The effect of forced exercise on knee-joints in Dio2$^{-/-}$-mice; type II iodothyronine deiodinase-deficient mice are less prone to develop OA-like cartilage damage upon excessive mechanical stress.

Nils Bomer [1,2†*], Frederique M.F. Cornelis [4†], Yolande F.M. Ramos [1], Wouter den Hollander [1], Lies Storms [4], Ruud van der Breggen [1], Nico Lakenberg [1], P. Eline Slagboom [1,2,3], Ingrid Meulenbelt [1,3‡], Rik J.L. Lories [4,5‡]

[1]Dept. of Molecular Epidemiology, LUMC, Leiden, Netherlands; [2]Integrated research of Developmental determinants of Ageing and Longevity (IDEAL), Leiden, Netherlands; [3]The Netherlands Genomics Initiative, sponsored by the NCHA, Leiden-Rotterdam, The Netherlands; [4] Laboratory of Tissue Homeostasis and Disease, Skeletal Biology and Engineering Research Centre, KU Leuven, Leuven, Belgium. [5] Division of Rheumatology, University Hospitals Leuven, Leuven, Belgium

[†]Shared first author; [‡]Shared last author

Running title: DIO2 and exercise-induced OA

Keywords: Dio2, Osteoarthritis, mechanotransduction, articular cartilage

Word count: 3027

*** Correspondening author:** Dept. Medical Statistics and Bioinformatics, Section Molecular Epidemiology, Leiden University Medical Center, LUMC Post-zone S-05-P, P.O. Box 9600, 2300 RC Leiden, The Netherlands.

Phone +31 71-5269786; Fax: +31 71-5268280; E-mail: n.bomer@lumc.nl

ABSTRACT

Objective: To further explore deiodinase iodothyronine type-2 (*DIO2*) as a therapeutic target in osteoarthritis by studying the effects of forced mechanical loading on *in vivo* joint cartilage tissue homeostasis and the modulating effect herein of *Dio2* deficiency.

Methods: Wild-type and C57BL/6-*Dio2* $^{-/-}$ -mice were subjected to a forced running regime for one hour per day for three weeks. Severity of osteoarthritis was assessed by histological scoring for cartilage damage and synovitis. Genome wide gene expression was determined in knee-cartilage by microarray analysis (Illumina MouseWG-6 v2). STRING-db analyses were applied to determine enrichment for specific pathways and to visualize protein-protein interactions.

Results: In total, 158 probes representing 147 unique genes showed significantly differential expression with a fold-change ≥ 1.5 upon forced exercise. Among these are genes known for their association with OA (e.g. *Mef2c, Egfr, Ctgf, Prg4* and *Ctnnb1*), supporting the use of forced running as an OA-model in mice. *Dio2*-deficient mice showed significantly less cartilage damage and signs of synovitis. Gene expression response upon exercise between wild-type and knockout-mice was significantly different for 29 genes.

Conclusion: Mice subjected to a running regime have significant increased cartilage damage and synovitis scores. Lack of *Dio2* protected against cartilage damage in this model and was reflected in a specific gene expression profile, and either mark a favorable effect in the Dio2-knockout (e.g. *Gnas*) or an unfavorable effect in wild-type cartilage homeostasis (e.g. *Hmbg2* and *Calr*). These data further support *DIO2* activity as a therapeutic target in OA.

INTRODUCTION

Osteoarthritis (OA) is a prevalent, complex, chronic and disabling disease of articular joints, characterized by progressive destruction of joint cartilage, remodeling of the subchondral bone, formation of osteophytes and synovitis [1, 2]. It causes pain and disability to an increasing proportion of the population and is associated with the obesity pandemic, aging of the population and not in the least by improved survival of patients with cardiovascular or oncological health problems. All together this imposes a large and growing social and economic burden [3, 4]. The development of novel therapeutic approaches is therefore urgently needed and should be based on insights into the underlying disease mechanisms [5-7]. Several genetic studies identified robust signals for OA susceptibility [8-13], that suggest specific genes, involved in cartilage development and growth, to play a key role in the OA disease process [14, 15]. Notable example of such OA susceptibility alleles, are the C-variant of the rs225014 single nucleotide polymorphism (SNP) located in the coding region of the deiodinase iodothyronine type-2 (D2) gene *(DIO2*) [8, 16] and the rs945006 SNP in the deiodinase iodothyronine type-3 (D3) gene *(DIO3*), another deiodinase with a counter regulatory function for *DIO2* [16]. For together, D2 and D3 primarily regulate the bio-availability of intracellular thyroid hormone in specific tissues such as the growth plate, but not systemically. The deiodinase type 2 protein (D2) catalyzes the conversion of intracellular inactive thyroxine (T4) to active thyroid hormone (T3). During skeletal development, this conversion plays a critical role in the process of endochondral bone formation by facilitating terminal maturation of hypertrophic chondrocytes subsequently leading to breakdown of the cartilage matrix and replacement by bone. This process is essential in skeletal

development and growth but loss of the chondrocyte's maturational arrested characteristics is considered deleterious for postnatal articular cartilage.[17, 18] *DIO2* mRNA and D2 protein levels are highly upregulated in human osteoarthritic cartilage as compared to healthy cartilage [19-21], suggesting that in disease *DIO2* contributes to the loss of the highly specialized maturational arrested state of articular chondrocytes [17]. Cartilage-specific over-expression of human *DIO2* in rats was associated with increased damage to the articular cartilage in a surgical OA model. However, this was without clear evidence that hypertrophy of chondrocytes plays an essential role and rather pointing towards increased tissue destructive enzyme activity and enhanced expression of IL-1 target genes [22]. Upregulation of *DIO2* expression in a human *in vitro* model resulted in a marked reduction of the capacity of chondrocytes to deposit ECM components, including type II and type X collagen, while inducing OA-specific markers of cartilage matrix degeneration and mineralization [18]. In contrast, pharmalogical inhibition of *DIO2* increased the expression of collagens and aggrecan without clear effect on hypertrophy or tissue destructive enzymes. These accumulating data suggest that D2 inhibition and/or modulation may become a therapeutic target, but the in vivo impact of D2 loss of function in joint biology and disease remains largely unknown in particular at the molecular level.

In this study, we set out to study the molecular network of *Dio2* in the healthy and challenged joint. We performed genome wide expression analyses in aging wild-type and *Dio2*$^{-/-}$ mice, including groups exposed to a moderately strenuous running regime. Our results indicate that *Dio2* is effectively involved in specific gene networks that can be associated with osteoarthritis and provide further insights into the complex molecular

94 interactions involved in healthy and diseases articular cartilage.

MATERIALS AND METHODS

Animal experiments

Dio2$^{-/-}$ mice were a kind gift of Dr. V. Galton (Dartmouth Medical School, NH, USA)[23] and were backcrossed onto the C57Bl/6 background. All experiments were approved by the Ethics Committee for Animal Research (KU Leuven, Belgium). Four to 6 months old male *Dio2*$^{-/-}$ (n=22) and wild-type mice (n=30) ran for 3 weeks 1 hour/day, 5 days/week, at a speed of 11 m/min and with an inclination of 5°. For additional details, see the Online Methods.

Histological assessment of osteoarthritis

Right knees were fixed overnight at 4°C in 2% formaldehyde, decalcified for 3 weeks in 0.5M EDTA pH 7.5 and embedded in paraffin. Severity of disease was determined by histological scores on hematoxylin/eosin or Safranin O stained sections (5µm) throughout the knee (5 sections at 100µm distance). For additional details on histological assessment and statistical analyses, see the Online Methods.

RNA isolation

Snap frozen cartilage of the left knees was powderized using a Retsch Mixer Mill 200 under cryogenic conditions. RNA was isolated and washed using the RNeasy mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. RNA quality was assessed using a Biocore lab-on-a-chip and quantity was assessed using a Nanodrop spectrophotometer. For additional details, see the Online Methods.

Microarray analysis

Complementary DNA synthesis, amplification, biotin labeling and hybridization onto the microarrays was performed using the Ambion TotalPrep-96 RNA amplification kit (Life Technologies, Bleiswijk, The Netherlands) according to manufacturer's protocol. After hybridization on Illumina MouseWG-6 v2 BeadChip microarrays (Illumina, Eindhoven, The Netherlands) the slides were scanned with the Illumina Beadscanner 500GX. For additional details on microarray handling and data analyses, see the Online Methods.

Pathway analysis and protein-protein interaction networks

Gene enrichment among the genes with significant differential expression was performed with STRING (Search Tool for the Retrieval of Interacting Genes/Proteins 9.1) [24]. Pathways with a P-value ≤ 0.05 after FDR correction were considered significant. Enrichment in protein-protein interactions was also analyzed using the STRING database. For additional details, see the Online Methods

Quantitative RT-PCR assays (validation)

Validation of the microarray results was performed by quantitative real-time PCR. 500 ng of total RNA was processed with the First Strand cDNA Synthesis Kit according to the manufacturer's protocol (Roche Applied Science, Almere, The Netherlands) upon which cDNA was diluted 5 times. RT-qPCR measurements were performed on the Roche Lightcycler 480 II, using Fast Start Sybr Green Master reaction mix according to the manufacturer's protocol (Roche Applied Science). For additional details, see the Online Methods.

RESULTS

Reduced severity of OA after forced running in *Dio2*$^{-/-}$ mice

To study the role of *Dio2* in joint homeostasis, *Dio2*$^{-/-}$ and wild-type mice were studied in a forced-exercise setup and compared to non-running mice (**Figure 1**). No striking developmental skeletal phenotype appears present in the *Dio2*$^{-/-}$ mice [25] and our observations in our mouse colony are in agreement with these findings. Wild-type and *Dio2*$^{-/-}$ mice displayed a similar running behavior. As shown in figure 1, overall group analysis indicated a significant difference between wild-type- and knockout-mice with respect to cartilage damage ($P = 0.0006$) and synovial hyperplasia ($P = 0.0536$). No specific effects within wild-type- or knoukout-mice of exercise were found, nor interaction between genotype and exercise. Sidak's multiple comparison test indicated that the effect was determined by the difference in genotype in the exercise group ($P < 0.0001$ for cartilage damage – 95% CI of the difference between the means (0.1820 to 0.5820) and $P = 0.0184$ for synovial hyperplasia – 95% CI of the difference between the means (0.03661 to 0.4627)) (**Figure 1**).

Microarrays: Differential expression in knee-joints upon forced exrecise

To identify genes responsive to the forced running regime in mice, genome wide gene expression in knee-cartilage was studied by microarray. Gene expression was detected before and after the running regime in interaction with the genetic background (wild-type and *Dio2*$^{-/-}$-mice). **Figure 2** shows a schematic overview of the study strategy.

After quality control and normalization, 20872 of the 45281 probes of the BeadChip array were used for analyses. Microarrays on human articular cartilage samples showed

corresponding numbers for specific expression [26]. In order to detect all articular cartilage genes that are responsive to the applied running regime, we performed differential expression analyses in 3 strata: total (A), *Dio2*$^{-/-}$ (B) and wild type (C) group (**Figure 2)**. For each strata significance was adjusted for multiple testing according to the "Benjamini and Hochberg" method. In the total and wild type strata, we independently detected respectively 1862 and 892 probes representing 1699 and 830 genes (558 overlapping) that were significantly differentially expressed between the forced exercise and the control group. In contrast, in the *Dio2*$^{-/-}$-stratum, this comparison did not result in any significantly differentially expressed probes after multiple testing. Among the differentially expressed probes in the total and wild-type groups, we observed respectively 102 and 97 probes with a fold-change of 1.5 and higher that together consisted of 158 significant unique probes, representing 147 unique transcripts that were responsive to the running regime (**Figure 2** and **Supplement table S1**). Notably, of the 158 differentially expressed probes, 31 were up-regulated (20%) and 127 showed down-regulation (80%). Among the 147 unique transcripts, we observed genes known for a potentially role in cartilage homeostasis and disease, such as Proteoglycan 4, also known as Lubricin (*Prg4*, 1.95-fold down; P=4.44 x 10^{-2})[27], myocyte enhancer factor 2C (*Mef2c*, 1.9-fold down; P=3.05 x 10^{-2})[28, 29] and connective tissue growth factor (*Ctgf*, also known as Ccn2, 1.79-fold down; P=1.32 x 10^{-2})[30].

Technical validation of our microarray results was carried out by RT-qPCR in the discovery cohort (20 samples previously included in the microarray analyses). Genes to be validated were selected based on P-value and fold difference (differential reaction on mechanical stress). Here, 19 out of the 20 genes tested showed similar effect sizes and

direction as the original data, only *Pfn1* did not show similar effect of expression (see **Supplementary table S2**).

Pathway analyses: Protein-protein interactions and gene enrichment analyses

To identify functional connections between the genes that were found as differentially expressed (Table S1) we determined the protein-protein interaction prediction using STRING-db. Analyzing the 147 differentially expressed genes, representing 124 unique proteins, showed enrichment of interaction in the subset of proteins (P=1.61x10^{-4}; 63 interactions; **Figure S1**), indicating that the gene-products that were identified are closely interacting as a response to the forced running regime.

Furthermore, we assessed whether the differentially expressed genes occurred more frequently in a specific pathways in mice. Gene enrichment analyses revealed enrichment for biological processes (GOTERM_BP_FAT) concerning, amongst others, "regulation of metabolic process" (P= 0.0063; GO: 0019222; N=46, e.g. *Calr, Igfbp5, Sox4* and *Tcf4*), "skeletal system development" (P= 0.00395; GO:0001501; N=12, e.g. *Ctnnb1, Ctgf, Sox4* and *Mef2c*), "anatomical structure development" (P= 0.039; GO:0048856; N=36, e.g. *Sox4, Notch3, Egfr, Ctnnb1* and *Mef2c*), and "regulation of response to stress" (P=0.0171; GO:0080134; N=14; *Setd8*, *Ankrd1*, *Mef2c, Egfr* and *Ctgf*) with application of a false discovery rate algorithm.

To connect thyroid hormone signaling with the effects of forced mechanical stress, we added genes involved in intracellular thyroid hormone signaling (*Dio2, Thra, Thrb* and *Rxra*) into the protein-protein interaction prediction. The network that is formed (**Figure 3**) shows thyroid signaling genes/proteins to be incorporated into the large network, as

previously seen (**Figure S1**). Thyroid signaling was found to interact directly with the differential expressed genes through, *Ctgf* (A) and *Egfr* (B), via thyroid receptor alpha (*Thra*) and retinoid x receptor (*Rxra*), both known factors in the development of OA[30-32].

Genes differentially expressed in *Dio2*$^{-/-}$ mice compared to wild type mice upon a forced running regime.

Since we found differences in differential expression patterns between knockout and wild type mice upon the forced running regime, we assessed which of the 147 differentially expressed genes (**Figure 1** and **Supplementary Table S1**) showed significant interaction between running regime and genetic background based on nominal P-values. In total 29 probes, representing 29 genes were found be significantly different expressed in knockout-mice as compared to wild type-mice when undergoing a forced running regime (**Table 1**). The significant differences in effect (beta-values), as a result of the running regime, between the knockout- and the wildtype-group can be devided in 3 sub-groups. Each group shows a different effect-size based on "genotype" for the 29 genes found to show a significant interaction. We identified 4 genes that showed no differential expression upon running in wild-type mice, but are differentially expressed (down-regulated) upon running in *Dio2*$^{-/-}$ mice (**Group 1**; e.g. *Gnas* and *Rhbdl2*). In total 16 genes only showed differential expression in wild-type mice and not in the *Dio2*$^{-/-}$ mice (**Group 2**; e.g. *Hmgb2, Calr* and *Lbh*) whereas, 9 genes showed significant differential effect-sizes between wild-type and knockout mice, but having the same direction of effect (**Group 3**; e.g. *Sox4* and *Socs2*). Depending on the gene-expression pattern the genes in

the different groups could mark a favorable effect in the *Dio2*-knockout mice (Group 1) or an unfavorable effect in wild type cartilage homeostasis (Group 2).

Tabel 1: Genes that show significant differential expression upon the running regime between wild-type- and knockout-mice (interaction)

	Run vs NoRun in Knockout			Run vs NoRun in Wildtype			Interaction
Symbol	**Beta**	**St. Error**	**P-value**	**Beta**	**St. Error**	**P-value**	**P-value**
Group 1: Genes with an effect of the running regime in knockout- but not in wild-type-mice							
Hist1h2an	-1,024	0,2603	0,000	-0,186	0,1088	0,087	**0,0030**
Gnas	-0,956	0,2318	0,000	-0,258	0,1473	0,080	**0,0111**
Rhbdl2	-1,135	0,2176	0,000	-0,349	0,2638	0,185	**0,0215**
E130112E08Rik	-1,041	0,2981	0,000	-0,26	0,2171	0,231	**0,0343**
Group 2: Genes with an effect of the running regime in wild-type- but not in knockout-mice							
Anxa11	0,02	0,0852	0,818	-0,636	0,0668	0,000	**0,0000**
Setd8	-0,006	0,0864	0,943	0,725	0,1044	0,000	**0,0000**
Phdla1	-0,152	0,1019	0,136	-0,591	0,1011	0,000	**0,0022**
Hmgb2	0,131	0,1439	0,363	0,666	0,1476	0,000	**0,0095**
Calr	-0,086	0,1561	0,581	-0,664	0,1658	0,000	**0,0111**
Gdi1	0,313	0,2297	0,173	0,923	0,1446	0,000	**0,0247**
Lbh	-0,13	0,0682	0,057	-0,889	0,0761	0,000	**0,0000**
Fmo5	-0,028	0,1028	0,784	-0,622	0,0848	0,000	**0,0000**
Igsf4a	-0,171	0,0978	0,081	-0,789	0,1221	0,000	**0,0001**
Fcho1	0,132	0,1144	0,250	0,665	0,1106	0,000	**0,0008**
Serpinb1a	0,396	0,3687	0,283	-0,877	0,1145	0,000	**0,0010**
Mtpn	-0,082	0,1084	0,451	-0,594	0,1121	0,000	**0,0010**
Rbm47	-0,09	0,075	0,229	-0,592	0,1011	0,000	**0,0013**
LOC433464	-0,086	0,1638	0,599	-0,675	0,0788	0,000	**0,0015**
Rer1	-0,185	0,1714	0,281	-0,611	0,1121	0,000	**0,0195**
Hist1h4i	0,113	0,2763	0,684	-0,811	0,1011	0,000	**0,0214**
Group 2: Genes with an effect of the running regime in both wild-type- and knockout-mice							
Mll5	-1,378	0,2306	0,000	-0,439	0,1184	0,000	**0,0003**
Chd1	-1,178	0,2311	0,000	-0,307	0,1125	0,006	**0,0007**
Septin11	-0,28	0,0887	0,002	-0,671	0,0918	0,000	**0,0022**
Sox4	-0,479	0,1399	0,001	-0,969	0,1064	0,000	**0,0053**
Gins1	0,771	0,305	0,011	1,596	0,0735	0,000	**0,0085**
Socs2	-0,337	0,1132	0,003	-0,713	0,0881	0,000	**0,0088**
C330023M02Rik	-0,308	0,1498	0,040	-0,678	0,054	0,000	**0,0202**
Hgsnat	-0,29	0,1274	0,023	-0,621	0,1001	0,000	**0,0410**
Rap2c	-0,385	0,0743	0,000	-0,628	0,0955	0,000	**0,0449**

[1]The beta-value is representing the difference in fold change between the running group and the non-running group. The direction of the beta-value is how the running-group is different from the non-running group.

[2]The standard error is showing the variation of the beta-value between samples.

[3]The nominal P-value is the P-value of that the single probe that was calculated to tell whether based on this single probe, the expression levels between runners is different of that of non-runners.

[4]The Interaction P-value is a summarizing value that tells whether the difference caused by exercise is significantly different between the knockout- and the wild-type-stratum

DISCUSSION

Dio2-deficient mice showed less cartilage damage and reduced severity of synovitis in a treadmill running model of OA. The absence of significant differential gene expression between the running and no-exercise group in *Dio2*$^{-/-}$ mice suggests that degenerative pathways are not activated in this knockout strain despite the biomechanical burden that is imposed. These data provide novel support for inhibition of *DIO2* as a therapeutic strategy in OA, in particular since no striking developmental skeletal phenotype appears present in the *Dio2*$^{-/-}$ mice (results not shown). Upon forced mechanical loading, wild type mice showed clear signs of OA and differential expression of genes associated with the disease, supporting the use of forced running as an OA-model in mice. A subset of the genes was found directly interacting with thyroid signaling through *Thra, Rxra* and *Dio2*, depicted in **Figure 3.** This indicates the importance of thyroid hormone signaling as a regulatory system in the response to stress and, when suppressed, for the maintenance of cartilage homeostasis.

With the differential expressed genes in the wild-type stratum and the combined knockout/wild-type stratum, pathway enrichment was found for expected biological processes, such as “skeletal system development”, but also for processes involved in “regulation of response to stress”. Notably, genes overlapping between these enriched processes as well as being ‘nodes’ in the recognized protein interaction networks (*Egfr (B) and Ctnnb1(C)*; **Figure 3**) are well known for their association with OA [30, 33, 34]. These could, therefore, point at important modulators affecting the propensity to develop OA upon mechanical stress and as such at potential drugable targets for novel therapeutic approaches.

In contrast, when assessing gene expression in the knockout-group alone, we found no significant differential gene expression upon severe mechanical loading, after multiple testing adjustment. Taken together with the pathology observations these data indicate that the repression of *Dio2* is beneficial against the development of cartilage damage upon mechanical stress. This effect could theoretically also be caused by a difference in power between the different strata (wild-type and knockout). However, since the comparison made in knockout animals (6 running versus 3 controls) is comparable to the wildtype animals (8 running versus 3 controls), this is unlikely to explain the complete absence of a significant differentially expressed gene in the knockout stratum.

Looking at the effect of *Dio2*-deficiency on gene expression, we found that the gene expression response upon exercise between wild-type and knockout-mice was significantly different for 29 genes with nominal significance, of the total set of 147 differentially expressed genes. Genes categorized in group 1 (**Table 1**), showing no differential expression upon forced running in wild-type mice, but are differentially expressed with nominal significance in *Dio2*$^{-/-}$ mice (*E130112E08Rik, Gnas, Hist1h2an and Rhbdl2*) could represent the respective favorable effects. *Gnas,* for example, is involved in skeletal development (GO:0001501) and complex skeletal disorders such as Albright Hereditary Osteodystrophy that can lead to early OA [35]. *Gnas* upregulation was found directly regulating hypertrophic differentiation of growth plate cartilage *in vivo* [36]. The knockout-specific down-regulation of *Gnas* upon forced running could be a protective mechanism within the *Dio2* knockout mouse.

We hypothesize that the sixteen genes within group 2, only showing differential expression in wild-type mice and not in the knockout-group (e.g. *Hmgb2* and *Calr*),

represent the unfavorable, damaging effects of forced running in wild type mice. *Hmgb2* was shown to be expressed at higher levels in human MSCs as compared to human articular chondrocytes and the expression declined during chondrogenic differentiation of MSCs [37]. The up-regulation of *Hmgb2* seen in wild type-mice upon forced running could be explained as a marker that changes in the differentiation status of the chondrocytes are occurring. A second interesting member of this group is calreticulin (*Calr*). *Calr* interacts with the glucocorticoid receptor (GR). It may also interact with other steroid receptors or thyroid receptors in a similar way [38]. Furthermore, *Calr* was shown to be involved in cartilage thinning of mandibular cartilage in a rat model that studied the effects of compressive mechanical loading [39]. In our forced running experiment we see a similar induction of *Calr* expression upon stress, but not in the *Dio2* knockout-mice, possibly giving the knockout-mouse an advantage against the formation of OA-like degradation or cartilage thinning.

Nine additional genes showed significant differential effect-sizes between wild-type and knockout mice, albeit the same direction of effect (e.g. *Sox4* and *Socs2*). Notable within this third group (**Table 3**) is *Sox4*, which was previously found to be expressed very early during chondrogenesis [40], much earlier than the well defined *Sox5* and *Sox6* [41]. Furthermore, it was shown that expression of *Sox4* could be stimulated by adding physiological concentrations of human parathyroid hormone (*PTH*), indicating involvement of the *PTH/PTHrP* receptor [42]. Here we show that *Sox4* expression is influenced by the absence of *Dio2*. In the knockout-mice the expression of *Sox4* is 2 times less down-regulated upon running when compared with the control group. How this

315 influences the structural integrity of articular cartilage remains unknown. Other genes in
316 the list have no straight-forward connection to OA based on current literature.
317 In conclusion, in the current paper we find that *Dio2* deficiency has a protective effect on
318 the homeostasis of articular cartilage in the knee-joints of mice undergoing a forced
319 running regime. This is consistent with our earlier findings, showing that
320 pharmacological inhibition of deiodinases in a human *in vitro* chondrogenesis model has
321 a beneficial effect on the early formation and maintenance of articular cartilage ECM [18].
322 It is therefore hypothesized that control of thyroid hormone signaling, both during
323 development and adult cartilage maintenance, is essential to ensure normal bone and
324 cartilage homeostasis, and that it could act as the master-switch that forces maturational
325 arrested chondrocytes to re-activate the endochondral ossification process, leading to
326 articular cartilage destruction. Our results show that interfering with intracellular thyroid
327 hormone levels could be a powerful way to oppose the pathological events that are
328 occurring in OA.
329

330 **ACKNOWLEDGEMENTS:**
331 We acknowledge support by Treat~OA and IDEAL, which are funded by the European
332 Union's Seventh Framework Program (FP7/2007-2011) under respective grant agreement
333 nos. 200800 and 259679. Furthernore, this work was supported by a grant from FWO
334 Vlaanderen (Flanders Research Foundation) G.0828.11N and by the Dutch Arthritis
335 Association. The funders had no role in study design, data collection and analysis,
336 decision to publish or preparation of the manuscript.
337

COMPETING INTEREST:

The authors declare no competing financial, personal, or professional interests.

AUTHOR CONTRIBUTIONS:

Conceived and designed the experiments: NB; FMFC; PES; IM; RJLL

Performed the experiments: NB; FMFC; LS; RvdB; NL;

Analyzed the data: NB; FMFC; YFMR; WdH; IM; RJLL

Wrote the manuscript: NB; FMFC; IM; RJLL

Critically reviewed the manuscript: All authors

REFERENCES:

Reference List

1 Bijlsma JW, Berenbaum F, Lafeber FP. Osteoarthritis: an update with relevance for clinical practice. *The Lancet* 1918;**377**:2115-26.

2 Loeser RF, Goldring SR, Scanzello CR, Goldring MB. Osteoarthritis: A disease of the joint as an organ. *Arthritis & Rheumatism* 2012;**64**:1697-707.

3 Goldring MB, Marcu KB. Cartilage homeostasis in health and rheumatic diseases. *Arthritis Res Ther* 2009;**11**:224.

4 Loeser RF, Goldring SR, Scanzello CR, Goldring MB. Osteoarthritis: A disease of the joint as an organ. *Arthritis & Rheumatism* 2012;**64**:1697-707.

5 Haq I, Murphy E, Dacre J. Osteoarthritis. *Postgrad Med J* 2003;**79**:377-83.

6 Aigner T, Rose J, Martin J, Buckwalter J. Aging theories of primary osteoarthritis: from epidemiology to molecular biology. *Rejuvenation Res* 2004;**7**:134-45.

7 Woolf AD, Pfleger B. Burden of major musculoskeletal conditions. *Bull World Health Organ* 2003;**81**:646-56.

8 Meulenbelt I, Min JL, Bos S, Riyazi N, Houwing-Duistermaat JJ, van der Wijk HJ, *et al.* Identification of DIO2 as a new susceptibility locus for symptomatic osteoarthritis. *Hum Mol Genet* 2008;**17**:1867-75.

366 9 Loughlin J, Dowling B, Chapman K, Marcelline L, Mustafa Z, Southam L, *et al.*
367 Functional variants within the secreted frizzled-related protein 3 gene are associated
368 with hip osteoarthritis in females. *Proc Natl Acad Sci U S A* 2004;**101**:9757-62.

369 10 Miyamoto Y, Mabuchi A, Shi D, Kubo T, Takatori Y, Saito S, *et al.* A functional
370 polymorphism in the 5' UTR of GDF5 is associated with susceptibility to
371 osteoarthritis. *Nat Genet* 2007;**39**:529-33.

372 11 Miyamoto Y, Shi D, Nakajima M, Ozaki K, Sudo A, Kotani A, *et al.* Common
373 variants in DVWA on chromosome 3p24.3 are associated with susceptibility to
374 knee osteoarthritis. *Nat Genet* 2008.

375 12 Southam L, Chapman K, Loughlin J. Genetic association analysis of BMP5 as a
376 potential osteoarthritis susceptibility gene. *Rheumatology (Oxford)* 2003;**42**:911-2.

377 13 Evangelou E, Valdes AM, Castano-Betancourt MC, Doherty M, Doherty S, Esko T,
378 *et al.* The DOT1L rs12982744 polymorphism is associated with osteoarthritis of the
379 hip with genome-wide statistical significance in males. *Ann Rheum Dis* 2013.

380 14 Reynard LN, Loughlin J. The genetics and functional analysis of primary
381 osteoarthritis susceptibility. *Expert Reviews in Molecular Medicine* 2013;**15**:null.

382 15 Cornelis FM, Luyten FP, Lories RJ. Functional effects of susceptibility genes in
383 osteoarthritis. *Discov Med* 2011;**12(63)**:129-39.

384 16 Meulenbelt I, Bos SD, Chapman K, van der BR, Houwing-Duistermaat JJ, Kremer
385 D, *et al.* Meta-analyses of genes modulating intracellular T3 bio-availability reveal

a possible role for the DIO3 gene in osteoarthritis susceptibility. *Ann Rheum Dis* 2011;**70**:164-7.

17 Lories RJ, Luyten FP. The bone-cartilage unit in osteoarthritis. *Nat Rev Rheumatol* 2011;**7**:43-9.

18 Bomer N, den Hollander W, Ramos YFM, Bos SD, van der Breggen R, Lakenberg N, *et al.* Underlying molecular mechanisms of DIO2 susceptibility in symptomatic osteoarthritis. *Annals of the Rheumatic Diseases* 2014.

19 Bos SD, Bovee JV, Duijnisveld BJ, Raine EV, van Dalen WJ, Ramos YF, *et al.* Increased type II deiodinase protein in OA-affected cartilage and allelic imbalance of OA risk polymorphism rs225014 at DIO2 in human OA joint tissues. *Ann Rheum Dis* 2012;**71**:1254-8.

20 Karlsson C, Dehne T, Lindahl A, Brittberg M, Pruss A, Sittinger M, *et al.* Genome-wide expression profiling reveals new candidate genes associated with osteoarthritis. *Osteoarthritis Cartilage* 2010;**18**:581-92.

21 Ijiri K, Zerbini LF, Peng H, Otu HH, Tsuchimochi K, Otero M, *et al.* Differential expression of GADD45beta in normal and osteoarthritic cartilage: potential role in homeostasis of articular chondrocytes. *Arthritis Rheum* 2008;**58**:2075-87.

22 Nagase H, Nagasawa Y, Tachida Y, Sakakibara S, Okutsu J, Suematsu N, *et al.* Deiodinase 2 upregulation demonstrated in osteoarthritis patients cartilage causes cartilage destruction in tissue-specific transgenic rats. *Osteoarthritis and Cartilage* 2013;**21**:514-23.

23 Schneider MJ, Fiering SN, Pallud SE, Parlow AF, St Germain DL, Galton VA. Targeted Disruption of the Type 2 Selenodeiodinase Gene (DIO2) Results in a Phenotype of Pituitary Resistance to T4. *Molecular Endocrinology* 2001;**15**:2137-48.

24 Szklarczyk D, Franceschini A, Kuhn M, Simonovic M, Roth A, Minguez P, *et al.* The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored. *Nucleic Acids Res* 2011;**39**:D561-D568.

25 Bassett JH, Boyde A, Howell PG, Bassett RH, Galliford TM, Archanco M, *et al.* Optimal bone strength and mineralization requires the type 2 iodothyronine deiodinase in osteoblasts. *Proc Natl Acad Sci U S A* 2010;**107**:7604-9.

26 Ramos YF, den HW, Bovee JV, Bomer N, van der Breggen R, Lakenberg N, *et al.* Genes Involved in the Osteoarthritis Process Identified through Genome Wide Expression Analysis in Articular Cartilage; the RAAK Study. *PLoS ONE* 2014;**9**:e103056.

27 Ruan MZC, Erez A, Guse K, Dawson B, Bertin T, Chen Y, *et al.* Proteoglycan 4 Expression Protects Against the Development of Osteoarthritis. *Science Translational Medicine* 2013;**5**:176ra34.

28 Arnold MA, Kim Y, Czubryt MP, Phan D, McAnally J, Qi X, *et al.* MEF2C Transcription Factor Controls Chondrocyte Hypertrophy and Bone Development. *Developmental Cell* 2007;**12**:377-89.

29 Mackie EJ, Ahmed YA, Tatarczuch L, Chen KS, Mirams M. Endochondral ossification: how cartilage is converted into bone in the developing skeleton. *Int J Biochem Cell Biol* 2008;**40**:46-62.

30 Omoto S, Nishida K, Yamaai Y, Shibahara M, Nishida T, Doi T, *et al.* Expression and localization of connective tissue growth factor (CTGF/Hcs24/CCN2) in osteoarthritic cartilage. *Osteoarthritis and Cartilage* 2004;**12**:771-8.

31 Shepard J, Jeong JW, Maihle N, O'Brien S, Dealy C. Transient anabolic effects accompany epidermal growth factor receptor signal activation in articular cartilage in vivo. *Arthritis Research & Therapy* 2013;**15**:R60.

32 Davidson ENB, Vitters EL, Mooren FM, Oliver N, Berg WB, Kraan PM. Connective tissue growth factor/CCN2 overexpression in mouse synovial lining results in transient fibrosis and cartilage damage. *Arthritis & Rheumatism* 2006;**54**:1653-61.

33 Shepard J, Jeong JW, Maihle N, O'Brien S, Dealy C. Transient anabolic effects accompany epidermal growth factor receptor signal activation in articular cartilage in vivo. *Arthritis Research & Therapy* 2013;**15**:R60.

34 Corr M. Wnt-beta-catenin signaling in the pathogenesis of osteoarthritis. *Nat Clin Pract Rheumatol* 2008;**4**:550-6.

35 Linglart A, Maupetit-M+¬houas S, Silve C. GNAS-Related Loss-of-Function Disorders and the Role of Imprinting. *Hormone Research in Paediatrics* 2013;**79**:119-29.

36 Bastepe M, Weinstein LS, Ogata N, Kawaguchi H, J++ppner H, Kronenberg HM, *et al.* Stimulatory G protein directly regulates hypertrophic differentiation of growth plate cartilage in vivo. *Proceedings of the National Academy of Sciences of the United States of America* 2004;**101**:14794-9.

37 Taniguchi N, Caram+¬s B, Hsu E, Cherqui S, Kawakami Y, Lotz M. Expression Patterns and Function of Chromatin Protein HMGB2 during Mesenchymal Stem Cell Differentiation. *Journal of Biological Chemistry* 2011;**286**:41489-98.

38 Burns K, Duggan B, Atkinson EA, Famulski KS, Nemer M, Bleackley RC, *et al.* Modulation of gene expression by calreticulin binding to the glucocorticoid receptor. *Nature* 1994;**367**:476-80.

39 Li H, Zhang XY, Wu TJ, Cheng W, Liu X, Jiang TT, *et al.* Endoplasmic Reticulum Stress Regulates Rat Mandibular Cartilage Thinning under Compressive Mechanical Stress. *Journal of Biological Chemistry* 2013;**288**:18172-83.

40 Sekiya I, Vuoristo JT, Larson BL, Prockop DJ. In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis. *Proceedings of the National Academy of Sciences* 2002;**99**:4397-402.

41 Dy P, Smits P, Silvester A, Penzo-M+¬ndez A, Dumitriu B, Han Y, *et al.* Synovial joint morphogenesis requires the chondrogenic action of Sox5 and Sox6 in growth plate and articular cartilage. *Developmental Biology* 2010;**341**:346-59.

42 Reppe S, Rian E, Jemtland R, Olstad OK, Gautvik VT, Gautvik KM. Sox-4 Messenger RNA Is Expressed in the Embryonic Growth Plate and Regulated via the Parathyroid Hormone/Parathyroid Hormone-Related Protein Receptor in Osteoblast-like Cells. *J Bone Miner Res* 2000;**15**:2402-12.

LEGENDS TO THE FIGURES:

Figure 1.

Histological scoring of osteoarthritis. Comparing wild-type and Dio2-/- mice as well as the effect of forced running. (A) Frontal Haematoxylin-Safranin O stained sections of C57Bl/6 wild-type and *Dio2*$^{-/-}$ knees (medial) of mice subjected to a running regime (run) and control mice (no run) (magnification 10x). (B) Cartilage damage was increased in wild-type mice as compared to Dio2-/- mice (2-way ANOVA p = 0.0006) and (C) a similar trend was observed for synovitis (2-way ANOVA p=0.0536). None of the other comparisons (e.g. 'WT no run' versus 'KO no run') were found significantly different. Data are shown as individual values, mean and 95% confidence intervals.

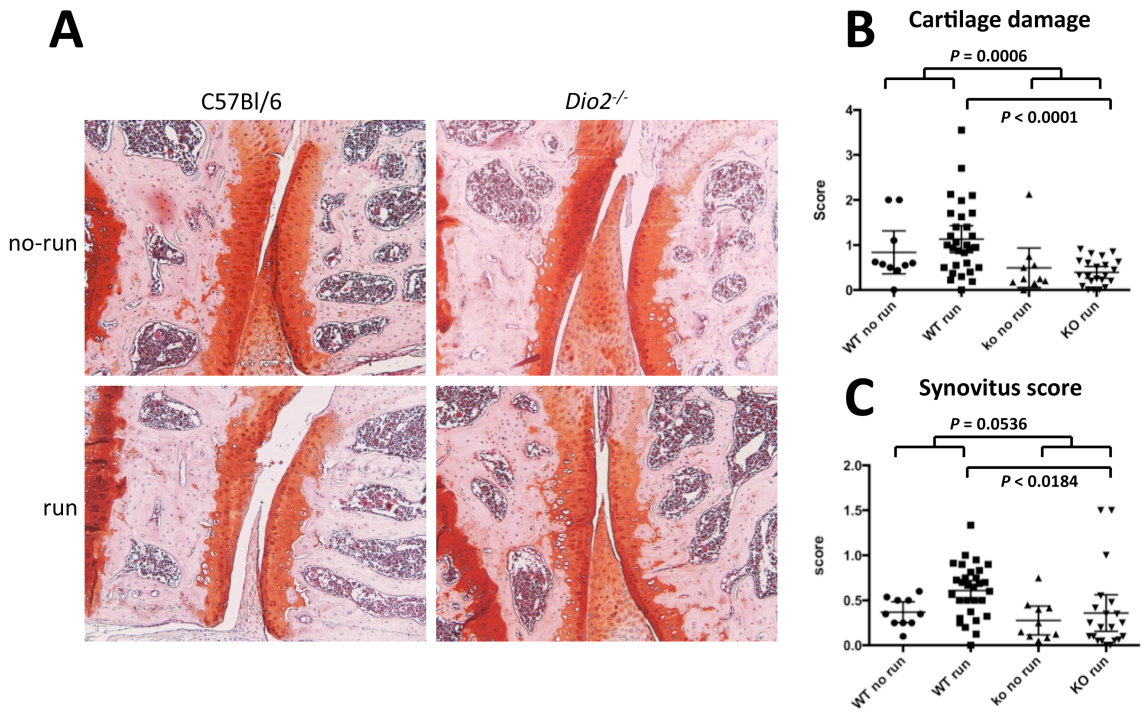
Figure 2.

Overview of the study strategy. Following microarray analysis, data were statistically analyzed. Secondary analyses using gene enrichment analysis and interaction analysis will lead to a set of genes which will be technically validated.

Figure 3.

Gene networks in search tool for the retrieval of interacting genes. Screenshot of the protein–protein interactions (STRING-db) for the 147 genes differing >1.5-fold between 'runners' and controls, including thyroid signaling members; Dio2, Thra, Thrb and Rxra (encircled by the dashed line). Mayor hubs in the network are depicted by A) ctgf, B) egfr, and C) ctnnb1. Disconnected proteins are hidden.

496



496

497 Figure 1

498

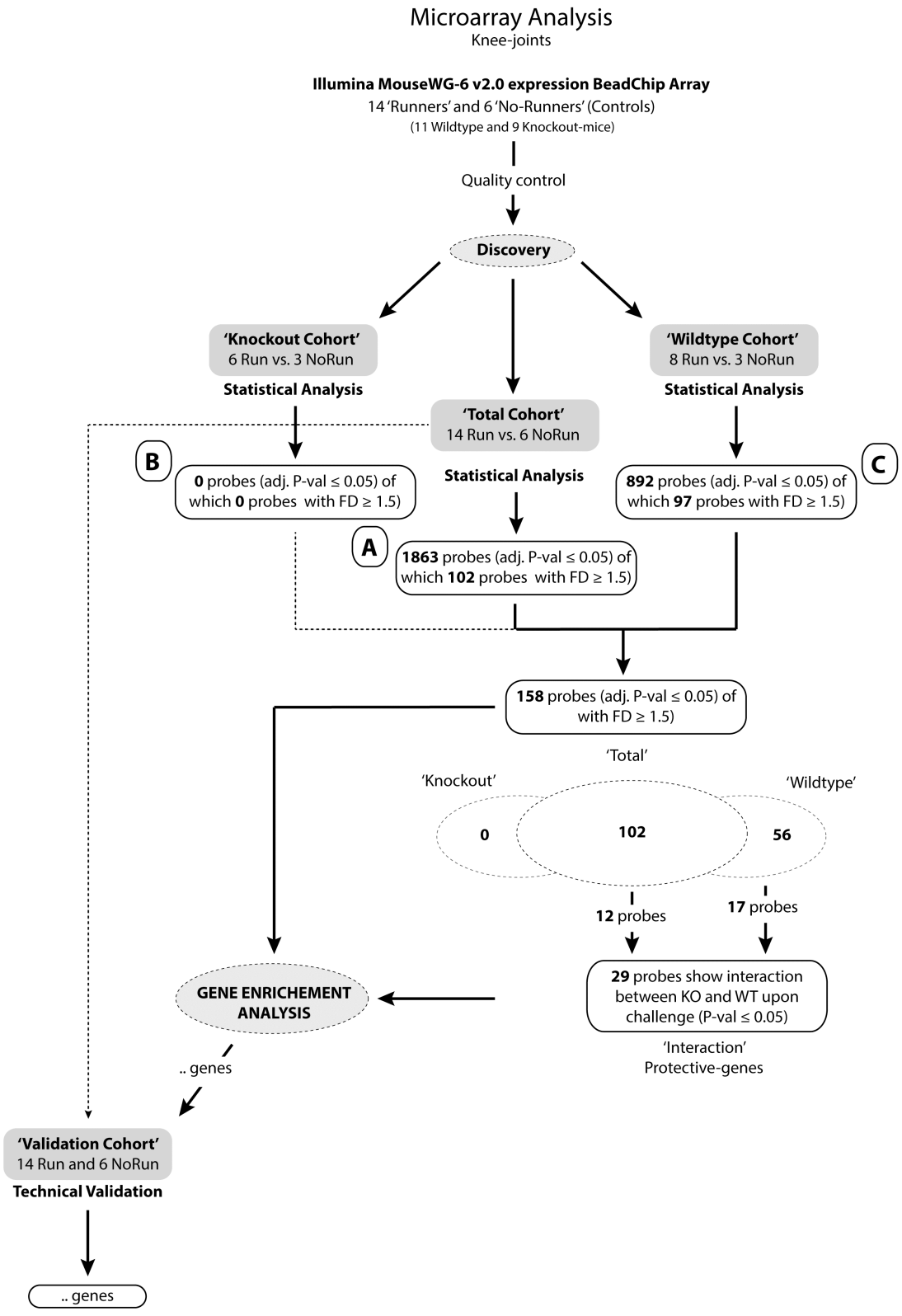
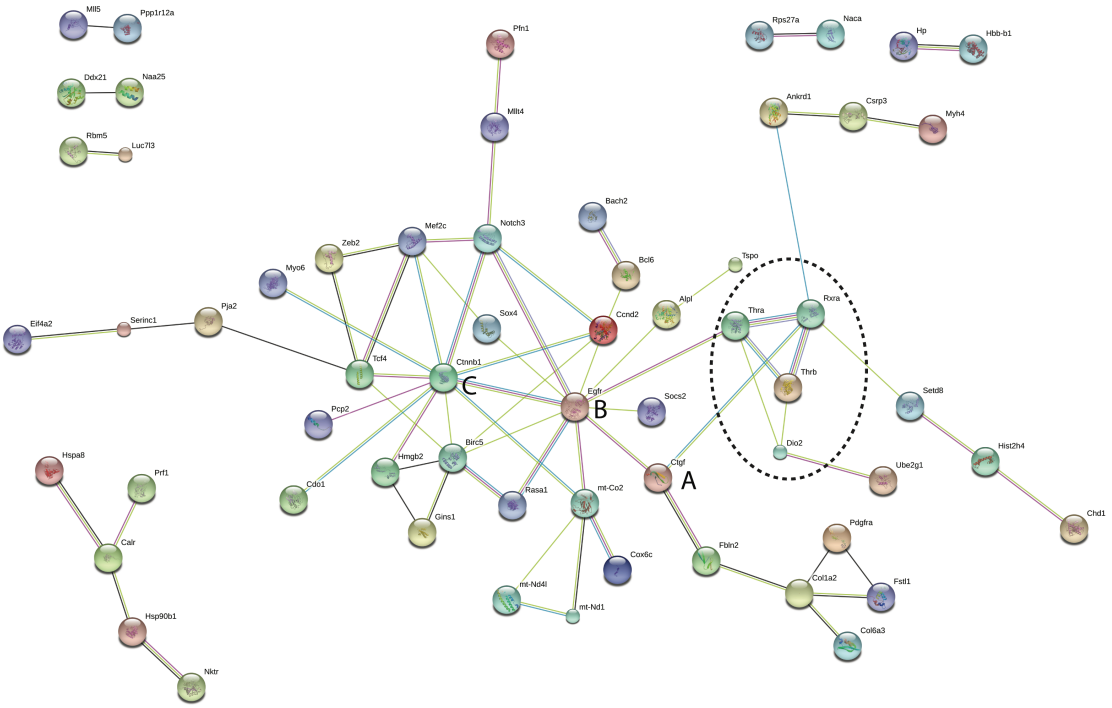

Microarray Analysis
Knee-joints
Illumina MouseWG-6 v2.0 expression BeadChip Array
14 'Runners' and 6 'No-Runners' (Controls)
(11 Wildtype and 9 Knockout-mice)
Quality control
Discovery
'Knockout Cohort'
6 Run vs. 3 NoRun
Statistical Analysis
'Total Cohort'
14 Run vs. 6 NoRun
Statistical Analysis
'Wildtype Cohort'
8 Run vs. 3 NoRun
Statistical Analysis
B
0 probes (adj. P-val ≤ 0.05) of which 0 probes with FD ≥ 1.5)
A
1863 probes (adj. P-val ≤ 0.05) of which 102 probes with FD ≥ 1.5)
C
892 probes (adj. P-val ≤ 0.05) of which 97 probes with FD ≥ 1.5)
158 probes (adj. P-val ≤ 0.05) of with FD ≥ 1.5)
'Total'
'Knockout'
'Wildtype'
0
102
56
12 probes
17 probes
29 probes show interaction between KO and WT upon challenge (P-val ≤ 0.05)
'Interaction'
Protective-genes
GENE ENRICHEMENT ANALYSIS
.. genes
'Validation Cohort'
14 Run and 6 NoRun
Technical Validation
.. genes

Figure 2



500
501 Figure 3

MATERIALS AND METHODS

Animal experiments

Dio2$^{-/-}$ mice were a kind gift of Dr. V. Galton (Dartmouth Medical School, NH, USA)[1] and were backcrossed onto the C57Bl/6 background. In the experiments reported here, mice were in the 6th generation of backcrossing. C57Bl/6 mice are known to develop spontaneous OA [2] and this is enhanced by forced treadmill running with incline[3]. Wild-type C57/Bl6 mice were purchased from Janvier (Le Genest St Isle, France). All experiments were approved by the Ethics Committee for Animal Research (KU Leuven, Belgium).

Four to 6 months old male *Dio2*$^{-/-}$ (n=22) and wild-type mice (n=30) were subjected to a forced-running exercise regimen on a four lane modular treadmill (Columbus Instruments, Columbus, OH, USA). These mice ran for 3 weeks 1 hour/day, 5 days/week, at a speed of 11 m/min and with an inclination of 5°. *Dio2*$^{-/-}$ (n=10) and wild-type (n=10) mice were used as non-running controls. All animals were littermates and were caged together and housed in the same facility.

Histological assessment of osteoarthritis

At the end of the experiment, mice were sacrificed; right knees were processed for histology and left knees for gene expression analysis. Right knees were fixed overnight at 4°C in 2% formaldehyde, decalcified for 3 weeks in 0.5M EDTA pH 7.5 and embedded in paraffin. Severity of disease was determined by histological scores on hematoxylin/eosin or Safranin O stained sections (5µm) throughout the knee (5 sections at 100µm distance). Both cartilage damage and synovial hyperplasia were assessed based

on OARSI guidelines [4, 5]. For cartilage damage, both depth of lesions (score 0-6) and extent of lesions (score 0-4) were evaluated in a combined score (depth x extent)[4]. Lesion grades represent the following features: 0 – surface and cartilage morphology intact; 1 – superficial zone fibrillation; 2 – surface discontinuity; 3 – vertical fissures; 4 – cartilage erosions; 5 – denudation of cartilage and exposure of subchondral bone; 6 remodelling including microfracture and osseus repair above the previous surface. Extent scores represent: 0 – no lesions; 1 – less than 10%; 2 – 10 to 25%; 3 – 25 – 50%; 4 – over 50%). Medial and lateral tibial and femoral cartilages were scored but lesions were only seen in the tibia. The score represents the sum of medial and lateral scores. For synovial hyperplasia, severity was determined on a 0-3 scale[5]. Scoring was done by 2 independent readers, blinded to the genotype.

For gene expression studies, cartilage was micro-dissected and separately stored, snap frozen in liquid nitrogen, at minus 80 degrees upon isolation.

RNA isolation

To gain isolation efficiency and thereby mRNA quality, the articular cartilage material of the knee-joints of two mice were pooled. The samples were subsequently pulverized using a Retsch MM200 apparatus under cryogenic conditions. After one run (20 seconds at 20 Hz), 1 ml of Trizol reagent (Life Technologies, Bleiswijk, The Netherlands) was added to the milling-chamber containing the pulverized joint. The joint-tissue and Trizol were mixed vigorously by performing a second run (20 seconds at 20 Hz). The frozen joint/Trizol mixture was scraped out of the milling-chamber and was then left to thaw. After addition of 200 µl of chloroform the sample was mixed and centrifuged for 15

minutes at 14.000g. The clear aqueous layer was transferred to a new vial and 1 volume of 70% ethanol/DEPC-treated water was added to precipitate RNA. The RNA was isolated and washed using the RNeasy mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. RNA quality was assessed using a Biocore lab-on-a-chip and quantity was assessed using a Nanodrop spectrophotometer. RIN values above 7 were considered of sufficient quality for further processing.

Mouse experiment analysis

All analyses were performed with GraphPad Prism. We hypothesized that the dependent variables (cartilage damage and synovial hyperplasia) could be determined by two independent variables (genotype and exercise) and therefore tested the effect of genotype, exercise and their interaction using 2-way ANOVA. The assumptions underlying the ANOVA were assessed: the dependent variables were measured at a ratio level and the independent variables were categorical, independent and based on independent observations. For synovial hyperplasia, data were normally distributed based on visual analysis and the D'Agostino – Pearson omnibus normality test. 4-group suggested homogeneity of variance for both datasets based on the Brown-Forsythe test. For cartilage damage analysis, data were not normally distributed and there was no homogeneity of variance. We therefore performed a log transformation of the scores resulting in positive assessment of the normal distribution and homogeneity of variance assessed as above. 2-way ANOVA and post-hoc Sidak test taking into account multiple comparisons were applied. Data are presented as mean + 95% CI and p-values < 0.05

were considered significant. In the post-hoc tests, adjusted p-values < 0.05 correspond with 95% CI for differences between means that do not contain the zero value.

Microarray analysis

For first- and second-strand reverse transcription, 500 ng of total RNA was used as template. Complementary DNA synthesis, amplification, biotin labeling and hybridization onto the microarrays was performed using the Ambion TotalPrep-96 RNA amplification kit (Life Technologies, Bleiswijk, The Netherlands) according to manufacturer's protocol. Samples were dispersed for experimental condition over the arrays of 6 chips from a single batch, and each chip contained a replicate sample that was also present on a second chip, to exclude batch effects across chips. After hybridization on Illumina MouseWG-6 v2 BeadChip microarrays (Illumina, Eindhoven, The Netherlands) the slides were scanned with the Illumina Beadscanner 500GX and after basic quality control (QC) using Beadstudio software the intensity values were normalized using the "rsn" option in the Lumi R-package. All samples were included in the dataset. Replicates measured over the different chips were taken into account when performing normalization. For further analysis, the replicate with the lowest number of calls was discarded from the dataset.

The raw probe-level data were exported for analyses using the Limma R-package. We checked the data for large-scale batch effects between chips using a principal component analysis. After correction for multiple testing as implemented in the "BH" (Benjamini and Holm) option on Limma, we listed the significantly differentially expressed probes between running and control mice (challenge), correcting for genotype, and for knockout

593 and *Dio2*$^{-/-}$ separately. The genes were then further filtered with a cut-off fold-difference
594 ≥ 1.5.
595 Using SPSS (Release 20.0.0, IBM) we used the 'Generalized Estimation Equation'
596 (GEE) option to investigate the influence of genotype on the significantly differentially
597 expressed probes between running and control mice thereby calculating the interaction-
598 term.
599
600 **Pathway analysis and protein-protein interaction networks**
601 Gene enrichment among the genes with significant differential expression was performed
602 with STRING (Search Tool for the Retrieval of Interacting Genes/Proteins 9.1) [6]. The
603 probe-list as stated in table S1 was entered into the indicated box to search for multiple
604 protein-protein interactions in mus musculus. Alternative names, provided by the
605 STRING program, given for 'LOC' and 'Riken' annotated genes were accepted as input
606 for the interaction prediction. Selecting for biological processes identified in the Gene
607 Ontology database (GOTERM_BP_FAT in the options menu implemented in STRING)
608 and using the genome background (mus musculus). Pathways with P-val ≤0.05 after FDR
609 correction for multiple testing were considered significant. Enrichment in protein-protein
610 interactions was also analyzed using the STRING database. Genes were classified as
611 network 'nodes' when having 10 or more interactions.
612
613 **Quantitative RT-PCR assays (validation)**
614 Validation of the micrarray results was performed by quantitative real-time PCR. 500 ng
615 of total RNA was processed with the First Strand cDNA Synthesis Kit according to the

manufacturer's protocol (Roche Applied Science, Almere, The Netherlands) upon which cDNA was diluted 5 times. RT-qPCR measurements were performed on the Roche Lightcycler 480 II, using Fast Start Sybr Green Master reaction mix according to the manufacturer's protocol (Roche Applied Science). Relative gene expressions of the Roche Lightcycler 480 II data were calculated by using the $2^{-\Delta\Delta Ct}$ method, in which Ct indicates cycle threshold, the fractional cycle number where the fluorescent signal reaches detection threshold.[7] Primer efficiencies were verified in advance; by performing a concentration curve experiment (primer sequences used are listed in **Table S3**). Only primers showing a perfect correlation between Ct-value and concentration and with a single peak in the melting-curve calculation (Lightcycler480 SW 1.5 software) were used. By selecting 20 genes covering the broad range of p-values and fold-changes as found with the microarray analyses we wanted to demonstrate the reliability of the microarray technique. The glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) household gene expression was used as internal standard for RNA quantity.[8, 9]

Reference List

1 Schneider MJ, Fiering SN, Pallud SE, Parlow AF, St Germain DL, Galton VA. Targeted Disruption of the Type 2 Selenodeiodinase Gene (DIO2) Results in a Phenotype of Pituitary Resistance to T4. *Molecular Endocrinology* 2001;**15**:2137-48.

2 SOKOLOFF L. Natural history of degenerative joint disease in small laboratory animals. II. Epiphyseal maturation and osteoarthritis of the knee of mice of inbred

640 strains. *American Medical Association Archives of Pathology* 1956;**Aug; 62(2)**:118-
641 28.

642 3 Lapvetelainen T, Nevalainen TF, Parkkinen JJ FAU, Arokoski JF, Kiraly KF,
643 Hyttinen MF, *et al.* Lifelong moderate running training increases the incidence and
644 severity of osteoarthritis in the knee joint of C57BL mice. *The anatomical record*
645 1995;**Jun;242(2)**:159-65.

646 4 Pritzker KPH, Gay S, Jimenez SA, Ostergaard K, Pelletier JP, Revell PA, *et al.*
647 Osteoarthritis cartilage histopathology: grading and staging. *Osteoarthritis and*
648 *Cartilage* 2006;**14**:13-29.

649 5 Glasson SS, Chambers MG, van den Berg WB, Little CB. The OARSI
650 histopathology initiative ΓÇô recommendations for histological assessments of
651 osteoarthritis in the mouse. *Osteoarthritis and Cartilage* 2010;**18, Supplement**
652 **3**:S17-S23.

653 6 Szklarczyk D, Franceschini A, Kuhn M, Simonovic M, Roth A, Minguez P, *et al.*
654 The STRING database in 2011: functional interaction networks of proteins, globally
655 integrated and scored. *Nucleic Acids Res* 2011;**39**:D561-D568.

656 7 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time
657 quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;**25**:402-8.

658 8 Toegel S, Huang W, Piana C, Unger F, Wirth M, Goldring M, *et al.* Selection of
659 reliable reference genes for qPCR studies on chondroprotective action. *BMC*
660 *Molecular Biology* 2007;**8**:13.

9 Fujii Y, Kitaura K, Matsutani T, Shirai K, Suzuki S, Takasaki T, *et al.* Immune-Related Gene Expression Profile in Laboratory Common Marmosets Assessed by an Accurate Quantitative Real-Time PCR Using Selected Reference Genes. *PLoS ONE* 2013;**8**:e56296.

SUPPLEMENTAL INFORMATION

Supplemental Figure Legends:

Figure S1: Study design. Graphical representation of the study design.

Figure S2: Gene networks in search tool for the retrieval of interacting genes (STRING). Screenshot of the protein–protein interactions determined with STRING-db for the 147 genes differing at least 1.5-fold between challenged and unchallenged mice (disconnected proteins are hidden).

Supplemental Figures:

Figure S1.

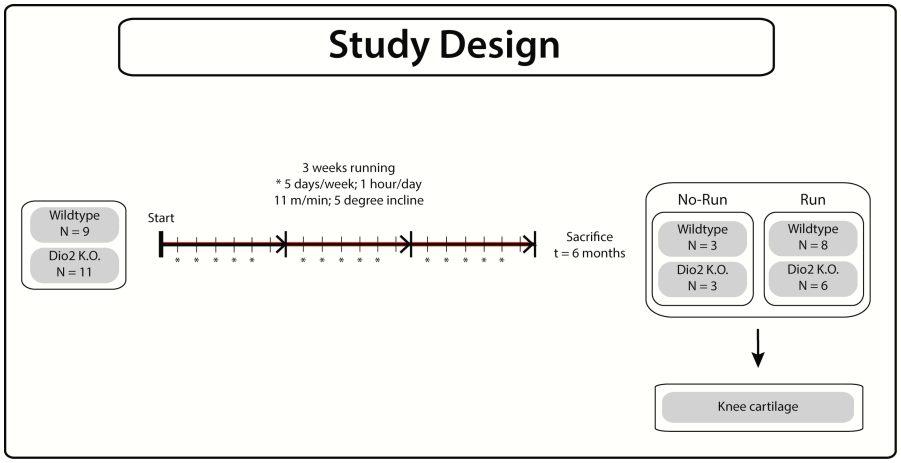
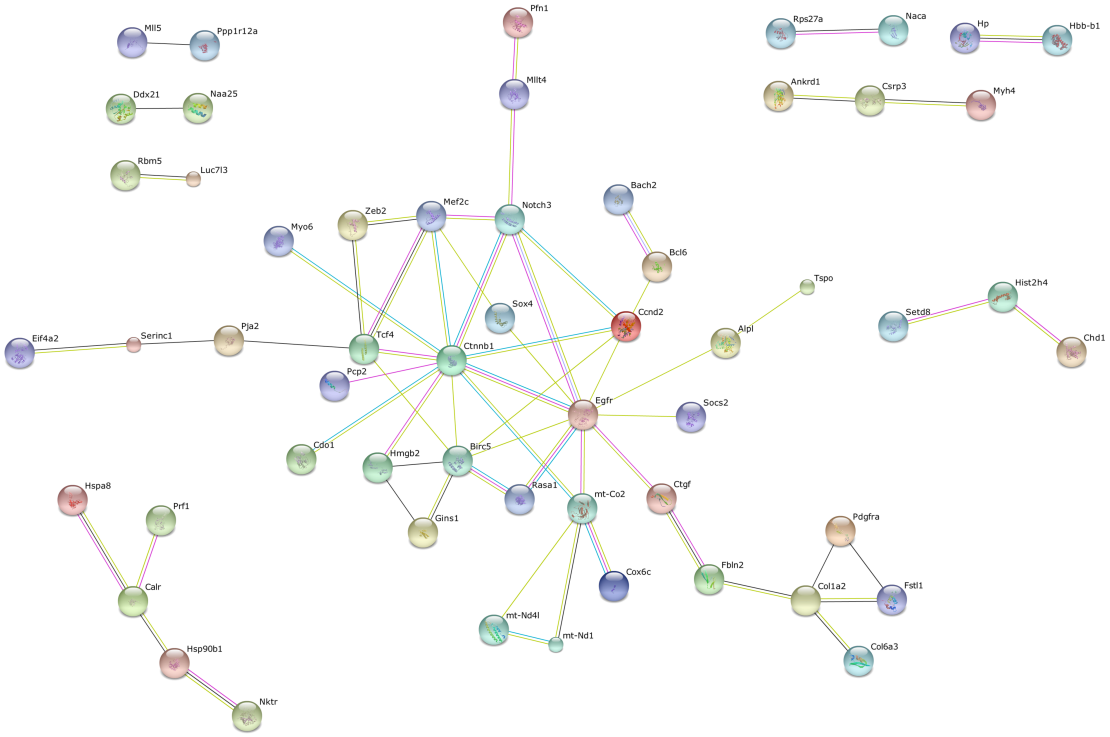


Figure S2.



Supplementary Table Legends:

Table S1: Overall differential expressed probes (N=158) upon exercise.

Table S2: Technical validation by RT-qPCR.

Table S3: RT-qPCR primer sequences.

688 **Supplemental Tables:**

689 **Table S1:**

Table S1: Differential expressed probes (N=158)
OVERALL DIFFERENTIAL EXPRESSED PROBES

SYMBOL	adj.P.Val	Abs FC
Gins1	3,77E-03	2,30
Purb	3,77E-03	-1,84
Apip	4,72E-03	1,51
Polr1d	4,72E-03	1,50
Rasa1	5,20E-03	-1,87
Ccnd2	6,52E-03	-1,97
Ccnd2	6,71E-03	-1,82
Sox4	6,93E-03	-1,66
Srr	7,55E-03	-1,90
mt-Co2	8,00E-03	-3,68
Dusp7	8,30E-03	-1,51
Rps27a	9,84E-03	1,56
Mll5	9,84E-03	-1,85
Hbb-b1	1,12E-02	-1,88
Mllt4	1,15E-02	-1,88
Bcl6	1,16E-02	-1,53
4933407C03Rik	1,23E-02	-1,55
Prf1	1,23E-02	-1,80
Srr	1,23E-02	-2,05
LOC665281	1,23E-02	-1,87
Ppp1r12a	1,25E-02	-1,54
LOC676974	1,26E-02	1,52
Tcf4	1,27E-02	-1,53
Naca	1,27E-02	1,50
LOC100047419	1,31E-02	-1,52
Csda	1,31E-02	-1,61
A230057M07Rik	1,32E-02	-1,79
Ctgf	1,32E-02	-1,79
Ankrd11	1,33E-02	-1,50
Rbm5	1,39E-02	-1,53
mt-Nd1	1,41E-02	-2,62
9030024J15Rik	1,50E-02	-1,67
LOC671878	1,53E-02	-1,50
Cnot4	1,54E-02	-1,67
Ube2l6	1,59E-02	1,68
LOC386021	1,59E-02	-1,57
Ide	1,61E-02	-1,51
B430305P08Rik	1,63E-02	-1,65
Pptc7	1,65E-02	-1,65
LOC100046770	1,69E-02	1,67

Chka	1,69E-02	-1,76
Gdi1	1,71E-02	1,55
Igfbp5	1,71E-02	-1,63
LOC386078	1,75E-02	-1,64
Rhbdl2	1,86E-02	-1,65
LOC385825	1,87E-02	-1,62
Ybx3	1,88E-02	-1,50
LOC386218	1,89E-02	-1,60
Ctgf	2,10E-02	-1,68
BC030476	2,14E-02	-2,14
Nfic	2,19E-02	-1,80
4732462B05Rik	2,21E-02	-1,57
LOC671641	2,29E-02	-1,84
Marveld1	2,34E-02	-1,53
mtDNA_COXII	2,53E-02	-2,10
Ankhd1	2,64E-02	-1,57
Igk-V33	2,72E-02	2,40
LOC546015	2,81E-02	-1,71
Fam134b	2,83E-02	-1,56
Cdo1	2,87E-02	-1,63
Ankrd1	2,95E-02	-1,65
Tde2	3,00E-02	-1,58
Mef2c	3,05E-02	-1,90
Hspa8	3,08E-02	1,55
Bach2	3,11E-02	-1,58
Col1a2	3,11E-02	-1,75
Hsp90b1	3,12E-02	-1,55
Htra4	3,12E-02	-1,84
IGHV1S119_L33961_Ig_heavy_variable_1S119_14	3,12E-02	1,78
Ddx21	3,14E-02	-2,00
Myh4	3,21E-02	-2,26
LOC674195	3,30E-02	-1,58
3300001P08Rik	3,31E-02	-1,78
B230387C07Rik	3,34E-02	-1,97
Chd1	3,38E-02	-1,65
Nfib	3,46E-02	-1,53
E130112E08Rik	3,46E-02	-1,55
Pfn1	3,52E-02	1,67
Gnas	3,54E-02	-1,51
Golga2	3,61E-02	-1,84
Cox6c	3,61E-02	-1,90
Mef2c	3,88E-02	-1,79
Zxda	4,07E-02	-1,53
Fbln2	4,07E-02	-1,57
mtDNA_ND4L	4,15E-02	-1,62
Amotl1	4,21E-02	-1,57
Tnxb	4,35E-02	-1,54
Zeb2	4,44E-02	-1,51
Phospho1	4,44E-02	-1,57
Prg4	4,44E-02	-1,95

Usp48	4,47E-02	-1,56
Stfa1	4,53E-02	1,80
Ermap	4,53E-02	-1,58
S3-12	4,61E-02	-1,52
Tcrb-V8.2	4,66E-02	1,51
LOC383077	4,69E-02	-1,53
LOC270589	4,69E-02	-1,60
2610024B07Rik	4,84E-02	-1,62
2010321I05Rik	4,85E-02	-1,52
Ctnnb1	4,90E-02	-1,51
Nktr	4,98E-02	-1,59
Hist1h2an	5,00E-02	-1,50

WILD-TYPE SPECIFIC PROBES

SYMBOL	adj.P.Val	FC
4930572J05Rik	2,87E-04	-1,56
Cd300lg	4,54E-04	-1,55
C330023M02Rik	1,22E-03	-1,60
Anxa11	1,22E-03	-1,55
LOC433464	1,58E-03	-1,60
Notch3	1,95E-03	-1,54
Myo6	4,33E-03	-1,55
Klf7	4,33E-03	-1,53
Lbh	4,44E-03	-1,85
Nrp1	4,89E-03	-1,52
2210023G05Rik	5,23E-03	-1,54
Socs2	5,60E-03	-1,64
Birc5	6,64E-03	1,54
Pja2	6,70E-03	-1,58
Chi3l3	6,70E-03	1,86
Fmo5	6,91E-03	-1,54
2400001E08Rik	7,00E-03	1,56
Rer1	8,57E-03	-1,53
scl0002617.1_582	8,75E-03	-1,52
EG433016	8,84E-03	1,90
Helb	9,77E-03	-1,54
Fcho1	1,00E-02	1,59
Setd8	1,05E-02	1,65
Hgsnat	1,06E-02	-1,54
Gadd45a	1,46E-02	-1,51
100041294	1,50E-02	1,53
Ube2g1	1,51E-02	-1,53
Sept11	1,59E-02	-1,59
Serpinb1a	1,62E-02	-1,84
Igsf4a	2,01E-02	-1,73
Hp	2,04E-02	1,78
Hp	2,29E-02	1,82
LOC669053	2,29E-02	2,74
Fstl1	2,31E-02	-1,60
Csrp3	2,47E-02	-1,68

Col6a3	2,52E-02	-1,68
Pja2	2,55E-02	-1,52
Hmgb2	2,66E-02	1,59
LOC380691	2,83E-02	1,58
Tspo	2,87E-02	1,54
Fhl1	3,09E-02	-1,52
Mtpn	3,12E-02	-1,51
Phlda1	3,20E-02	-1,51
Rbm47	3,23E-02	-1,51
Akp2	3,35E-02	-1,53
E030026I10Rik	3,47E-02	-1,53
Rap2c	3,53E-02	-1,55
Pdgfra	4,08E-02	-1,50
D16Ertd472e	4,11E-02	-1,56
Calr	4,18E-02	-1,58
Hist1h4i	4,19E-02	1,75
Eif4a2	4,31E-02	-1,54
Pcp2	4,44E-02	1,60
Pgk1	4,76E-02	-1,61
Tspo	4,88E-02	1,51
Bat2	4,97E-02	-1,51

690
691
692 **Table S2**
693

Table S2: Technical validation by RT-qPCR

	Microarray		RT-qPCR
	FC	**adj. P-val**	**FC**
Sox4	-1,66	6,93E-03	-1,62
Srr	-1,90	7,55E-03	-1,35
Mll5	-1,85	9,84E-03	-1,13
Mllt4	-1,88	1,15E-02	-1,53
Bcl6	-1,53	1,16E-02	-1,06
Tcf4	-1,53	1,27E-02	-1,24
Ctgf	-1,79	1,32E-02	-1,62
Rbm5	-1,53	1,39E-02	-1,56
9030024J15Rik (Egfr)	-1,67	1,50E-02	-1,83
Igfbp5	-1,63	1,71E-02	-1,59
Col1a2	-1,75	3,11E-02	-1,46
Pfn1	1,67	3,52E-02	1,00
Gnas	-1,51	3,54E-02	-1,10
Fbln2	-1,57	4,07E-02	-1,90
Tnxb	-1,54	4,35E-02	-2,13
Prg4	-1,95	4,44E-02	-2,30
Ctnnb1	-1,51	4,90E-02	-1,09
Nktr	-1,59	4,98E-02	-1,58
Col6a3	-1,68	2,52E-02	-1,85
Calr	-1,58	4,18E-02	-1,14

694

Table S3:

Table S3: RT-qPCR primer sequences

GAPDH	Forward	5'-TGCCATGTAGACCCCTTGAAG-3'
	Reverse	5'-ATGGTACATGACAAGGTGCGG-3'
Sox4	Forward	5'-AAAGAAGGCTCTGGGGTGAT-3'
	Reverse	5'-ATGCTCCCCTAAGCTCAACA-3'
Srr	Forward	5'-GTGCTGTCTCAGCATTTCCA-3'
	Reverse	5'-CACCCAGTTCAGGGAGGTTA-3'
Mll5	Forward	5'-AAGGCCTCTGCGCATAACTA-3'
	Reverse	5'-ACATGGGGGCTGTAAGTGAG-3'
Mllt4	Forward	5'-ACCTGGAGCTGTTCAGGAGA-3'
	Reverse	5'-AGGACACCCTGTCACTGTCC-3'
Bcl6	Forward	5'-CCTGAGGGAAGGCAATATCA-3'
	Reverse	5'-TTCACGGGGAGGTTTAAGTG-3'
Tcf4	Forward	5'-CGAATCACATGGGACAGATG-3'
	Reverse	5'-AAACGGGGTTAAGGAGCAGT-3'
Ctgf	Forward	5'-CACTCTGCCAGTGGAGTTCA-3'
	Reverse	5'-GTAATGGCAGGCACAGGTCT-3'
Rbm5	Forward	5'-GGGGACAATGGGCTTTTTAT-3'
	Reverse	5'-CCCAGGAAGTAGGGACACAA-3'
9030024J15Rik (Egfr)	Forward	5'-ACACTGCTGGTGTTGCTGAC-3'
	Reverse	5'-TGCCTTGGCAGACTTTCTTT-3'
Igfbp5	Forward	5'-GACCCCGGAAATGTATTCCT-3'
	Reverse	5'-CCAACGCTTTGCTTTCTTTC-3'
Col1a2	Forward	5'-GTGTTCAAGGTGGCAAAGGT-3'
	Reverse	5'-GACCGAATTCACCAGGAAGA-3'
Pfn1	Forward	5'-GCAAGACGGGGAATTTACAA-3'
	Reverse	5'-GGACTAGCGTCTTGGCAGTC-3'
Gnas	Forward	5'-TTTTTCCCCCAGTGATTCTG-3'
	Reverse	5'-AGGGAACTTTTGTGGCCTTT-3'
Fbln2	Forward	5'-GGCCAGTCCTACTTTGTGGA-3'
	Reverse	5'-TTCAATGCAGTTGGGTGGTA-3'
Tnxb	Forward	5'-AGCCTAACACAGGCTGGAGA-3'
	Reverse	5'-CATGGTAGCCCTCAAGGTGT-3'
Prg4	Forward	5'-AGAACCATGGGGTGGAAAAT-3'
	Reverse	5'-CAAGACAACAGGAAGCAGCA-3'
Ctnnb1	Forward	5'-ACCTTTCAGATGCAGCGACT-3'
	Reverse	5'-TATCATCGGAACCCAGAAGC-3'
Nktr	Forward	5'-TGCCTGCTATCACTGTGGAG-3'
	Reverse	5'-TCCTTTGATTTTCCGTCCAG-3'
Col6a3	Forward	5'-GATCGTTCGCAGACTCAACA-3'
	Reverse	5'-CTAAACTGCACGACCCCAAT-3'
Calr	Forward	5'-GAATACAAGGGCGAGTGGAA-3'
	Reverse	5'-GGGGGAGTATTCAGGGTTGT-3'