Pparγ2 Is a Key Driver of Longevity in the Mouse

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Abstract

Aging involves a progressive physiological remodeling that is controlled by both genetic and environmental factors. Many of these factors impact also on white adipose tissue (WAT), which has been shown to be a determinant of lifespan. Interrogating a transcriptional network for predicted causal regulatory interactions in a collection of mouse WAT from F2 crosses with a seed set of 60 known longevity genes, we identified a novel transcriptional subnetwork of 742 genes which represent thus-far-unknown longevity genes. Within this subnetwork, one gene was Pparγ (Nr1c3), an adipose-enriched nuclear receptor previously not associated with longevity. In silico, both the PPAR signaling pathway and the transcriptional signature of Ppary agonist rosiglitazone overlapped with the longevity subnetwork, while in vivo, lowered expression of Pparg1/2-hypomorphic and the Pparg2-deficient mice. These results establish Pparγ2 as one of the determinants of longevity and suggest that lifespan may be rather determined by a purposeful genetic program than a random process.

Introduction

Aging is not a disease, but a natural evolution characterized by declining biological function, whose timeline is sensitive to both environmental and genetic factors. Several longevity candidate genes have been identified, including the insulin/IGF1 signaling pathway [1–3]. With the use of dietary regimens, such as caloric restriction (CR) and by modulating core body temperature, the control of energy metabolism has been implicated as a critical determinant of the aging phenotype [4–6]. A central physiological component of energy metabolism, involved in energy preservation, is the white adipose tissue (WAT), which has also been directly associated with the determination of lifespan [7,8]. However, it is still uncertain whether WAT modulates aging via its ability to e.g. store fat, sensitize towards insulin, or produce adipocyte hormones. Also unknown is the nature of the involved genetic players and importantly, whether they function in a purposeful program or as random genetic events.

Using a systems approach we identified a novel subnetwork of genes in mouse WAT, which potentially impacts longevity, suggesting that aging is the result of a determined transcriptional network program and not entirely accidental. Furthermore, the most significantly enriched biological pathway revealed within this aging subnetwork was the PPAR signaling pathway. The aging subnetwork also contained the nuclear receptor Pparg (Nr1c3), a transcription factor well associated with adipocyte biology [9,10], but whose contribution to longevity has not been previously assessed. In this study, we support our network theory of aging by demonstrating a significantly altered lifespan in 2 independent genetic mouse models expressing reduced levels of Pparg. Thus, in addition to providing novel candidate ‘longevity genes’ such as Pparg2, this study also provides further insight into the potential role of WAT biology and genetics as determinants of lifespan.

Results/Discussion

We hypothesized that the age-dependent physiological remodeling that leads to phenotypic aging is caused by concerted changes in a longevity-determining genetic network rather than by random changes at the level of individual genes. This hypothesis was tested using a mouse transcriptional network that consists of a union of 4 individual Bayesian networks of predicted causal regulatory interactions in the WAT generated from individual F2 crosses. We interrogated this network of 13088 genes with a seed set of 60 genes, derived from public resources, which either increase or reduce lifespan when genetically perturbed in the
mouse (Table S1). Out of these 60 ‘known’ longevity genes, 33 were also present within the adipose tissue network (Table S1; Figure 1A). The pair-wise shortest path analysis against 106 randomly selected sets of 33 genes showed that these 33 genes on average were much more tightly connected than expected by chance ($p = 0.00149$) (Figure 1B). Furthermore, the distribution of the shortest paths within the set of 33 ‘known’ longevity genes was significantly tighter than that for the randomly selected sets as $>99\%$ of all Kolmogorov-Smirnov two-sided test $p$-values were less than 0.05. This tight, non-random interconnection of known aging-linked genes suggests that the associated biological phenomena are deliberate such that other ‘unknown’ age-related genes and/or biological processes may be predicted. This network theory is reminiscent of the transcriptional consequences of single genetic perturbations, such as knock-out mouse models or DNA polymorphisms, which result in concentrations of transcriptional changes in the genes functionally relating to the perturbed gene rather than altering genes diffusely distributed across the whole network [11]. Following the concept of using the ‘known’ to discover the ‘unknown’, we thus expanded the subnetwork beyond the 33 longevity genes to other genes most highly connected to them, and obtained a larger subnetwork, containing 742 genes (Table 1, Table S2). By assigning importance to the closeness of connection with known longevity genes, we were thus able to suggest several hundred additional genes that may influence the aging process. One such example, among the top 20 genes for the closeness of connectivity with the 33 ‘known’ longevity genes (Table 1), was the eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (Eif4ebp1, or 4E-BP1) which, in the unphosphorylated state, represses mRNA translation by binding to eIF4E. Since it regulates adipogenesis and metabolism [12], and one of the mediators of its phosphorylation is insulin signaling

Figure 1. A subnetwork of likely longevity genes in mouse adipose tissue. (A) Longevity-related subnetwork of 775 genes, extracted from the mouse adipose transcriptional network of 13,088 genes. The 33 ‘known’ longevity genes used as a seed set are depicted as green diamonds, and the 213 genes overlapping from the mouse WAT rosiglitazone signature in blue circles. The 5 gene overlap of ‘known’ longevity genes and rosiglitazone signature is shown as blue diamonds. Pparγ, shown as a red circle, is part of the rosiglitazone signature. (B) The distribution of mean shortest path lengths ($\mu$) for the set of 33 ‘known’ longevity genes and 106 randomly selected sets of 33 genes within the mouse consensus network. Red arrow marks the mean shortest path ($\mu = 6.7102$) for the ‘known’ longevity genes.

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[13], Eif4ebp1 can be linked to the established effects of insulin signaling on longevity. Moreover, in Drosophila 4E-BP plays an important role in lifespan extension upon dietary restriction [14]. Eif4ebp1 has furthermore been identified as a “funnel factor” in cancer, through which several oncogenic pathways converge [15].

Biological pathway enrichment analysis is a powerful tool to uncover functional associations within an a priori selected set of genes. When applied to the aging subnetwork of 742 genes (excluding the 33 ‘known’ longevity genes from the full set of 775 genes to eliminate bias), significant enrichment was revealed in several ontology classes with established links to aging such as complement and coagulation cascade (i.e. inflammation), insulin signaling, and ubiquinone pathway (i.e. oxidative stress) (Table 2). Importantly, however, several pathways lacking previously demonstrated association with longevity also appeared among the significantly enriched ontologies. One of these, the PPAR signaling pathway, was actually ranked the highest for the significant enrichment (Table S2). This finding thus validates the association of \( \text{Pparg} \) with the aging subnetwork and further implicates it as a potential determinant of the aging phenotype.

To test this hypothesis in vivo, we investigated the role of \( \text{Pparg} \) in longevity in two mouse models with genetically altered levels of \( \text{Pparg} \) expression: the hypomorphic \( \text{Pparg}^{1/2} \) knock-out mouse, which lacks \( \text{Pparg} \) exclusively in WAT (Figure S1A) and is severely lipodystrophic and remains insulin resistant throughout life [19]; and the \( \text{Pparg}^{2} \) deficient mouse that lacks \( \text{Pparg}^{2} \) in all tissues (Figure S1B) and shows some features of moderate lipodystrophy and insulin resistance at a young age [23], but which fully compensates upon aging (see below). The nearly complete knockdown of \( \text{Pparg}^{1/2} \) in the WAT of male \( \text{Pparg}^{low/lop} \) mice resulted in a reduction in lifespan by approximately 16 weeks when compared to the wild type mice (93.7 ± 4.4 vs 109.6 ± 3.4 weeks, p = 0.03) (Figure 2A). In some respects this observation goes against the prediction that reduced fat mass, as seen during CR [4,5], would increase longevity; however, if the known insulin sensitizing effects of \( \text{Pparg}^{2} \) were key to mediating the effects of CR, then one would expect reduced longevity in the \( \text{Pparg}^{low/lop} \) mice, where whole body insulin resistance is prominent. However, one potentially confounding factor in this experiment is the profound lipodystrophy exhibited by the \( \text{Pparg}^{low/lop} \) mice, which may not represent ‘normal’ metabolic environment due to the amount of metabolic compensation by the upregulation of other signaling pathways that these mice need for survival [19]. Also, although differences in the amount of gross

**Table 1.** Top 20 genes most highly connected to the set of 33 “known” longevity genes in male mouse adipose tissue.

| Gene symbol | Rank | Distance | Gene name |
|-------------|------|----------|-----------|
| Hoxa7       | 1    | 4.636    | homeo box A7 |
| Npr3        | 2    | 4.697    | natriuretic peptide receptor 3 |
| Tmem182     | 3    | 4.697    | transmembrane protein 182 |
| Plikb2      | 5    | 4.758    | plexin B2 |
| Fads3       | 6    | 4.788    | fatty acid desaturase 3 |
| Mcam        | 7    | 4.788    | melanoma cell adhesion molecule |
| Mmd         | 8    | 4.818    | monocyte to macrophage differentiation-associated |
| 1110006G14Rik | 9   | 4.818    | RIKEN cDNA 1110006G14 gene |
| Palmd       | 10   | 4.848    | palmdelphin |
| Fry         | 11   | 4.879    | furry homolog (Drosophila) |
| Apcdd1      | 12   | 4.879    | adenomatosis polyposis coli down-regulated 1 |
| Gpt1        | 13   | 4.909    | glutamic pyruvic transaminase 1, soluble |
| Prelp       | 14   | 4.909    | proline arginine-rich end leucine-rich repeat |
| Eif4ebp1    | 15   | 4.909    | eukaryotic translation initiation factor 4E binding protein 1 |
| MMG00345348 | 17  | 4.909    | NA |
| Echdc3      | 18   | 4.909    | enoyl Coenzyme A hydratase domain containing 3 |
| Fzd4        | 19   | 4.970    | frizzled homolog 4 (Drosophila) |
| Apol6       | 20   | 4.970    | apolipoprotein L, 6 |
| Rtn2        | 21   | 4.970    | reticulin 2 (Z-band associated protein) |
| Smoc1       | 23   | 4.970    | SPARC related modular calcium binding 1 |

*Rank within the whole male mouse adipose tissue network of 10,388 genes.

aDistance to the subnetwork of 33 “known” longevity genes.

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tumors were not observed upon macroscopic necropsy, we cannot exclude the possible contribution of more discrete tumors to the decreased longevity of the Pparghyp/hyp mice. Interestingly though, the males of an equally lipodystrophic A-ZIP/F-1 mouse model have more than 40% mortality rate before 30 weeks of age [8], in comparison to the Pparghyp/hyp mice which survived 85% of the average 2 year lifespan of wild type mice. In this sense, Pparghyp/hyp mouse model is one of the longest living severely lipodystrophic models reported.

In order to assess more directly the effects of Ppar on longevity, without the added complication of reduced adiposity or insulin sensitivity, we made use of Pparg22/2 mice that we generated in the laboratory and which lack Pparc, the WAT enriched Pparc isoform, in all tissues. Although young Pparg22/2 mice are lean [23], our 2 year old Pparg22/2 mice had the same total and lean body mass, body fat content (Figure S2A and S2B), and caloric intake (12.33±1.53 vs. 14.24±1.53 kcal/day/mouse, p = 0.421) as their age-matched littermate controls. Young Pparg22/2 mice have also been reported to be insulin resistant [23]. Again in contrast, there were no differences in glucose tolerance, the HOMA index for insulin resistance, nor in circulating insulin or adiponectin levels between our Pparg22/2 and Pparg2+/- mice at 2 years of age (Figure S2C, S2D, S2E, S2F). Thus, our aging Pparg22/2 mice represent a very metabolically ‘clean’ model for investigating the role of Ppar2 in longevity.

Consistent with reduced longevity in the Pparghyp/hyp mouse, we noted a significant decrease in lifespan in Pparg22/2 mice. The female Pparg22/2 mice lived, on average, 8.8 weeks less than their wild type controls (p = 0.02 when limiting the analysis to those living no more than 120 weeks), although this difference seemed to

Table 2. Pathway analysis of the predicted novel longevity genes in male mouse adipose tissue.

| Pathway                              | Gene count | p      | Overlap genes                  |
|--------------------------------------|------------|--------|--------------------------------|
| PPAR signaling pathway               | 11         | 0.00066| Apoa5, Apoc3, Acp7, Cpt2, Fabp3, Gyk, Pck1, Pparg, Rgs9, Slc27a2, Sorbs1 |
| Ubiquinone biosynthesis              | 4          | 0.00069| Coq5, MMG0237617, ND5, Ndufa12 |
| Valine, leucine and isoleucine       | 8          | 0.00078| Acat1, Aldh6a1, Auh, Dld, Ech1, Hiba1, Mcc1, Mcee |
| degradation                          | 6          | 0.00133| Aldob, Fbp1, Fbp2, Hspd, Pgm2, Tkt |
| Pentose phosphate pathway            | 10         | 0.00153| Clr, C2, F2, Fgb, Fgg, Hc, Kng1, Plg, Serpine1, Serpin2 |
| Complement and coagulation cascades  | 11         | 0.00230| Cd44, Col2a1, Col3a1, Col4a1, Col5a3, F1n, Fndc1, Itga6, Itga7, Spp1, Thbs2 |
| ECM-receptor interaction             | 6          | 0.00300| Acab, Acat1, Aldh6a1, Ech1, Ldhc, Mcee |
| Propanoate metabolism                | 5          | 0.00536| Aldob, Fbp1, Fbp2, Gpt1, Tkt |
| Carbon fixation                      | 14         | 0.00760| Acab, Eif4ebp1, Fasn, Fbp1, Fbp2, Pck1, Pde3b, Ptk3r5, Ppargc1a, Ppp1r3b, Pygb, Slc2a4, Socs2, Sorbs1 |
| Insulin signaling pathway            | 7          | 0.01434| Aldh1a3, Aldob, Dld, Fbp1, Fbp2, Ldhc, Pgm2 |
| Glycolysis/Gluconeogenesis           | 6          | 0.01514| Acab, Acat1, Dld, Ldhc, Pck1, Pcx |
| Pyruvate metabolism                  | 5          | 0.0206 | Addsl1, Ansl, Dld, Gpt1, Pcx |
| Alanine and aspartate metabolism     | 2          | 0.03434| Acab, Fasn |
| Fatty acid biosynthesis              | 3          | 0.03845| Cars2, Cdo1, Ldhc |
| Cysteine metabolism                 | 4          | 0.04157| Dld, Idh1, Pck1, Pcx |
| Citrate cycle (TCA cycle)            |             |        |                                 |

The input set of 197 genes was determined by the overlap of the full set of 742 potentially novel longevity genes and the set of 3853 genes for which functional data was available in the KEGG repository at the time of analysis. Note that the 33 “known” longevity genes were excluded from the determination of the input set to remove bias. Only those pathways with p<0.05 are shown.

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Figure 2. Pparg determines longevity. (A) Lifespan of hypomorphic (hyp) Pparg deficient mice (n = 38 wild type and 24 Pparghyp/hyp mice). ** p = 0.003. (B) Lifespan of Pparg2 knock-out mice (n = 25 wild type and 26 Pparg2/- mice). * p = 0.020 when mice >120 weeks were excluded from the test.

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disappear towards extreme age (Figure 2B). Gross morphological differences that could contribute to mortality were not observed between the genotype groups, although again the contribution of more discrete tumors can not be excluded. Since the Pparg$^{+/+}$ mice had reduced longevity, comparable to that in Pparg$^{hypo/hypo}$ mice, but were not lipodystrophic or insulin resistant, our observations point more towards a specific role for Ppary2 and any of its downstream pathways in the regulation of longevity, rather than mere changes in fat content and/or insulin signaling. Together our studies thus reveal another genetic factor, Pparg2, that affects the basic mechanisms of aging, independent of changes in fat mass or insulin sensitivity [1,2,7]. Interestingly, a potential molecular mechanism linking aging and Ppary has recently been suggested to involve a steroid receptor coactivator-1 (SRC-1) as the age-induced loss of PPARγ/SRC-1 interactions increased the binding of PPARγ to the promoter of a model adipogenic gene for fatty acid binding protein 4 (FABP4, also called aP2) [24].

Both our in silico and in vivo results in the mouse tie longevity tightly together with signaling through Ppary, and especially the Ppary2 isoform. We have recently shown increased longevity in knock-in mice carrying the Ala12 allele of the common human genetic variant Pro12Ala variant of PPARγ2 [29], which associates with leanness and improved insulin sensitivity in both man and mouse [25-27]. The species gap between mice and humans for the role of Ppary2 in longevity is bridged by the observation that lifespan is increased also in human carriers of the Ala12 allele of the Pro12Ala variant of PPARγ2 [29]. In the clinical setting, therefore, the links we show between longevity and both Pparg and the rosiglitazone signature suggest that thiazolidinediones [29] (TZDs), like rosiglitazone or pioglitazone which are widely used Ppary agonists and insulin sensitizers in the treatment of type 2 diabetes mellitus (T2D), could be beneficial for longevity. On the face of it, this may in fact seem paradoxical, considering that impaired insulin signaling through insulin receptor or its substrates increases, rather than decreases lifespan in a number of mouse models [1,2,7]. However, this can be reconciled by the fact that these models are primarily protected from the detrimental effects of age-induced increase in plasma insulin levels as TZDs lower circulating insulin levels [30,31]. Fittingly, low insulin levels and maintained insulin sensitivity characterize human centenarians [32]. In light of the above, the results from ongoing outcome trials evaluating the long-term health benefits of treatments with PPARγ agonists, i.e. TZDs, are eagerly awaited.

In summary, we have identified a substantial set of potential novel longevity genes in mouse adipose tissue, and demonstrate, as a case study, the significant effects of perturbed Ppary activity on mouse lifespan. Furthermore, our network analysis suggests that, at least in the context of adipose tissue, the determination of longevity may not be a random process, but governed by a concerted effort of a distinct subnetwork of genetic players.

Materials and Methods

Ethics statement

Animal experiments were approved by the local ethics committee and performed according to governmental guidelines.

Compilation of the seed set of 60 “known” longevity genes

To obtain a list of genes with known association to longevity, we used the Phenotypes section of the Mouse Genome Informatics (MGI) resource of The Jackson Laboratory (http://www.informatics.jax.org/) [33], the GenAge Model Organisms pages for mouse within the Human Ageing Genomic Resources (HAGR) [34], and a literature search. The list was compiled in October, 2007.

Generation of the transcriptional network for mouse adipose tissue

Detailed description of these methods is given in Text S1. In summary, we obtained male adipose tissue gene expression data from 4 different mouse F2 crosses [35,36] using Agilent microarrays, and generated a Bayesian network for each cross by integrating genetic and gene expression data [37–39]. The combined network, containing 13088 nodes and 22809 edges, was obtained as the union of all these 4 separate Bayesian networks.

Connectivity of “known” longevity genes within the adipose transcriptional network

To assess the degree of connectivity of the 33 ‘known’ longevity genes that were present in the adipose consensus network, mean shortest paths were computed using Dijkstra’s algorithm [40] for our set of 33 nodes (= genes) as well as 10$^4$ randomly selected sets of 33 nodes. Briefly, the algorithm finds the smallest number of edges we have to “walk” in order to “travel” from a source node (= gene) to another node (= gene) of interest within the map/network. The probability of finding random sets of 33 nodes with shorter mean paths than with our set was obtained by counting the number of such eventualities within the randomized sets, and amounted to a $p$-value of 0.00149, demonstrating that indeed our 33 genes are much more connected within the adipose tissue consensus network than expected by chance. Kolmogorov-Smirnov (KS) test was used to further assess whether there were any significant differences between the shortest path distribution within our longevity gene-set and those within each of the 10$^4$ random sets. The resulting $p$-value distribution demonstrated that indeed the longevity genes shortest path distribution is not a normal occurrence in the network.

Generation of Pparg$^{hypo/hypo}$ and Pparg2$^{+/−}$ mice

Pparg2$^{+/−}$ mice were generated from Pparg$^{hypo/hypo}$ [19] mice by successive matings with transgenic C57Bl/6J mice expressing FLP and Cre recombinases to remove the Pparg2 specific exon B. All mice studied were backcrossed a minimum of 9 generations to achieve an essentially pure C57Bl/6J background.

Survival

The original survival cohorts consisted of 38 wild type and 24 Pparg$^{hypo/hypo}$ male, and 25 wild type and 26 Pparg2$^{+/−}$ female mice which were maintained on a 12 hour light/dark cycle, fed regular chow, had free access to H$_2$O and received standard animal care. The mice were bred locally and were entered into the survival cohort over the course of 23 weeks for male Pparg$^{hypo/hypo}$ mice, and 19 months for female Pparg2$^{+/−}$ mice. For all groups, deaths were recorded weekly. Mice observed as moribund were euthanized and recorded as dead on that week. All Pparg$^{hypo/hypo}$ reached the end-point, but a few Pparg2$^{+/−}$ mice survived at the time of analysis.

Metabolic exploration of Pparg2$^{+/−}$ mice

Approximately 2 year old wild type (n = 5) and Pparg2$^{+/−}$ (n = 9) mice were subjected to the following analysis according to standardized Eumorphia/EMPRess (http://empress.har.mrc.ac.uk/) protocols: body composition by quantitative nuclear magnetic resonance on a Minispec analyzer (Bruker Optics, The Woodlands, TX), food intake, intraperitoneal glucose tolerance test (IPGTT), and fasting plasma insulin and adiponectin measure-
ments using Ultrasensitive Mouse Insulin ELISA kit (Merodia, Uppsala, Sweden) and Quantikine Mouse Adiponectin/Adiponectin R&D systems Inc., Minneapolis, MN), respectively. HOMA index for insulin resistance was calculated from fasting glucose and insulin values [41].

RNA analysis

Pparg1 and Pparg2 gene expression in WAT, BAT, liver and skeletal muscle of Pparghyp/hyp mice was previously reported [19] and is presented for comparative purposes. For Pparg2−/− mice, total RNA was extracted from WAT, BAT, liver and skeletal muscle either with RNaseasy for Lipid Tissues Mini Kit (Qiagen, Valencia, CA) or Trizol (Invitrogen, Carlsbad, CA), and reverse transcribed to cDNA using SuperScript II System (Invitrogen) and random hexamer primers. Pparγ1 and Pparγ2 gene expression was quantified by qRT-PCR using isoform-specific primers and SYBR Green chemistry on a LightCycler 480 (Roche, Penzberg, Germany).

Statistical analyses

Statistical methods pertaining to the network and other associated analysis of gene expression and gene set data were as detailed above. Kaplan-Meier survival analysis, which allows for censored cases, was used to analyze the survival data in SPSS (version 14). Metabolic and molecular data for Pparghyp/hyp and Pparg2−/− mice were analyzed using Student’s t-test and are presented as means ± s.e.m.

Supporting Information

Figure S1 Pparg1 and Pparg2 gene expression in WAT, BAT, liver and skeletal muscle in mouse models with altered Pparg locus. Data are presented relative to mean WAT expression in the wild type (Pparg+/+) for each Pparg isoform. Note the much lower expression levels in liver and muscle. (A) Hypomorphic Pparg deficient mouse. (B) Pparg2 knock-out mouse. Note that only one per group was analyzed.

Table S1 The seed set of 60 “known” longevity genes in mouse for the identification of a novel transcriptional longevity subnetwork. The gene list was derived from public resources (see Materials and Methods). The overlaps with the consensus white adipose tissue network (33 genes) and the rosiglitazone signature in the mouse WAT (3 genes) are indicated by a plus sign.

Figure S2 Metabolic phenotype of ~2 year old Pparg2 knock-out mice. For all tests, n = 4–9 per group. (A) Unaltered body weight and (B) fat content were analyzed by QNMR and are presented in % of fat of total body weight. (C) Intraportal glucose tolerance test. The mean areas under the curve above baseline (AUC) are shown in the inset. (D) HOMA index for insulin resistance, calculated from fasting glucose and insulin values. (E) Fasting insulin and (F) adiponectin levels. None of the comparisons showed statistical significance.

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Author Contributions

Conceived and designed the experiments: CA SH TAC EES JA. Performed the experiments: CA RD SH AA LP TAC HK. Analyzed the data: CA RD SH. Contributed reagents/materials/analysis tools: HK JZ. Performed the experiments: CA RD SH AA LP TAC HK. Analyzed the data: CA RD SH. Contributed reagents/materials/analysis tools: HK JZ. Wrote the paper: CA RD SH EES JA.

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