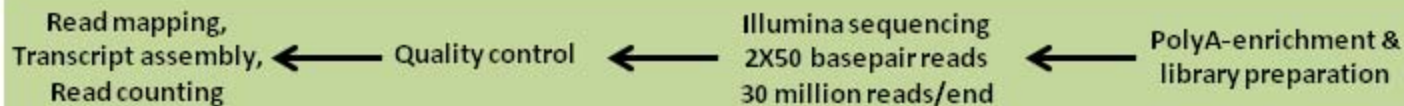
Animal breeding and stereotaxic surgery



Tissue collection

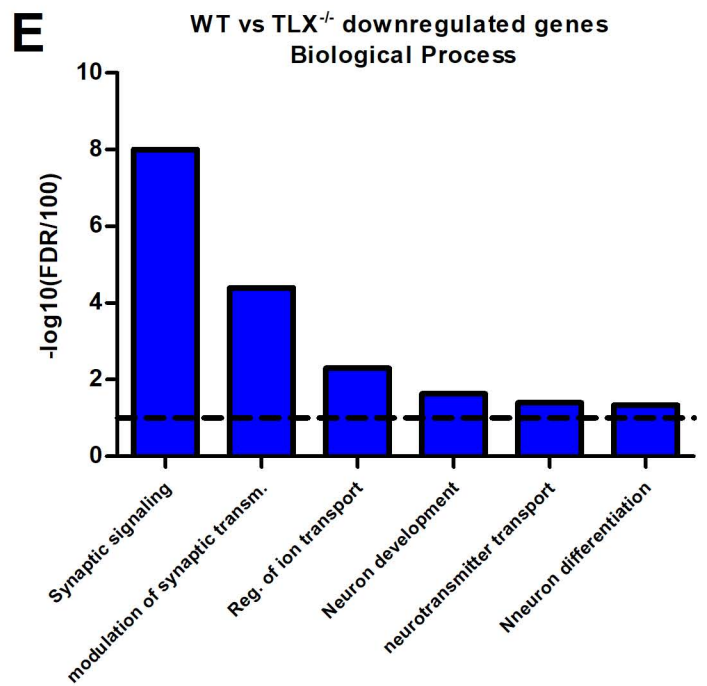
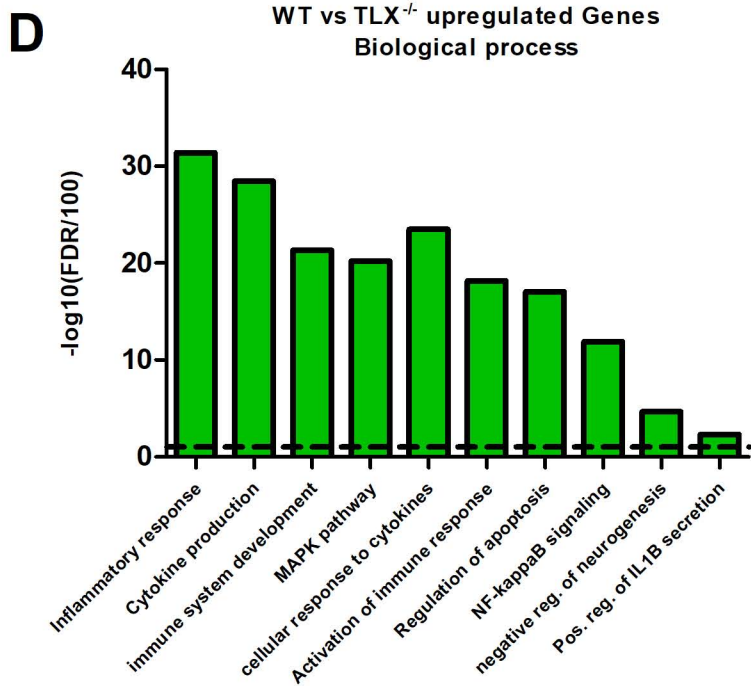
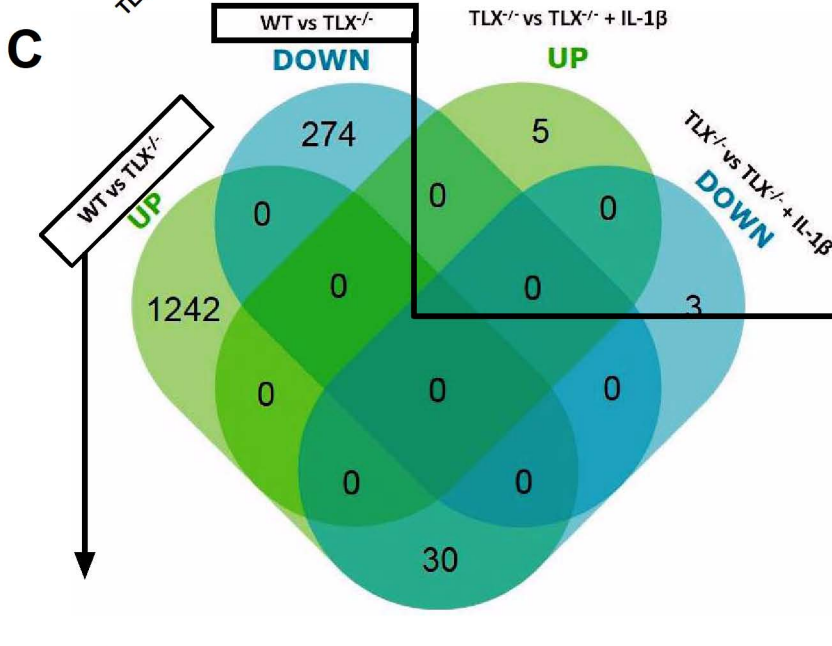
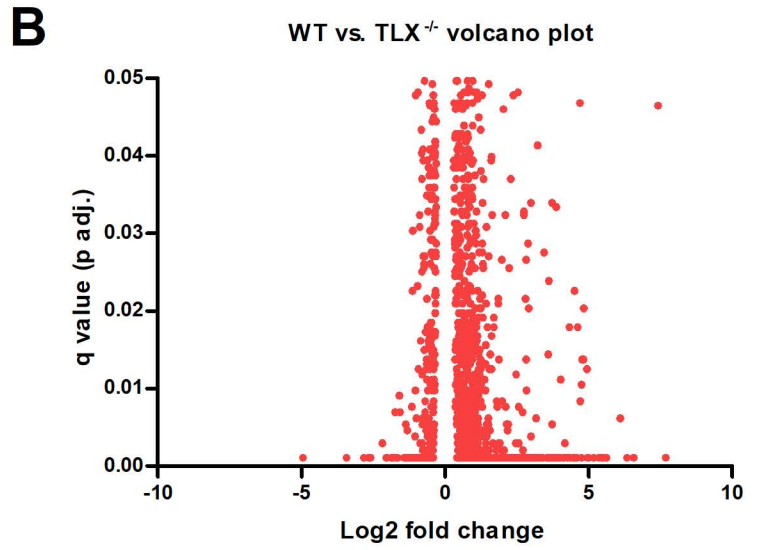
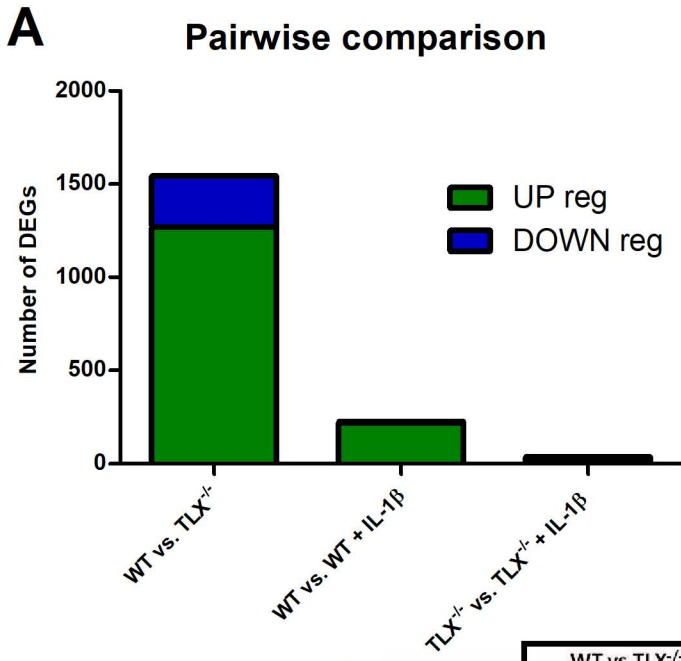


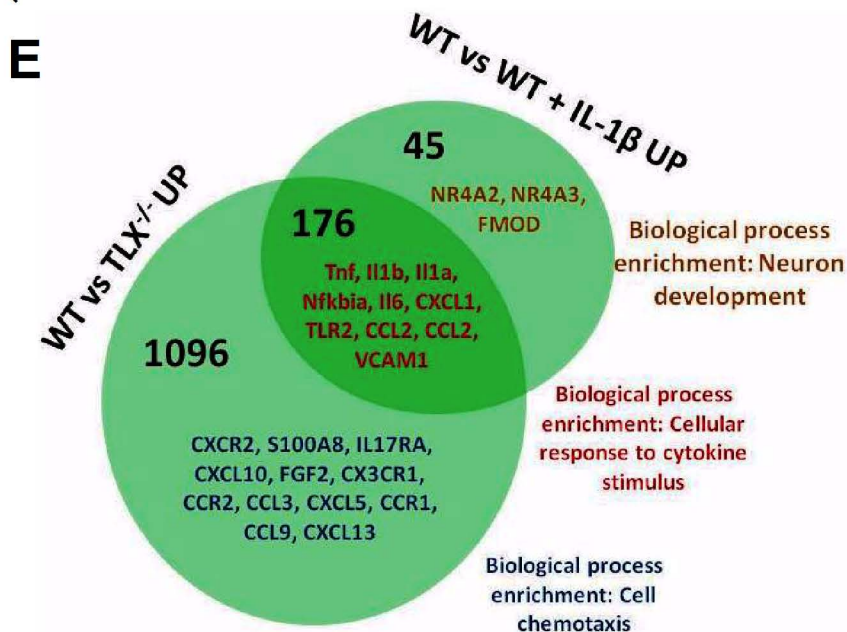
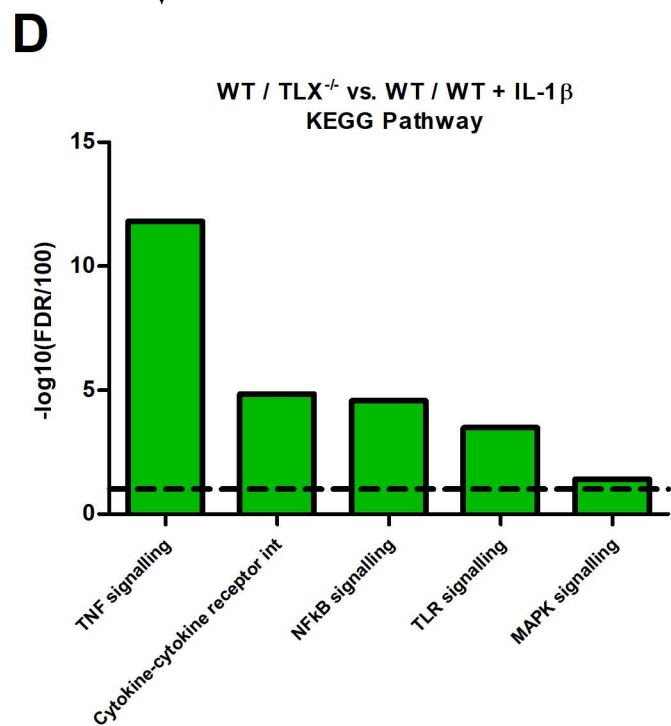
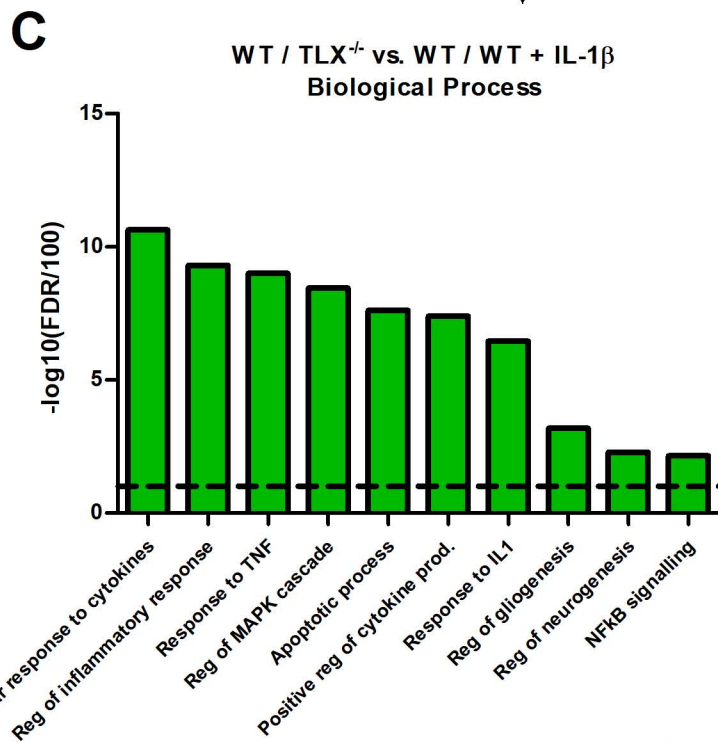
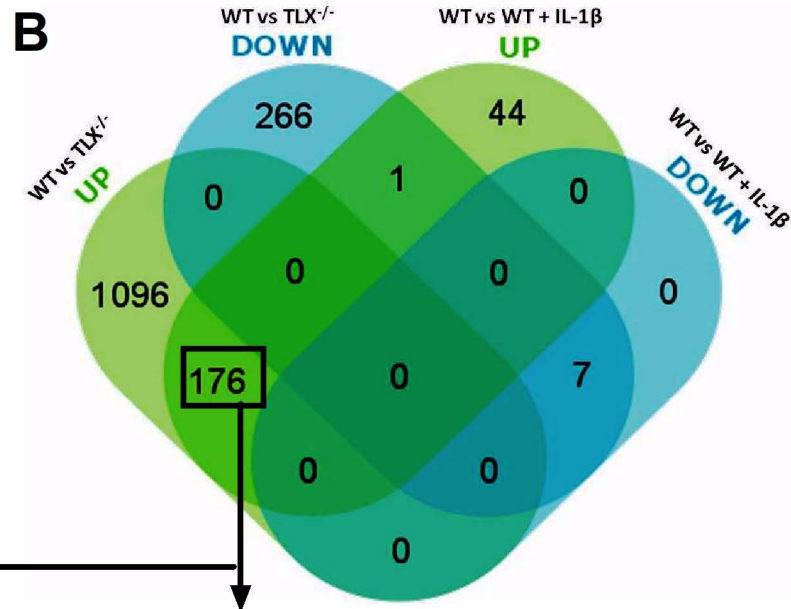
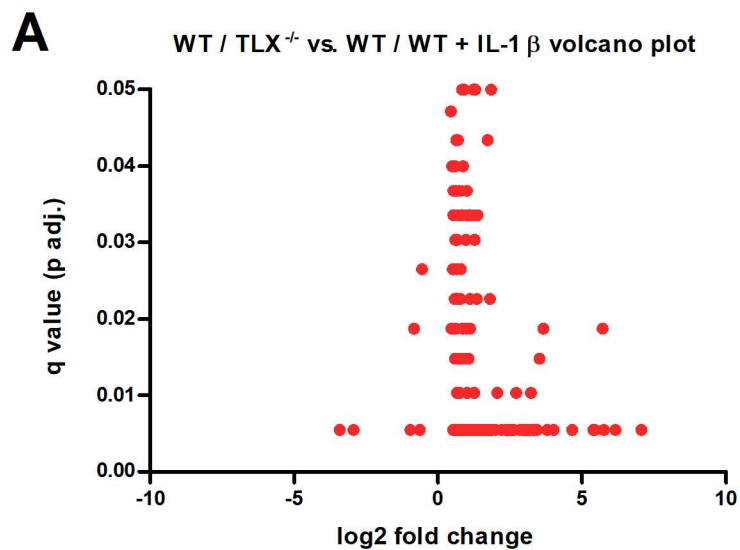
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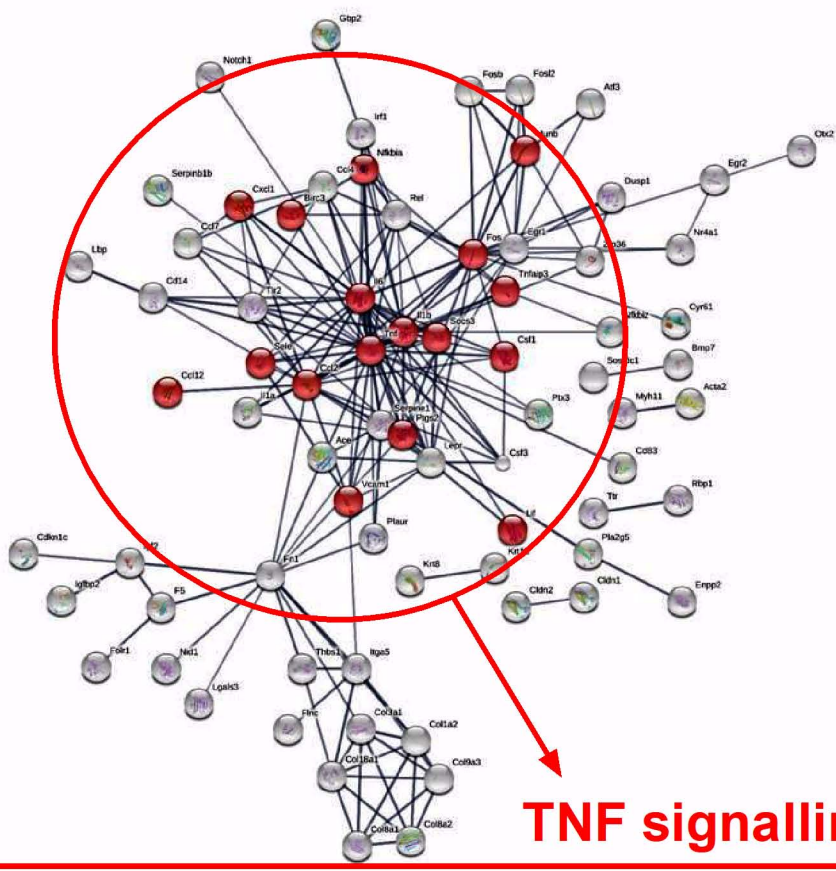
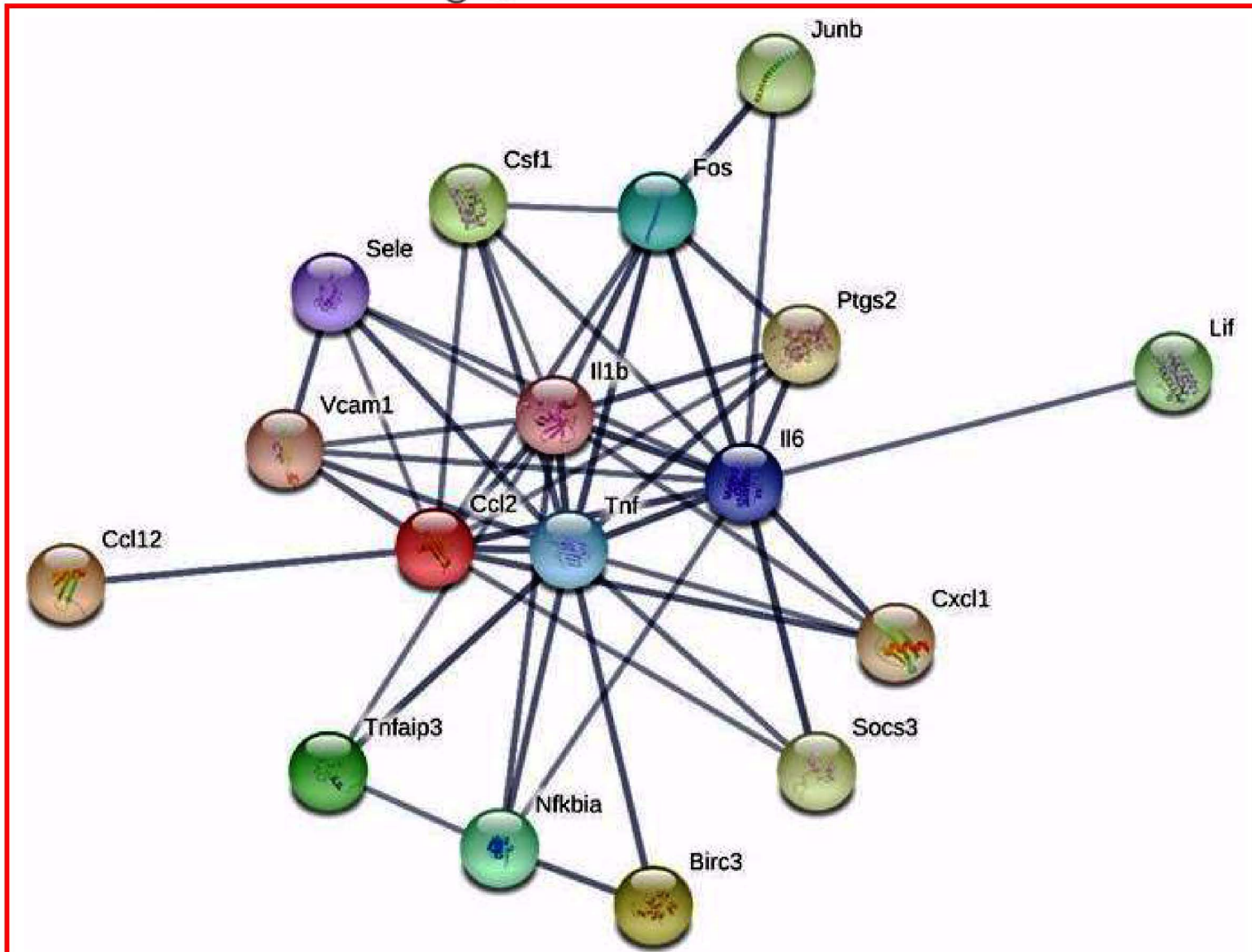


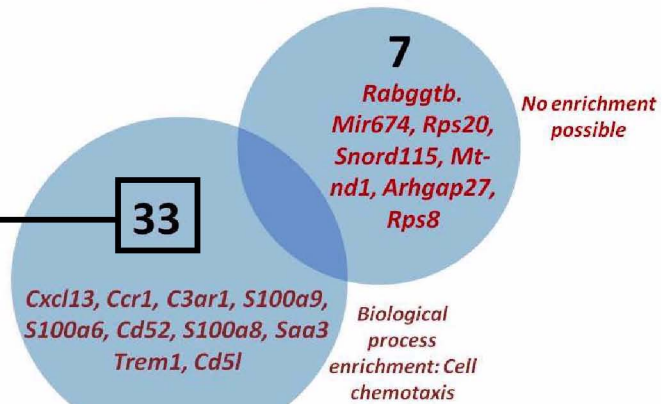
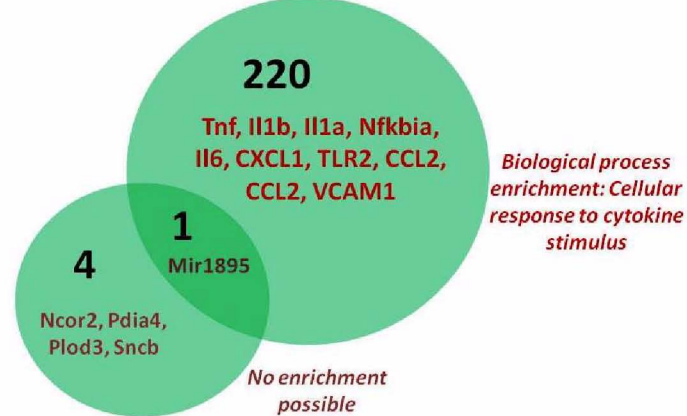
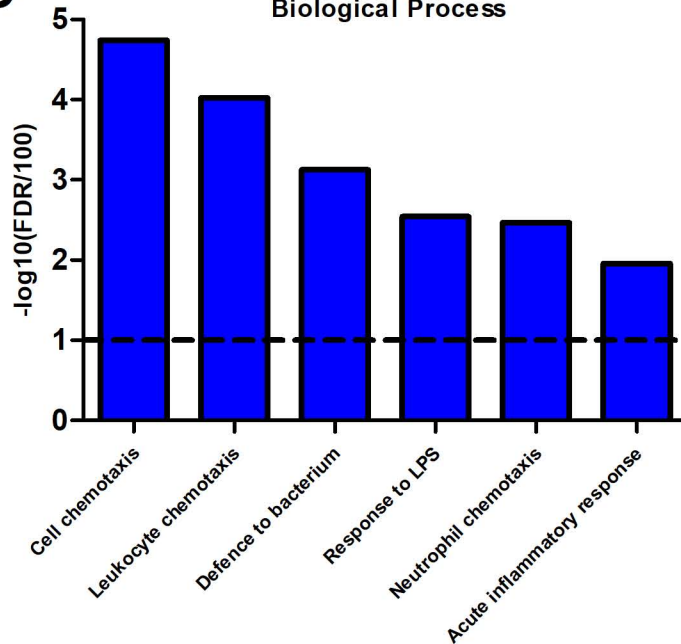
Analysis of results

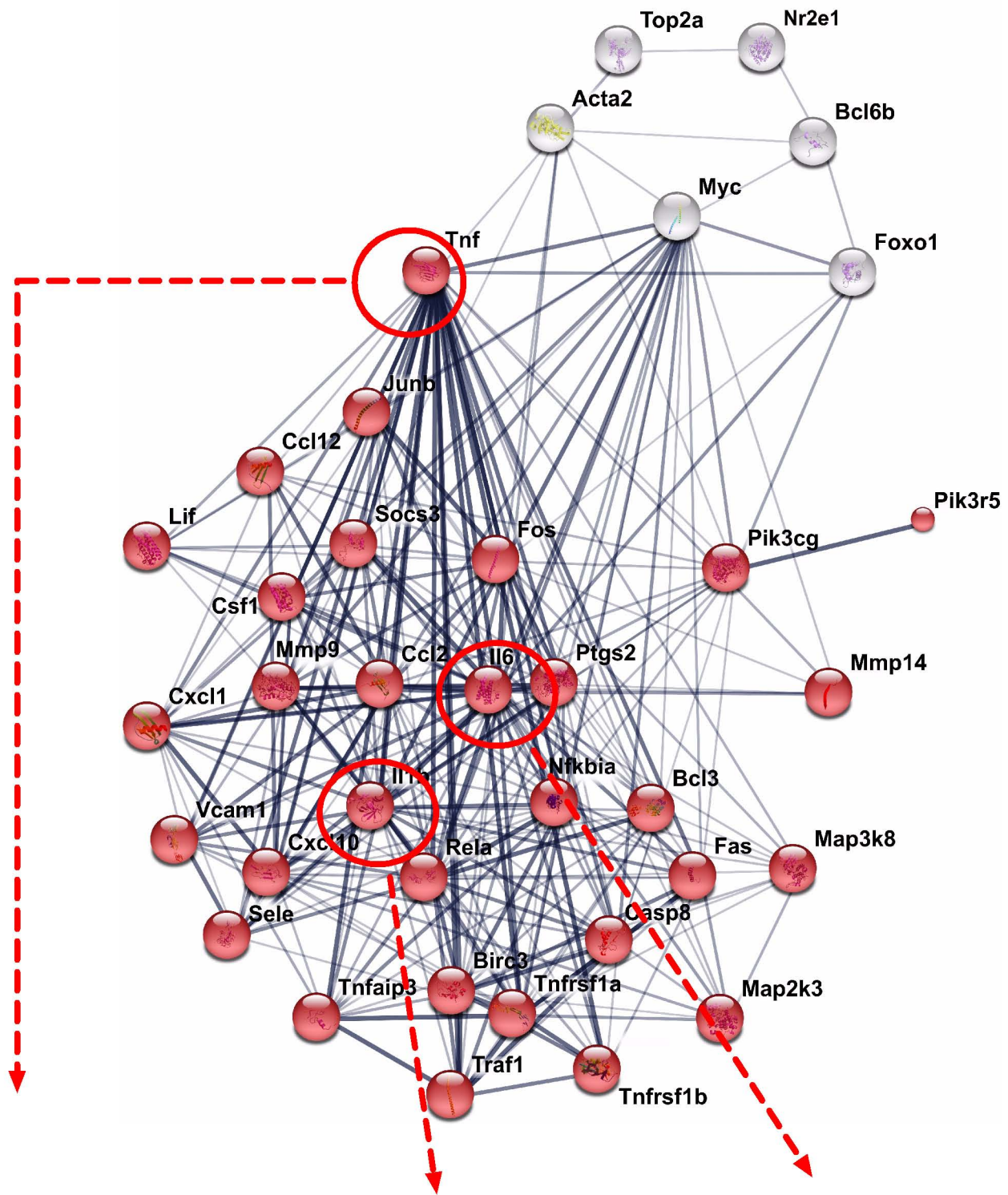
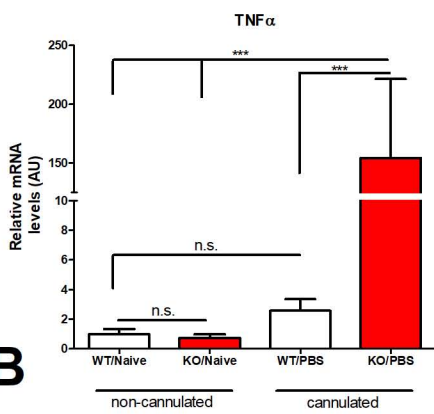
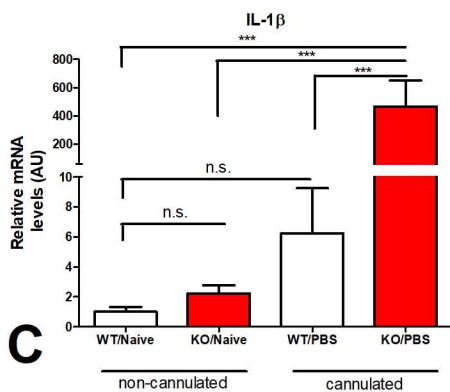






A**B****TNF signalling pathway**

AWT vs WT + IL-1 β DOWNTLX^{-/-} vs TLX^{-/-} + IL-1 β DOWN**B**WT vs WT + IL-1 β UPTLX^{-/-} vs TLX^{-/-} + IL-1 β UP**C**TLX^{-/-} vs TLX^{-/-} + IL-1 β down regulated
Biological Process

A**B****C****D**