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On the Expansion of "Dangerous" Gene Repertoires by Whole-Genome Duplications in Early Vertebrates

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SUMMARY

The emergence and evolutionary expansion of gene families implicated in cancers and other severe genetic diseases is an evolutionary oddity from a natural selection perspective. Here, we show that gene families prone to deleterious mutations in the human genome have been preferentially expanded by the retention of "ohnolog" genes from two rounds of whole-genome duplication (WGD) dating back from the onset of jawed vertebrates. We further demonstrate that the retention of many ohnologs suspected to be dosage balanced is in fact indirectly mediated by their susceptibility to deleterious mutations. This enhanced retention of "dangerous" ohnologs, defined as prone to autosomal-dominant deleterious mutations, is shown to be a consequence of WGD-induced speciation and the ensuing purifying selection in post-WGD species. These findings highlight the importance of WGD-induced nonadaptive selection for the emergence of vertebrate complexity, while rationalizing, from an evolutionary perspective, the expansion of gene families frequently implicated in genetic disorders and cancers.

INTRODUCTION

Just as some genes happen to be more "essential," owing to their deleterious loss-of-function or null mutations, some genes can be classified as more "dangerous," due to their propensity to acquire deleterious gain-of-function mutations. This is, in particular, the case for oncogenes and genes with autoinhibitory protein folds, whose mutations typically lead to constitutively active mutants with dominant deleterious phenotypes (Pufall and Graves, 2002).

Dominant deleterious mutations, that are lethal or drastically reduce fitness over the lifespan of organisms, must have also impacted their long term evolution on timescales relevant for genome evolution (e.g., >10–100 million years [MY]). In fact, dominant disease genes in humans have been shown to be under strong purifying selection (Furney et al., 2006; Blekhman et al., 2008; Cai et al., 2009). Yet, "dangerous" gene families implicated in cancer and severe genetic diseases have also been greatly expanded by duplication in the course of vertebrate evolution. For example, the single orthologous locus, *Ras85D* in flies and *Let-60* in nematodes, has been duplicated into three *RAS* loci in typical vertebrates, *KRAS*, *HRAS*, and *NRAS*, that present permanently activating mutations in 20%–25% of all human tumors, even though *HRAS* and *NRAS* have also been shown to be dispensable for mouse growth and development (Ise et al., 2000; Esteban et al., 2001).

While the maintenance of essential genes is ensured by their lethal null mutations, the expansion of dangerous gene families remains an evolutionary puzzle from a natural selection perspective. Indeed, considering that many vertebrate disease genes are phylogenetically ancient (Domazet-Loso and Tautz, 2008; Cai et al., 2009; Dickerson and Robertson, 2012), and that their orthologs also cause severe genetic disorders in extant invertebrates (Berry et al., 1997; Ciocan et al., 2006; Robert, 2010), it is surprising that dangerous gene families have been duplicated more than other vertebrate genes without known dominant deleterious mutations. While gene duplicates can confer mutational robustness against loss-of-function mutations, multiple copies of genes prone to gain-of-function mutations are expected to lead to an overall aggravation of a species' susceptibility to genetic diseases and thus be opposed by purifying selection.

Two alternative hypotheses can be put forward to account for the surprising expansion of dangerous gene families. Either, the propensity of certain genes to acquire dominant deleterious mutations could be a mere by-product of their presumed advantageous functions. In that case, only the overall benefit of gene family expansion should matter, irrespective of the mechanism of gene duplication. Alternatively, gene susceptibility to dominant deleterious mutations could have played a driving role in the striking expansion of dangerous gene families. But what could have been the selection mechanism?

In this article, we report converging evidences supporting the latter hypothesis and propose a simple evolutionary model to explain the expansion of such dangerous gene families. It is based on the observation that the majority of human genes prone to dominant deleterious mutations, such as oncogenes and genes with autoinhibitory protein folds, have not been duplicated through small scale duplication (SSD). Instead, the expansion of these dangerous gene families can be traced back to two rounds of whole-genome duplication (WGD), that occurred at the

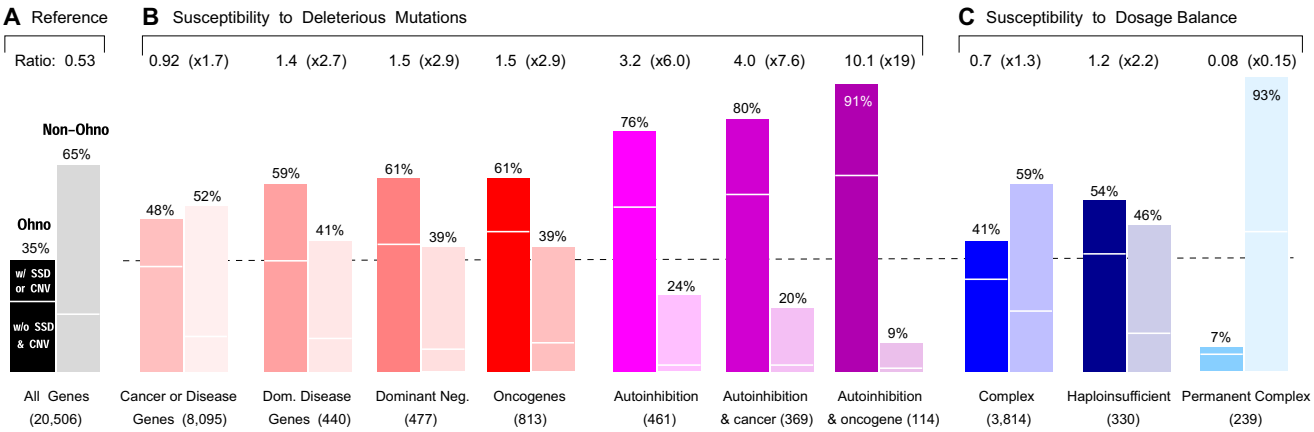

Figure 1. Prevalence of Retained Ohnologs in the Human Genome within Different Gene Classes

(A and B) Prevalence of retained ohnologs either "w/ SSD or CNV" or "w/o SSD & CNV" for all 20,506 human protein-coding genes (A), and gene classes susceptible to deleterious mutations (B). Note that gene classes with higher susceptibility to deleterious mutations retained more ohnologs.

(C) Ohnolog retention in gene classes susceptible to dosage balance constraints. Fold changes in ohnolog/nonohnolog ratios are given relative to the reference from all human genes in (A).

See also Figure S1.

onset of jawed vertebrates, some 500 MY ago (Ohno, 1970; Putnam et al., 2008).

These two rounds of WGD in the early vertebrate lineage are frequently credited with creating the conditions for the evolution of vertebrate complexity. Indeed, WGD-duplicated genes, so-called "ohnologs" in honor of Susumu Ohno (Ohno, 1970; Wolfe, 2000), have been preferentially retained in specific gene classes associated with organismal complexity, such as signal transduction pathways, transcription networks, and developmental genes (Maere et al., 2005; Blomme et al., 2006; Freeling and Thomas, 2006; Sémon and Wolfe, 2007; Makino and McLysaght, 2010; Huminiecki and Heldin, 2010). By contrast, gene duplicates coming from SSD are strongly biased toward different functional categories, such as antigen processing, immune response, and metabolism (Huminiecki and Heldin, 2010). SSD paralogs and WGD ohnologs also differ in their gene expression and protein network properties (Hakes et al., 2007; Guan et al., 2007). Furthermore, recent genome-wide analysis have shown that ohnologs in the human genome have experienced fewer SSD than "nonohnolog" genes and tend to be refractory to copy number variation (CNV) caused by polymorphism of small segmental duplications in human populations (Makino and McLysaght, 2010). These antagonist retention patterns of WGD and SSD/CNV gene duplicates in the human genome have been suggested to result from dosage balance constraints (Makino and McLysaght, 2010) on the relative expressions of multiple protein partners (Veitia, 2002), as proposed earlier for other organisms like yeast (Papp et al., 2003) and the paramecium (Aury et al., 2006).

In this article, we investigate the evolutionary causes responsible for the expansion of gene families prone to deleterious mutations in vertebrates and propose a simple evolutionary model accounting for their antagonistic retention pattern after WGD and SSD events. The retention of ohnologs in the human genome is shown to be more strongly associated with their susceptibility to deleterious mutations, than their functional importance or "essentiality." We also demonstrate using a causal inference analysis, that the retention of many ohnologs suspected to be dosage balanced is in fact an indirect effect of their higher susceptibility to deleterious mutations. We argue that the enhanced retention of dangerous ohnologs is a somewhat counterintuitive yet simple consequence of the speciation event triggered by WGD and the ensuing purifying selection in post-WGD species.

These findings rationalize, from an evolutionary perspective, the WGD expansion of gene families frequently implicated in genetic disorders, such as cancer, and highlight the importance of nonadaptive selection on the emergence of vertebrate complexity.

RESULTS

Genes Prone to Deleterious Mutations Retain More Ohnologs

We first analyzed a possible association between the susceptibility of human genes to deleterious mutations and their retention of ohnologs, as proposed in Gibson and Spring (1998) for multidomain proteins. To this end, we considered multiple classes of genes susceptible to deleterious mutations from experimentally verified databases and literature. These classes include cancer genes (from multiple sources including COSMIC [Forbes et al., 2011] and CancerGenes [Higgins et al., 2007]), genes mutated in other genetic disorders, dominant negative genes from OMIM, and genes with autoinhibitory protein folds (Experimental Procedures). We looked at the relative contributions of WGD and SSD in the expansion of these "dangerous" gene classes.

The results, depicted in Figures 1 and S1, demonstrate indeed a strong association between the retention of human ohnologs from vertebrate WGD and their reported susceptibility to deleterious mutations, as compared to nonohnologs, whereas an

opposite pattern is found for SSD/CNV gene duplicates. Overall, the 8,095 human genes associated with the occurrence of cancer and other genetic diseases have retained significantly more ohnologs than expected by chance, 48% versus 35% (48%; 3,844/8,095; $p = 1.3 \times 10^{-129}$, χ^2 test). Furthermore, these associations, which do not take into account the actual severity of the gene mutations, are clearly enhanced when the analysis is restricted to genes with direct experimental evidence of dominant deleterious mutations, such as dominant disease genes (59%; 261/440; $p = 1.7 \times 10^{-27}$, χ^2 test), dominant negative mutants (61%; 292/477; $p = 3.9 \times 10^{-34}$, χ^2 test), oncogenes (61%; 493/813; $p = 1.4 \times 10^{-54}$, χ^2 test), or genes exhibiting autoinhibitory constraints (76%; 350/461; $p = 2.7 \times 10^{-77}$, χ^2 test). The biased retention of ohnologs is even stronger for genes combining several factors associated with an enhanced susceptibility to deleterious mutations, such as cancer genes with autoinhibitory folds, (80%; 294/369; $p = 1.0 \times 10^{-73}$, χ^2 test), or oncogenes with autoinhibitory folds, (91%; 104/114; $p = 6.9 \times 10^{-37}$, χ^2 test).

This retention of dangerous ohnologs is illustrated on Table 1 that presents an up-to-date list of 76 hand-curated gene families of up to four ohnologs, exhibiting both autoinhibitory folds and oncogenic properties (see Table S1 for oncogenic and autoinhibitory details and references). These dangerous ohnologs are typically found along signal transduction cascades, from receptor tyrosine kinases and cytoplasmic or nuclear kinases to guanine exchange factors (GEF), GTPase activating proteins (GAP), and transcription factors (Table 1, gene classes A–E). In addition, autoinhibited oncogenes are also found in other ohnolog families with diverse functions (Table 1, gene class F). By contrast, we obtained a hand-curated list of only ten nonohnolog genes exhibiting both autoinhibitory and oncogenic properties, Table 1, gene class G (see Table S2 for oncogenic and autoinhibitory details and references). Interestingly, half of them (4/10) can be traced back to SSD events, which occurred after or at the same period of the two WGD in early vertebrate lineages (Table S2). All in all, this implies that >90% of known oncogenes with autoinhibitory folds have retained at least one ohnolog pair in the human genome (as well as, possibly, a few additional duplicates from more recent SSD events).

Ohnologs Are Conserved but More "Dangerous" than "Essential"

We then investigated whether the susceptibility of ohnologs to deleterious mutations could be directly quantified through comparative sequence analysis. We used Ka/Ks ratio estimates, which measure the proportion of nonsynonymous substitutions (Ka) to the proportion of synonymous substitutions (Ks) (Extended Results and Table S3). Ohnologs exhibit statistically lower Ka/Ks ratios, Figures 2, S2, and S3, which provides direct evidence of strong conservation, consistent with a higher susceptibility of ohnologs to deleterious mutations. Similar trends have also been reported for ohnologs specific to teleost fishes (Brunet et al., 2006) or to the more recent WGD in *Xenopus laevis* lineage (Sémon and Wolfe, 2008). Note, however, that the functional consequences of such deleterious mutations, leading either to a gain or a loss of function, cannot be directly inferred from Ka/Ks distributions. Yet, as outlined below, we found marked differences in the retention of "dangerous" ohnologs prone to dominant gain-of-function mutations and "essential" ohnologs exhibiting lethal loss-of-function or null mutations.

While autosomal-dominant disease genes exhibit a strong ohnolog retention bias (Figure 1B), 59% versus 35% (59%; 261/440; $p = 1.7 \times 10^{-27}$, χ^2 test), autosomal-recessive disease genes are not significantly enriched in ohnologs 37% versus 35% (37%; 221/598; $p = 0.24$, χ^2 test). Similarly, human orthologs of mouse genes, reported as being "essential" genes from large-scale null mutant studies in mouse, are not strongly enriched in ohnologs 56% versus 54% (56%; 1,537/2,729; $p = 3.8 \times 10^{-3}$, χ^2 test), where 54% = 3,190/5,956 is the global proportion of ohnologs among the 5,956 genes tested for null mutation in mouse (Experimental Procedures). In fact, this small enrichment becomes even nonsignificant once genes with dominant allelic mutants are removed from the list of 5,956 genes tested for essentiality in mouse, i.e., 50% versus 48% (50%; 760/1,525; $p = 0.09$, χ^2 test), where 48% = 1,782/3,739 is the global proportion of ohnologs among the 3,739 genes tested for essentiality in mouse, after removing dominant disease genes, oncogenes, and genes with dominant negative mutations or autoinhibitory folds.

All in all, this shows that the retention of ohnologs has been most enhanced for genes prone to autosomal-dominant deleterious mutations and not autosomal-recessive deleterious mutations. This suggests that the retention of ohnologs is more strongly related to their "dangerousness," as defined by their high susceptibility to dominant deleterious mutations, than their functional importance or "essentiality," as identified through large-scale null mutation studies in mouse.

Ultimately, we will argue that the "dangerousness" of ohnologs effectively controls their individual retention in the genomes of post-WGD species, as will be shown below in the section Model for the Retention of Dangerous Ohnologs.

Mixed Susceptibility of Human Ohnologs to Dosage Balance

An alternative hypothesis, focusing instead on the collective retention of interacting ohnologs, has been frequently invoked to account for the biased retention of ohnologs in unicellular organisms like yeast (Papp et al., 2003) or the paramecium (Aury et al., 2006) and in higher eukaryotes (Birchler et al., 2001; Makino and McLysaght, 2010).

This "dosage balance" hypothesis posits that interacting protein partners tend to maintain balanced expression levels in the course of evolution, in particular, for protein subunits of conserved complexes (Birchler et al., 2001; Veitia, 2002; Papp et al., 2003; Veitia, 2010; Makino and McLysaght, 2010). Thus, SSD of dosage balanced genes are thought to be generally detrimental through the dosage imbalance they induce, thereby raising the odds for their rapid nonfunctionalization (Papp et al., 2003; Maere et al., 2005). By contrast, rapid nonfunctionalization of ohnologs after WGD has been suggested to be opposed by dosage effect, in particular, for highly expressed genes and genes involved in protein complexes or metabolic pathways (Aury et al., 2006; Evlampiev and Isambert, 2007; Gout et al., 2010; Makino and McLysaght, 2010). This is because WGD initially preserves correct relative dosage between

Table 1. Ohnolog Families with Both Autoinhibitory and Oncogenic Properties

A. Ohnolog Receptor Tyrosine Kinases and Other Receptor Kinases							
ALK	*LTK*			*KIT*	*CSF1R*	*FLT3*	
EGFR	*ERBB2*	*ERBB3*	*ERBB4*	*MET*	*MST1R*		
FGFR1	*FGFR2*	*FGFR3*	*FGFR4*	*NPRA*	*NPRB*		
IGF1R	*INSR*	*INSRR*		*PDGFRA*	*PDGFRB*		
B. Ohnolog Cytoplasmic and Nuclear Protein Kinases							
ABL1	*ABL2*			*PKN1*	*PKN2*	*PKN3*	
ARAF	*BRAF*	*RAF1*		*PRKAA1*	*PRKAA2*		
AKT1	*AKT2*	*AKT3*		*PRKCA*	*PRKCB*	*PRKCG*	
CAMK1	*CAMK1D*	*CAMK1G*	*PNCK*	*PRKCE*	*PRKCH*		
CAMKK1	*CAMKK2*			*PRKCI*	*PRKCZ*		
CSNK1D	*CSNK1E*			*PRKD1*	*PRKD2*	*PRKD3*	
GSK3A	*GSK3B*			*PRKG1*	*PRKG2*		
GRK4	*GRK5*	*GRK6*		*PTK2*	*PTK2B*		
JAK1	*JAK2*	*JAK3*	*TYK2*	*RSK1*	*RSK2*	*RSK3*	*RSK4*
SRC	*FGR*	*FYN*	*YES1*	*MSK1*	*MSK2*		
HCK	*LCK*	*BLK*	*LYN*	*NDR1*	*NDR2*		
MKNK1	*MKNK2*			*SYK*	*ZAP70*		
NEK6	*NEK7*						
C. Ohnolog GEF							
ARHGEF3	*NET1*			*RALGDS*	*RGL1*	*RGL2*	*RGL3*
ARHGEF6	*COOL1*			*SOS1*	*SOS2*		
DBL	*DBS*	*MCF2L2*		*TIAM1*	*TIAM2*		
FGD1	*FGD2*	*FGD3*	*FGD4*	*TIM*	*WGEF*	*SGEF*	*NGEF*
PDZ-RHOGEF	*LSC*	*LARG*		*VAV1*	*VAV2*	*VAV3*	
P114-RHOGEF	*GEF-H1*						
D. Ohnolog GAP							
ASAP1	*ASAP2*	*ASAP3*		*PLXNA1*	*PLXNA2*	*PLXNA3*	*PLXNA4*
IQGAP1	*IQGAP2*	*IQGAP3*		*PLXNB1*	*PLXNB2*	*PLXNB3*	*PLXND1*
E. Ohnolog DNA Binding and Transcription Factors							
CEBPA	*CEBPB*	*CEBPE*		*IRF4*	*IRF8*	*IRF9*	
CUX1	*CUX2*			*MEIS1*	*MEIS2*	*MEIS3*	
ELK1	*ELK3*	*ELK4*		*p53*	*p63*	*p73*	
ETS1	*ETS2*			*RUNX1*	*RUNX2*	*RUNX3*	
ETV1	*ETV4*	*ETV5*		*SOX1*	*SOX2*	*SOX3*	
ETV6	*ETV7*						
F. Other Ohnolog Genes with Both Autoinhibitory and Oncogenic Properties							
ANP32A	*ANP32B*	*ANP32E*		*nNOS*	*eNOS*		
ATP2B1	*ATP2B2*	*ATP2B3*	*ATP2B4*	*NOTCH1*	*NOTCH2*	*NOTCH3*	
cIAP1|2	*XIAP*			*PLCB1*	*PLCB2*	*PLCB3*	
CCNT1	*CCNT2*			*PLCD1*	*PLCD3*	*PLCD4*	
FLNA	*FLNB*	*FLNC*		*PLCG1*	*PLCG2*		
FURIN	*PCSK4*			*PTPN1*	*PTPN2*		
KPNA2	*KPNA7*			*SMURF1*	*SMURF2*		
NEDD4	*NEDD4L*			*TRPV1|3*	*TRPV2*	*TRPV4*	*TRPV5|6*
NOXA1	*NOXA2*						
G. Nonohnolog Genes with Both Autoinhibitory and Oncogenic Properties							
CAMK4	*ELF3*	*MELK*	*MOS*	*PDPK1*	*BRK*	*PTPN11*	*RET*	*RPS6KB1*	*TTN*

GEF, guanine exchange factors; GAP, GTPase activating proteins.
See also Tables S1 and S2.

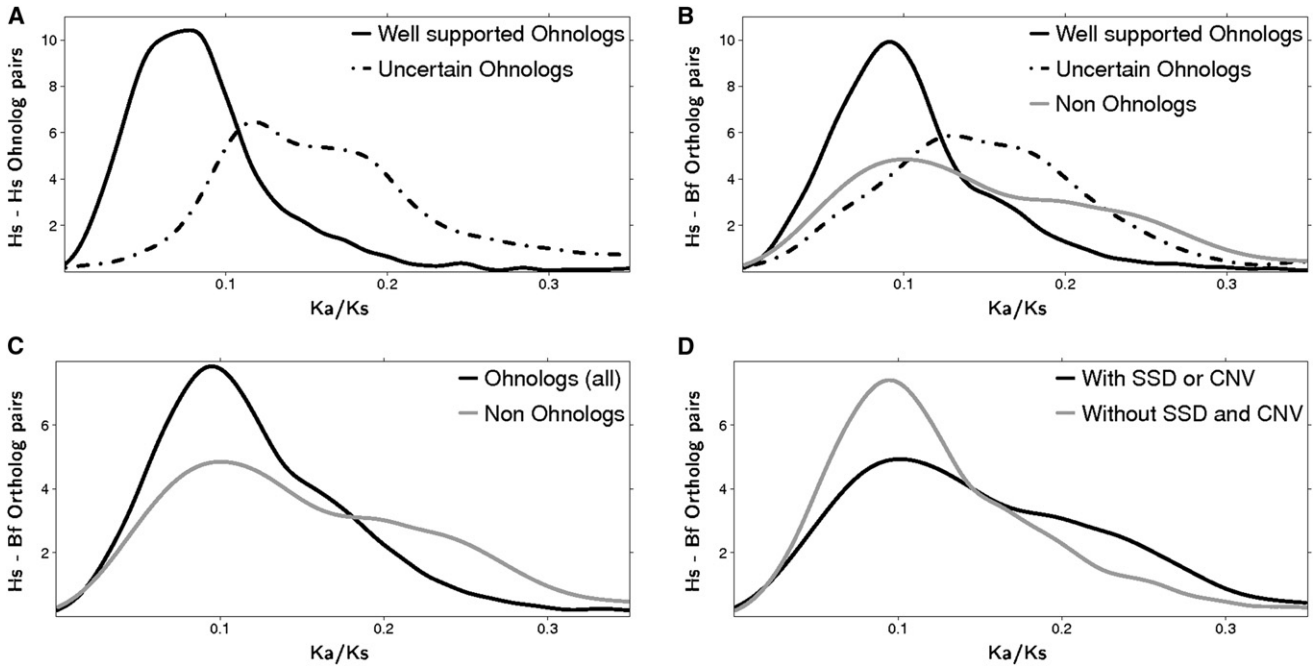

Figure 2. Ka/Ks Distributions for WGD and SSD or CNV Duplicates in the Human Genome

(A–D) Ka/Ks distributions for human-human (Hs-Hs) ohnolog pairs (A) and human-amphioxus (Hs-Bf) ortholog pairs (B) with different confidence status (see Extended Results). Ka/Ks distributions for human-amphioxus (Hs-Bf) ortholog pairs involving a human ohnolog (C) and for human-amphioxus (Hs-Bf) ortholog pairs exhibiting either SSD or CNV (D).

See also the Extended Results, Figures S2 and S3, and Table S3 for statistical significance and comparison with other invertebrate outgroups.

expressed genes, whereas subsequent random nonfunctionalization of individual ohnologs disrupts this initial dosage balance. For instance, yeast *Saccharomyces cerevisiae* has retained 76% of its ribosomal gene ohnologs from a 150 MY old WGD (Kellis et al., 2004; Lin et al., 2007), although the maintenance of these ohnologs has been suggested to require frequent gene conversion events (Kellis et al., 2004; Evangelisti and Conant, 2010) as well as fine-tuned dosage compensation to ensure a balanced expression with the remaining 24% ribosomal genes having lost their ohnologs (Zeevi et al., 2011).

Following on this dosage balance hypothesis, we performed statistical analysis on multiprotein complexes from HPRD (Keshava Prasad et al., 2009) and CORUM (Ruepp et al., 2010) databases and a hand-curated list of permanent complexes (Zanivan et al., 2007) (Experimental Procedures) to investigate for a possible association between the retention of human ohnologs and their susceptibility to dosage balance constraints.

The results depicted in Figure 1C demonstrate, in agreement with (Makino and McLysaght, 2010), that genes implicated in multiprotein complexes have retained significantly more ohnologs than expected by chance, 41% versus 35% (41%; 1,567/3,814; $p = 8.7 \times 10^{-17}$, χ^2 test). This trend is also enhanced when focusing on haploinsufficient genes, that are known for their actual sensitivity to dosage balance constraints (Qian and Zhang, 2008) (54%; 179/330; $p = 8.0 \times 10^{-14}$, χ^2 test).

Yet, surprisingly, an opposite trend corresponding to the elimination of ohnologs is observed for genes implicated in permanent complexes, that are presumably strongly sensitive to dosage balance constraints (7.5%; 18/239; $p = 1.2 \times 10^{-18}$, χ^2 test) (Figure 1C). In fact, looking more closely at the few human ohnologs, that have not been eliminated from permanent complexes (Table 2), we found that they are likely under less stringent dosage balance constraints than most proteins in permanent complexes, as they typically coassociate with mitochondrial proteins or form large multimeric subcomplexes with intrinsic stoichiometry disequilibrium.

This suggests that the elimination of most ohnologs from permanent complexes is, in fact, strongly favored under dosage imbalance and becomes likely inevitable once a few of those ohnologs have been accidentally lost following WGD. Indeed, the uneven elimination of ohnologs in permanent complexes is expected to lead to the assembly of nonfunctional, partially formed complexes detrimental to the cell, unless dosage compensation mechanisms effectively re-establish proper dosage balance at the level of gene regulation (Birchler et al., 2001), as for yeast ribosomal proteins (Zeevi et al., 2011). By contrast, transient complexes, which are typically more modular than permanent complexes, are expected to accommodate such dosage changes more easily, as they do not usually require the same strict balance in the expression levels of their protein partners.

These findings on the differences in retention of human ohnologs between permanent and more transient complexes suggest the relevance of different underlying causes. Although dosage balance presumably remains the primary evolutionary constraint in permanent complexes (<2% of human genes), which lead to the elimination of ohnologs in permanent complexes in

Table 2. Low Retention of Ohnologs in Permanent Complexes

Permanent Complexes[a]	Number of Ohnologs	Intrinsic Stoichiometry Disequilibrium of Ohnologs in Permanent Complexes
ATP F0	3/12	the 3 ohnologs ATP5G1-3 form the 10-mer C-ring of the F-type ATP synthase
ATP F1	0/5	
COX	2/11	the 2 ohnologs COX4I1,2 coassemble with 3 mitochondrial encoded genes
SRS	2/32	Ohnologs are X-linked RPS4X (with no X-inactivation) and Y-linked RPS4Y1
Mitochondrial SRS	0/30	
LRS	2/50	RPL3 and RPL39 have ohnologs RPL3L and RPL39L with unknown functions
Mitochondrial LRS	0/48	
Proteasome	2/31	ohnologs PSMA7 or PSMA7L are included in the 2 rings of 7 α subunits
Pyruvate dehydrogenase	0/5	
RNA Pol II	0/12	
RNA Pol III	0/9	

COX, cytochrome c oxidase; LRS, large ribosomal subunit; SRS, small ribosomal subunit.
[a]Zanivan et al., 2007.

vertebrate genomes, gene susceptibility to deleterious mutations may be more relevant for the retention of ohnologs within the 17% of human genes participating in more transient complexes. For instance, transient complexes involved in phosphorylation cascades or GTPase signaling pathways are known to be more sensitive to the level of activation of their protein partners than to their total expression levels. Thus, although the active forms of multistate proteins typically amount to a small fraction of their total expression level, hence providing a large dynamic range for signal transduction, it also makes them particularly susceptible to gain-of-function mutations. Such mutations can shift protein activation levels 10- to 100-fold without changes in expression levels and likely underlie stronger evolutionary constraints than the 2-fold dosage imbalance caused by gene duplication.

Indirect Cause of Ohnolog Retention in Protein Complex

To further investigate the relative effects of dosage balance and gene susceptibility to deleterious mutations, we analyzed whether the overall enhanced retention of ohnologs within multiprotein complexes (Figure 1C) could indirectly result from an enhanced susceptibility to deleterious mutations. Indeed, as outlined in Figure 3A, cancer and disease genes are more prevalent within complexes than expected by chance, 29% versus 19% (29%; 2,362/8,095; $p = 3.7 \times 10^{-132}$, χ^2 test) and this trend is enhanced for genes with stronger susceptibility to deleterious mutations, such as oncogenes (39%; 320/813; $p = 2.9 \times 10^{-52}$, χ^2 test) or oncogenes with autoinhibitory folds (59%; 67/114; $p = 2.9 \times 10^{-28}$, χ^2 test). By contrast, ohnologs are only slightly, although significantly, more prevalent in complexes than expected by chance, 22% versus 19% (22%; 1,567/7,110; $p = 9.0 \times 10^{-14}$, χ^2 test), whereas the proportion implicated in cancer or disease genes is clearly enhanced 54% versus 39% (54%; 3,844/7,110; $p = 9.5 \times 10^{-140}$, χ^2 test).

To go beyond these simple statistical associations and quantify the direct versus indirect effects of deleterious mutations and dosage balance constraints on the biased retention of human ohnologs, we have performed a Mediation analysis following the approach of Pearl (Pearl, 2001, 2011). The Mediation framework, developed in the context of causal inference analysis, aims at uncovering, beyond statistical correlations, causal pathways along which changes in multivariate properties are transmitted from a cause, *X*, to an effect, *Y*. More specifically, a Mediation analysis assesses the importance of a mediator, *M*, in transmitting the indirect effect of *X* on the response $Y \equiv Y(x,m(x))$ (Figure 3B).

Mediation analyses have been typically used in social sciences research (Baron and Kenny, 1986) as, for instance, in the context of legal disputes over alleged discriminatory hiring. In such cases, the problem is to establish that gender or race (*X*) have directly influenced hiring (*Y*) and not simply indirectly through differences in qualification or experience (*M*). Mediation analyses have also been used in epidemiology, as in a formal study (Robins and Greenland, 1992) that establishes the direct effect of smoking (*X*) on the incidence of cardiovascular diseases (*Y*), while taking into account the indirect effect of other aggravating factors, such as hyperlipidemia (*M*).

In this report, we have applied the Mediation analysis to genomic data to discriminate between direct effect (*DE*) and indirect effect (*IE*) of deleterious mutations (*X* or *M*) and dosage balance constraints (*M* or *X*) on the biased retention of human ohnologs (*Y*). The results, derived in Extended Experimental Procedures (Table S4) and summarized in Figure 3C and Table S5, demonstrate that the retention of ohnologs in the human genome is more directly caused by their susceptibility to deleterious mutations than their interactions within multiprotein complexes.

Indeed, the direct causal effect of a change from "noncomplex" to "complex" proteins only accounts for 23% of a small total effect (*TE*) of complex on the retention of ohnologs (*DE*/*TE* = 23% with *TE* = 0.079), whereas 82% of this small total effect is indirectly mediated by their susceptibility to deleterious mutations (*IE*/*TE* = 82% with 5% nonlinear coupling between direct and indirect effects) (Extended Results). By contrast, the alternative hypothesis, assuming a direct effect of deleterious mutations, accounts for 99% of a three times larger total effect on ohnolog retention (*DE*/*TE* = 99% with *TE* = 0.23), whereas the "complex" versus "noncomplex" status of human genes

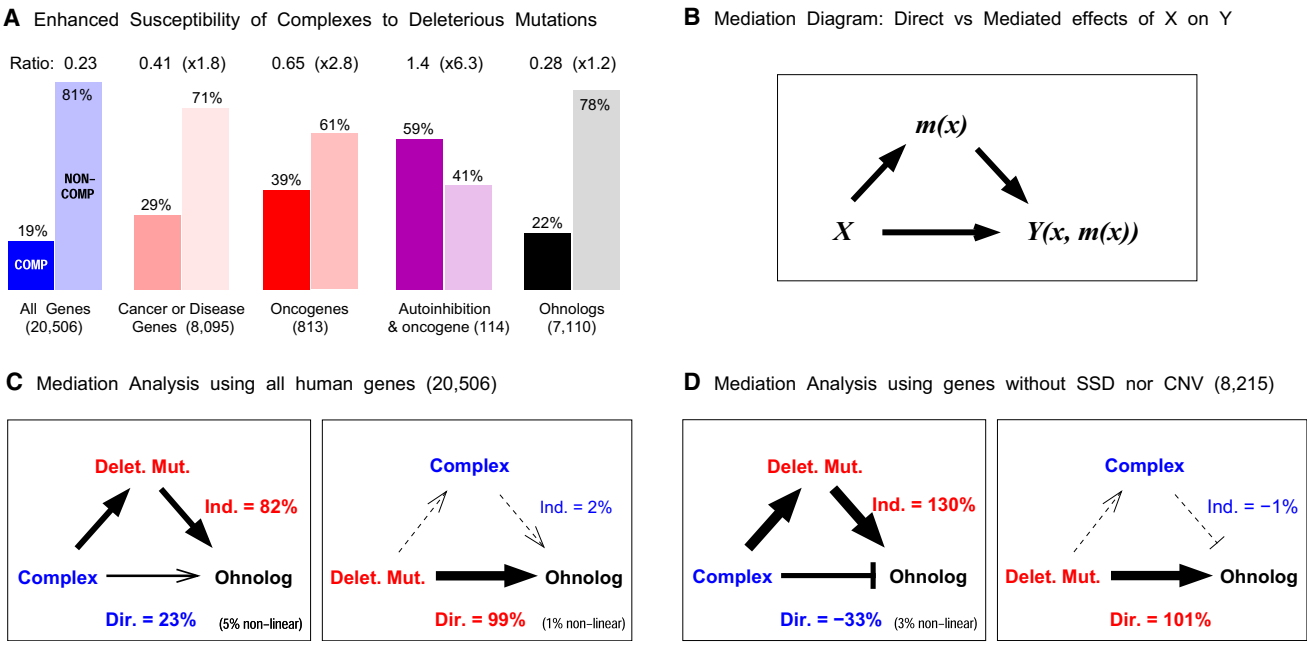

Figure 3. Mediation Analysis of the Indirect Effect of Deleterious Mutations on the Retention of Ohnologs in Multiprotein Complexes

(A) Enhanced susceptibility of complexes to deleterious mutations.

(B) Mediation diagram depicting the direct versus indirect (i.e., mediated) effects of the cause *X* on the outcome *Y*(*x*,*m*(*x*)) (Pearl, 2011). See also Extended Experimental Procedures.

(C and D) Quantitative Mediation analysis of direct versus indirect effects of deleterious mutations and dosage balance on the retention of human ohnologs using (C) all human genes (20,506) or (D) all human genes without SSD nor CNV (8,215). The thickness of the arrows outlines the relative importance of the corresponding direct or indirect effects. These results are consistent with those obtained from partial correlation analysis.

See also the main text, Extended Results, and Tables S4, S5, and S6.

has a negligible indirect effect on ohnolog retention in this case (*IE*/*TE* = 2%) (Extended Results). These trends are also further enhanced when the analysis is restricted to the 40% of human genes (8,215) without SSD and CNV duplicates (Figure 3D; Table S5; Extended Results). In fact, the direct effect of multiprotein complexes then tends to oppose the retention of ohnologs (*DE*/*TE* = −33% with *TE* = 0.064), as in the case of permanent complexes detailed above, but on an increased sample size of 8,215 genes without SSD or CNV duplicates (i.e., more than a third of human genes) in place of 239 genes from permanent complexes. By contrast, there is a five times larger total effect due to the direct effect of deleterious mutations on the retention of ohnologs (*DE*/*TE* = 101% with *TE* = 0.32), Figure 3D. This is an instance of Simpson's paradox, where two effects oppose each other, thereby, revealing the existence of conflicting underlying causes, namely, a strong positive effect of deleterious mutations and a small negative effect of dosage balance constraints on the retention of human ohnologs without SSD and CNV duplicates.

We have also examined the effects of other alternative properties on the retention of ohnologs (Extended Results; Table S5). In particular, we have found that gene expression levels and Ka/Ks ratios do not significantly mediate the effect of deleterious mutations on the retention of ohnologs. In fact, gene expression levels (Extended Experimental Procedures) have a negligible total effect on the retention of human ohnologs (*TE* = 0.003), by contrast to what has been reported for the paramecium (Gout et al., 2009). The total effects of Ka/Ks on ohnolog retention are also lower than the total effects of deleterious mutations, as *TEs* from deleterious mutations are ~2- to 3-fold stronger than *TEs* from Ka/Ks and become >10-fold stronger for genes without SSD and CNV (Extended Results).

In addition, we have performed a complementary systematic study of all these genomics properties using partial correlation analysis, which aims at "removing" the effect of a third property (*Z*) on the standard pair correlations between two variables (*X*) and (*Y*). The results detailed in Extended Results and Table S6 are entirely consistent with those obtained through mediation analysis, although the two approaches are not equivalent. Indeed, although mediation effects require partial correlation, partial correlation does not imply mediation, in general (Extended Results).

All in all, these results support the fact that the retention of ohnologs in the human genome is more strongly associated with their "dangerousness" (i.e., susceptibility to dominant deleterious mutations) than with their functional importance ("essentiality"), sensitivity to dosage balance, absolute expression levels or sequence conservation (i.e., Ka/Ks).

Model for the Retention of "Dangerous" Ohnologs

As demonstrated above, human genes with a documented sensitivity to dominant deleterious mutations have retained statistically more ohnologs from the two WGD events at the

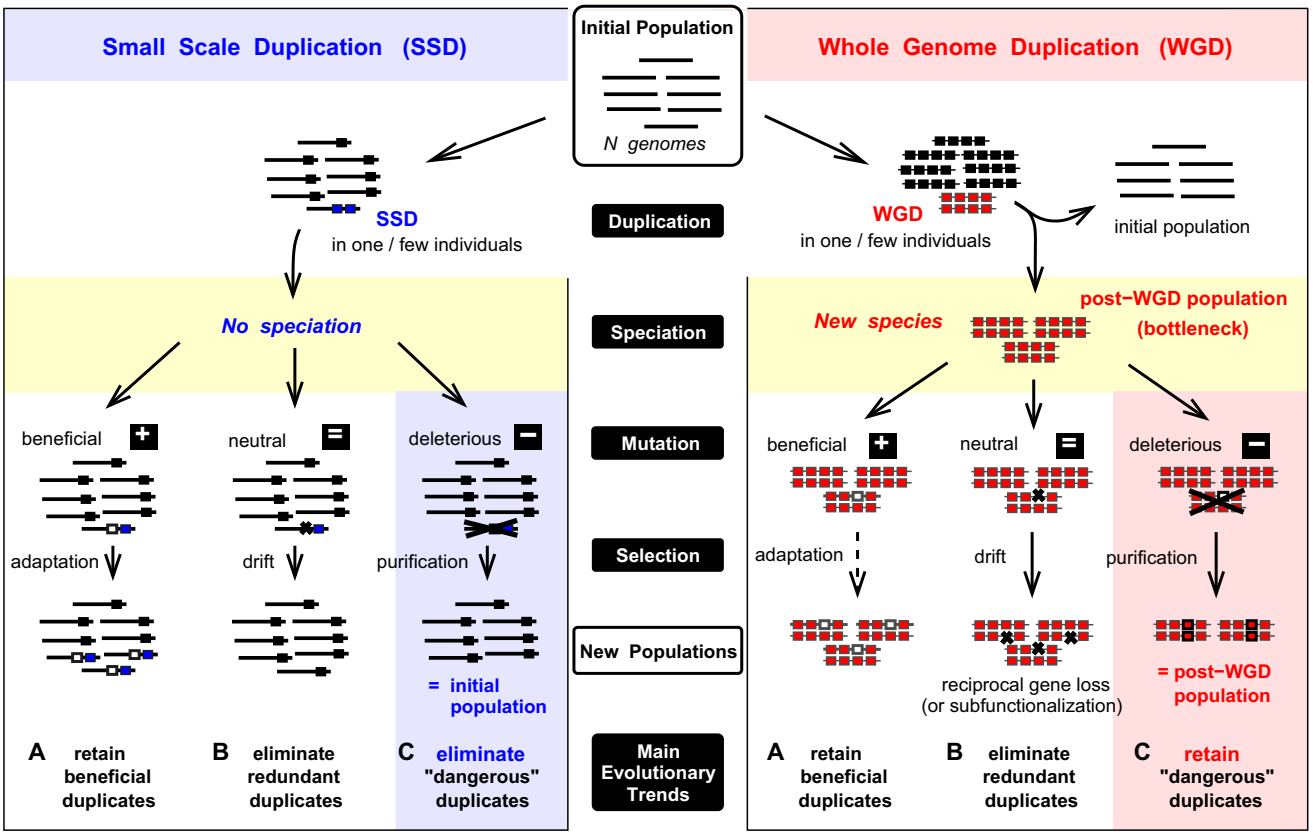

Figure 4. Evolutionary Trends of Duplicated Genes following SSD or WGD

(A–C) Horizontal lines represent the genome of different individuals. Square blocks symbolize the genes, duplicated (SSD: blue; WGD: red) or not (black). Black crosses highlight the loss of one gene (small crosses) or the elimination of an individual (larger crosses), whereas bordered square blocks emphasize retained mutated copies. Evolutionary scenarios are depicted at the population genetics level following either a SSD (left panel) or a WGD (right panel) in one or a few individuals of an initial population. Unlike SSD, WGD is invariably coupled to a speciation event, owing to the difference in ploidy between pre- and post-WGD individuals. Three possible scenarios—beneficial (A), neutral or nearly neutral (B), or deleterious mutations (C) in one gene duplicate—are outlined in post-SSD and post-WGD populations. The main difference concerns the mutation/selection process of "dangerous" genes, i.e. genes prone to autosomal-dominant deleterious mutations (C). See main text for a detailed description.

onset of jawed vertebrates. This suggests that ohnologs have been retained in vertebrate genomes, not because they initially brought selective advantages following WGD, but because their mutations were more likely detrimental or lethal than nonfunctional, thereby preventing their rapid elimination from the genomes of surviving individuals following WGD transitions, as outlined in the evolutionary model depicted in Figure 4.

For completeness and clarity, Figure 4 examines all possible evolutionary scenarios following either a SSD or a WGD duplication event in the genome of one or a few individuals in an initial population. The first and critical difference between SSD and WGD duplication events occurs at the population genetics level with an obligate speciation following WGD event, owing to the difference in ploidy between pre- and post-WGD individuals. As a result, all individuals in the post-WGD population carry twice as many genes as their pre-WGD relatives, whereas only a few individuals in the post-SSD population carry a single small duplicated region. Figure 4 then outlines the three mutation/selection scenarios focusing on a single gene duplicate in the genomes of post-SSD or post-WGD populations: (A) Beneficial mutations after SSD or WGD are expected to spread and become eventually fixed in the new populations, although the bottleneck in population size following WGD limits in practice the efficacy of adaptation in post-WGD species. (B) Neutral or nearly neutral mutations mainly lead to the random nonfunctionalization of one copy of most redundant gene duplicates and, therefore, to their elimination following both SSD and WGD events. In post-WGD populations, this results in the "reciprocal gene loss" of most gene duplicates, which is also known to lead to further speciations in post-WGD species, owing to the interbreeding incompatibility between post-WGD individuals with different "reciprocal gene loss" pattern (Lynch and Force, 2000a). Alternatively, neutral or nearly neutral mutations can also result in the eventual retention of both duplicate copies through subfunctionalization (Hughes, 1994; Lynch and Force, 2000b), that is, by rendering each duplicate copy unable to perform all the functions of their ancestral gene (see Discussion). (C) Finally, dominant deleterious mutations favor the elimination of the individuals

(or their descendants) harboring them through purifying selection. However, this typically leads to opposite outcomes in post-SSD and post-WGD populations. In post-SSD populations, dominant deleterious mutations will tend to eliminate SSD duplicates before they have the time to reach fixation (see below). By contrast, in post-WGD populations, where all ohnologs have been initially fixed through WGD-induced speciation, purifying selection will effectively favor the retention of dangerous ohnologs, as all surviving individuals still present (nondeleterious) functional copies of these dangerous genes.

Note, in particular, that this somewhat counterintuitive evolutionary model for the retention of ''dangerous'' ohnologs hinges on two unique features:

(1) It requires an autosomal dominance of deleterious mutations, in agreement with our observation, above, that retained ohnologs are more ''dangerous'' than ''essential.''
(2) It relies on the fact that successful WGD events start with a concomitant speciation event, which immediately fixes all ohnolog duplicates in the initial post-WGD population (Figure 4).

Note, also, that the same evolutionary trend is expected for dangerous SSD duplicates that would have the time (t) to become fixed through genetic drift in a population of size N before deleterious mutations can arise at a rate K, i.e., $t = 4N < 1/K$. This corresponds to a population bottleneck effect with $N < 1/(4K) \approx 5{,}000–10{,}000$ for typical vertebrates.

DISCUSSION

Beyond human and vertebrate genomes, WGD events have now been established in all major eukaryote kingdoms (Sémon and Wolfe, 2007; Evlampiev and Isambert, 2007). Unlike SSD events, WGD transitions provide a unique evolutionary mechanism, enabling the simultaneous duplication of entire genetic pathways and multiprotein complexes, followed by long periods of functional divergence and extensive loss of ohnologs (Aury et al., 2006). Moreover, although both WGD and SSD events have expanded the gene repertoires and resulting protein networks (Evlampiev and Isambert, 2007; Evlampiev and Isambert, 2008) of eukaryotes, it has become increasingly clear that WGD and SSD events actually lead to the expansion of different gene classes in the course of evolution, (Maere et al., 2005; Aury et al., 2006; Sémon and Wolfe, 2007; Makino and McLysaght, 2010; Huminiecki and Heldin, 2010; and this study).

In this article, we report that WGD have effectively favored the expansion of gene families prone to deleterious mutations in the human genome, such as genes implicated in cancer and genes with autoinhibitory interactions. In particular, we found that the retention of many ohnologs suspected to be dosage balanced is in fact indirectly mediated by their susceptibility to deleterious mutations.

From a broader perspective, a number of studies have now shown that many genomic properties, such as gene essentiality, duplicability, functional ontology, network connectivity, expression level, mutational robustness, divergence rates, etc., all appear to be correlated to some extent. In the light of the present study, we expect that many of these statistically significant correlations mainly result from indirect rather than direct associations, which may even frequently oppose each other. This highlights the need to rely on more advanced inference methods to analyze the multiple, direct, and indirect causes underlying the evolution of specific gene repertoires.

In the present study, we have quantitatively analyzed the direct versus indirect effects of the susceptibility of human genes to deleterious mutation and dosage balance constraints on the retention of ohnologs and proposed a simple evolutionary mechanism to account for the initial retention of ''dangerous'' ohnologs after WGD (Figure 4). On longer timescales, we expect that this initial retention bias of ''dangerous'' ohnologs effectively promote a prolonged genetic drift and, thus, a progressive functional divergence between ohnolog pairs. This eventually favors the subfunctionalization (Hughes, 1994; Lynch and Force, 2000b) of ancestral functions between ohnolog pairs, which ultimately warrants their long-term maintenance following WGD events.

Note, however, that this subfunctionalization process requires that the expression of ohnologs is not rapidly suppressed by large-scale deletion or silencing mutations in regulatory regions. As ohnolog pairs are not arranged in tandem, large-scale deletions through unequal crossing-over cannot typically remove entire ohnolog duplicates while preserving the integrity of nearby genes. Furthermore, as the size of promoter or enhancer regions is typically much smaller than UTRs and coding regions, one expects that the rate of transcriptional silencing does not exceed the rates of functional silencing and divergence in UTRs and coding regions. In fact, early estimates (Nadeau and Sankoff, 1997) showed that gene loss and functional divergence after genome duplications in early vertebrates occurred at comparable rates in gene families including at least two ohnologs. This is also directly evidenced by pseudotetraploid species like the vertebrate *Xenopus laevis*, which still retains ~40% of its initial ohnologs from a 30-million-year-old WGD (Sémon and Wolfe, 2008). All in all, this suggests that ohnologs prone to dominant deleterious mutations have at least a few million years to diverge and become nonredundant genes before they have a chance to be deleted or transcriptionally silenced.

Yet, we found that the retention of these dangerous ohnologs remains intrinsically stochastic by nature as many of them have also been eliminated following WGD events. This presumably occurred through loss-of-function mutations, transcriptional silencing, or large-scale deletion before ohnolog pairs could diverge and become nonredundant genes. More quantitatively, a simple theoretical estimate, derived from the long-term retention statistics of Figure 1, shows that only 6%–10% of the initial ohnolog duplicates have been retained on average at each round of WGD, Figure 5 (see Extended Results for details). By comparison, ~23%–30% of the initial ohnologs prone to gain-of-function mutations have been retained on average at each WGD. This implies that genes susceptible to deleterious mutations are two to five times more likely to retain ohnologs on long evolutionary timescales. Moreover, genes combining several factors associated with enhanced susceptibility to autosomal-dominant deleterious mutations are shown to be more than ten times more

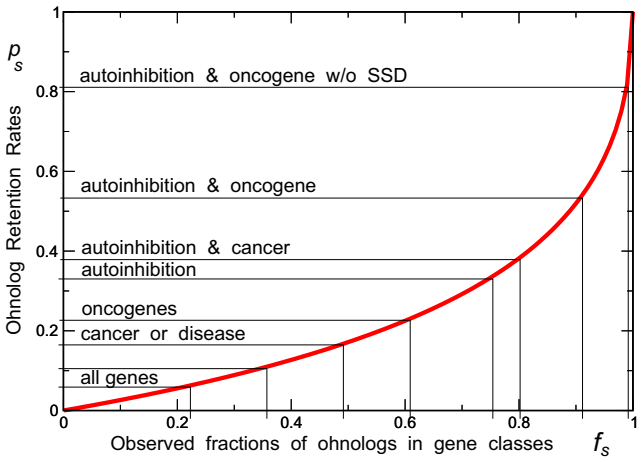

Figure 5. Estimates of Ohnolog Retention Rates

Estimates of ohnolog retention rates p_s in early vertebrates from the observed fraction f_s of ohnologs in the human genome for gene classes, s, with increasing susceptibility to deleterious mutations. The theoretical estimate (red curve) is obtained assuming that the retentions of ohnologs were comparable for each of the two WGD at the onset of vertebrates, and reads $P_s = 2/f_s - 1 - \sqrt{(2/f_s - 1)^2 - 1}$ as detailed in the Extended Results and Tables S7 and S8.

likely to retain ohnologs than genes lacking gain-of-function mutations (Figure 5), as illustrated on the examples of oncogenes with autoinhibitory folds (Table 1).

In turn, the elimination of ohnologs has been shown to drive further speciation events within post-WGD (sub)populations, due to the emergence of recombination barriers from the accumulation of differences in ohnolog deletion patterns between post-WGD individuals (Lynch and Force, 2000a). The resulting fragmentation of post-WGD subpopulations is then expected to sustain negative selection pressure that favors the retention of the remaining ohnolog pairs prone to deleterious mutations, as outlined in Figure 4. Hence, although most WGDs are unlikely to bring much fitness benefit on short evolutionary timescales (if only due to the population bottlenecks associated with WGD-induced speciations; Figure 4), they provide a unique evolutionary mechanism to experiment virtually unlimited combinations of regulation/deletion patterns from redundant ohnolog genes. Over long timescales (>100–500 MY), such trial and error combinations have visibly led to the evolutionary success and radiation of WGD species.

In summary, we present evidence supporting an evolutionary link between the susceptibility of human genes to dominant deleterious mutations and the documented expansion of these "dangerous" gene families by two WGD events at the onset of jawed vertebrates. We propose that deleterious mutations, responsible for many cancers and other severe genetic diseases on the lifespan of human individuals, have also underlain purifying selection over long evolutionary timescales, which effectively favored the retention of vertebrate ohnologs prone to dominant deleterious mutations, as outlined in Figure 4. From a population genetics perspective, we argue that this counterintuitive retention of dangerous ohnologs hinges in fact on WGD-induced speciation events, which are largely credited for the genetic complexity and successful radiation of vertebrate species.

These findings highlight the importance of purifying selection from WGD events on the evolution of vertebrates and, beyond, exemplify the role of nonadaptive forces on the emergence of eukaryote complexity (Fernández and Lynch, 2011).

EXPERIMENTAL PROCEDURES

WGD Duplicated Genes or "Ohnologs"

Human ohnolog genes were obtained from (Makino and McLysaght, 2010). Makino and McLysaght compared different vertebrate and six nonvertebrate outgroup genomes to identify ohnologs in the human genome. The final data set consists of 8,653 ohnolog pairs and 7,110 unique ohnologs. We further divided ohnologs into well supported (3,963), plausible (894), and more uncertain (2,253) ohnologs (see Extended Experimental Procedures).

SSD Duplicated Genes

We identified paralogous genes within the human genome from sequence similarity search. We obtained a total of 11,185 SSD genes. In particular, paralogs that were not annotated as ohnologs were taken to be SSD genes (see Extended Experimental Procedures).

Genes with CNV

CNV regions were obtained from Database of Genomic Variants (Zhang et al., 2006). A total of 5,709 genes were identified to be CNV genes as their entire coding sequence fell within one of the CNV regions.

Cancer and Disease Genes

We obtained cancer genes from multiple databases, including COSMIC (Forbes et al., 2011) and CancerGenes (Higgins et al., 2007), listed in Table S7. The detailed list of 6,917 cancer genes is given in Table S8 with a hand-curated list of 813 verified or predicted (Bozic et al., 2010) oncogenes (see Extended Experimental Procedures). We obtained 2,580 disease genes from the "Morbidmap" database of OMIM and hand curated subsets of 440 autosomal-dominant and 598 autosomal-recessive disease genes from Blekhman et al. (2008).

Genes with Autoinhibitory Folds

To obtain genes coding for proteins with autoinhibitory folds we searched PubMed with keyword "autoinhibitory domain" and retrieved relevant autoinhibitory genes and domains manually. Further gene candidates with autoinhibitory folds were obtained from databases, OMIM, SwissProt, NCBI Gene, and GeneCards using the parsing terms: auto/self-inhibit*. Careful manual curation of this list of gene candidates with the available literature finally yielded a total of 461 genes with autoinhibitory folds (94% of initial candidates).

Essential Genes

Mouse essential genes were obtained from Mouse Genome Informatics database. Essential genes were defined as genes having lethal or infertility phenotypes on loss-of-function or knockout mutations (2,729 genes) (see Extended Experimental Procedures).

Genes in Complexes and Permanent Complexes

Protein complexes were obtained from Human Protein Reference Database (HPRD) (Keshava Prasad et al., 2009) and CORUM database (Ruepp et al., 2010). In addition, a manually curated data set of permanent complexes (239 genes) was obtained from Zanivan et al. (2007). The final data set consists of 3,814 protein complex genes (see Extended Experimental Procedures).

Haploinsufficient and Dominant Negative Genes

Haploinsufficient and dominant negative candidate genes were obtained from parsing OMIM text files with Perl regular expressions. The resulting gene lists were manually curated with the available literature, yielding a total of

330 haploinsufficient genes (80% of initial candidates) and 477 dominant-negative genes (63% of initial candidates).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Results, Extended Experimental Procedures, three figures, and eight tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.09.034.

LICENSING INFORMATION

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