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REVIEW

Constitutive heterochromatin: a surprising variety of expressed sequences

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Abstract The organization of chromosomes into euchromatin and heterochromatin is amongst the most important and enigmatic aspects of genome evolution. Constitutive heterochromatin is a basic yet still poorly understood component of eukaryotic chromosomes, and its molecular characterization by means of standard genomic approaches is intrinsically difficult. Although recent evidence indicates that the presence of transcribed genes in constitutive heterochromatin is a conserved trait that accompanies the evolution of eukaryotic genomes, the term heterochromatin is still considered by many as synonymous of gene silencing. In this paper, we comprehensively review data that provide a clearer picture of transcribed sequences within constitutive heterochromatin, with a special emphasis on *Drosophila* and humans.

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Historical features

Heterochromatin was originally defined cytologically as the set of chromosomal regions that stain deeply at prophase and maintain a compact organization throughout all stages of the mitotic cell cycle (Heitz 1928). In a wide variety of eukaryotes, large chromosomal portions, or even entire chromosomes, are made up of heterochromatin. Heterochromatin was further classified into facultative or constitutive (Brown 1966). Facultative heterochromatin corresponds to euchromatic regions (chromosome portions, entire chromosomes, or whole genomes), the structure and activity of which are subjected to control in that they can be alternatively functional or silenced during specific phases of development (Plath et al. 2002). In contrast, constitutive heterochromatin is commonly found in large blocks near centromeres and telomeres; it consists mostly of repetitive DNA sequences and maintains its characteristic organization on both homologous chromosomes.

Constitutive heterochromatin is a basic component of eukaryotic genomes in that it forms about 5% of the genome in *Arabidopsis thaliana*, 30% in *Drosophila* and humans, and up to 70–90% in certain nematodes and plants (Moritz and Roth 1976; Peterson et al. 1998; *Arabidopsis* genome initiative 2000; Dimitri et al. 2005a, b), yet the

reasons for its widespread occurrence are still unclear. A set of distinctive properties, antagonistic compared to the rest of the genome, have historically been recognized for constitutive heterochromatin in virtually all animal and plant species: (1) strongly reduced level of meiotic recombination; (2) low gene density; (3) mosaic inactivation of the expression of euchromatic genes when moved nearby, a phenomenon termed position effect variegation; (4) late replication during S phase; (5) transcriptional inactivity; and (6) enrichment in highly repetitive satellite DNAs and transposable element remnants.

Together, these properties led to the view of constitutive heterochromatin as a "desert" or a "graveyard" of genetic functions (reviewed by John 1988). In the last three decades, however, studies primarily conducted in *Drosophila melanogaster* have shown that constitutive heterochromatin does in fact play roles in important cellular functions, such as chromosome organization and inheritance, and contains genes essential for viability and fertility (Gatti and Pimpinelli 1992; Williams and Robbins 1992; Weiler and Wakimoto 1995; Dernburg et al. 1996; Elgin 1996; Karpen et al. 1996; Dimitri and Junakovic, 1999; Eissenberg and Hilliker 2000; Henikoff et al. 2001; Coulthard et al. 2003; Dimitri et al. 2005a; Fitzpatrick et al. 2005; Villasante et al. 2007). Thus, the idea that constitutive heterochromatin is merely a genomic wasteland has become obsolete.

Drosophila heterochromatin genes

Essential genes early defined by genetic and cytological analysis

D. melanogaster is the model organism in which the greatest progress in the study of heterochromatin functions has been made due to the ability to combine genetic, cytological, and genomic approaches. Using chromosome banding techniques, the mitotic heterochromatin of *D. melanogaster* has been subdivided into 62 regions with distinctive cytological properties (Gatti and Pimpinelli 1983; Pimpinelli and Dimitri 1989; Dimitri 1991). Genes essential for viability and fertility were initially identified in *D. melanogaster* by recessive mutations genetically linked to regions of constitutive heterochromatin (Brosseau 1960; Hilliker 1976; Marchant and Holm 1988). The identification of such mutations was followed by complementation analysis using chromosome rearrangements with cytologically determined breakpoints that mapped to mitotic heterochromatin, which yielded significant insight into the location and structural organization of the genetic loci located in autosomal and sex heterochromatin of *D. melanogaster* (Gatti and Pimpinelli 1983; Pimpinelli et al. 1985; Dimitri 1991; Koryakov et al. 2002).

Sex chromosome heterochromatin genes

Some loci located in the sex chromosome heterochromatin are physically very large and mainly consist of high- and middle-repetitive DNAs. The kl-5, kl-3, and kl-1 fertility factors on the Y-chromosome are estimated to contain about 4 Mb of DNA; they require structural integrity for function and form giant loops that are actively transcribed in primary spermatocytes (reviewed by Gatti and Pimpinelli 1992). These gigantic loci, which were originally suggested to perform structural functions, have in fact turned out to harbor protein-coding genes. For example, *kl-5* encodes an axonemal–dynein heavy chain that is expressed in the testis (Gepner and Hays 1993). These genes are made up of small unique exons and transposable element-rich mega-introns that can account for 1 or 2 Mb of DNA (Kurek et al. 2000; Carvalho et al. 2001).

Other loci found on the Y and X heterochromatin, such as *bobbed*, encoding the ribosomal genes (Ritossa and Spiegelman 1965) are not inactivated by breakpoints of translocations or inversions, like the Y-fertility factors, but only by deletions; they consist of an array of middle-repetitive sequences whose number is critical for their activity. A similar organization is exhibited by *Suppressor of Stellate [Su(Ste)]* locus (Litvak 1984; Bozzetti et al. 1995) which is involved in a natural case of RNA silencing-mediated regulation. *Su(Ste)* repeats produce short sense and antisense RNAs that cause the repression of testis-expressed homologous *Stellate* genes on the X-chromosome (Aravin et al. 2004). In addition, the X-chromosome heterochromatin carries a group of still poorly characterized genetic loci that are thought to be all composed of repeated elements (reviewed by Gatti and Pimpinelli 1992): compensatory response (*cr*), *ABO*, *collochore* (*col*), and *Ribosomal exchange* (*Rex*) with its suppressor. The *collochore* locus mediates proper sex chromosome pairing and disjunction during the first meiotic division; *cr* controls rDNA gene dosage compensation; the *ABO* elements rescue the maternal defects caused by a recessive maternal effect mutation called abnormal oocyte (*abo*). Finally, mutations in *Rex* cause a high frequency of exchanges and deletions in the rDNA (Rasoly and Robbins 1991).

It is worth noting that both *ABO* and *Su(Ste)* loci are part of genetic systems that involve specific interactions between heterochromatin and euchromatin genetic elements. Such heterochromatin elements were defined "criptic" in that they escape genetic analysis and their effect can be detected only in the presence of mutations in the euchromatic counterpart (Palumbo et al. 1994).

Autosomal heterochromatin essential genes

Thus far, at least 32 genes essential for viability have been mapped to mitotic heterochromatin of chromosomes 2 and

3 (Dimitri 1991; Koryakov et al. 2002), but only a few of those are clearly defined at the molecular level: *RpL5, light, concertina, rolled, RpL38, Nipped-B, Nipped-A, Parp and RpL15* (Hilliker 1976; Devlin et al. 1990a, b; Parks and Wieschaus 1991; Biggs et al. 1994; Rollins et al. 1999; Tulin et al. 2002; Myster et al. 2004; Marygold et al. 2005; Schulze et al. 2005). Most of the genes detected thus far were mapped cytologically to heterochromatin regions of moderate fluorescence after staining with 4,6-diamino-2-phenylindole-dihydrochloride (DAPI); as example, see the mapping of essential gene shown in Fig. 1. These regions harbor clusters of transposable elements and are devoid of highly repetitive satellite DNAs (Pimpinelli et al. 1995; Lohe et al. 1993).

Putative genes defined by heterochromatin sequence annotation

In the last decade, the completion of genome sequencing projects has yielded a great amount of information on DNA sequences in several organisms. The release of the sequence of *D. melanogaster* heterochromatin by the Berkeley Drosophila Genome Project (http://www.fruitfly.org/) and *Drosophila* Heterochromatin Genome Project (DHGP; http://www.dhgp.org/index_release_notes.html) has greatly facilitated studies of mapping, molecular organization, and function of genes located in pericentromeric heterochromatin. Initially, 3.8 Mb of about 120 Mb of the *D. melanogaster* euchromatic genome sequence included in

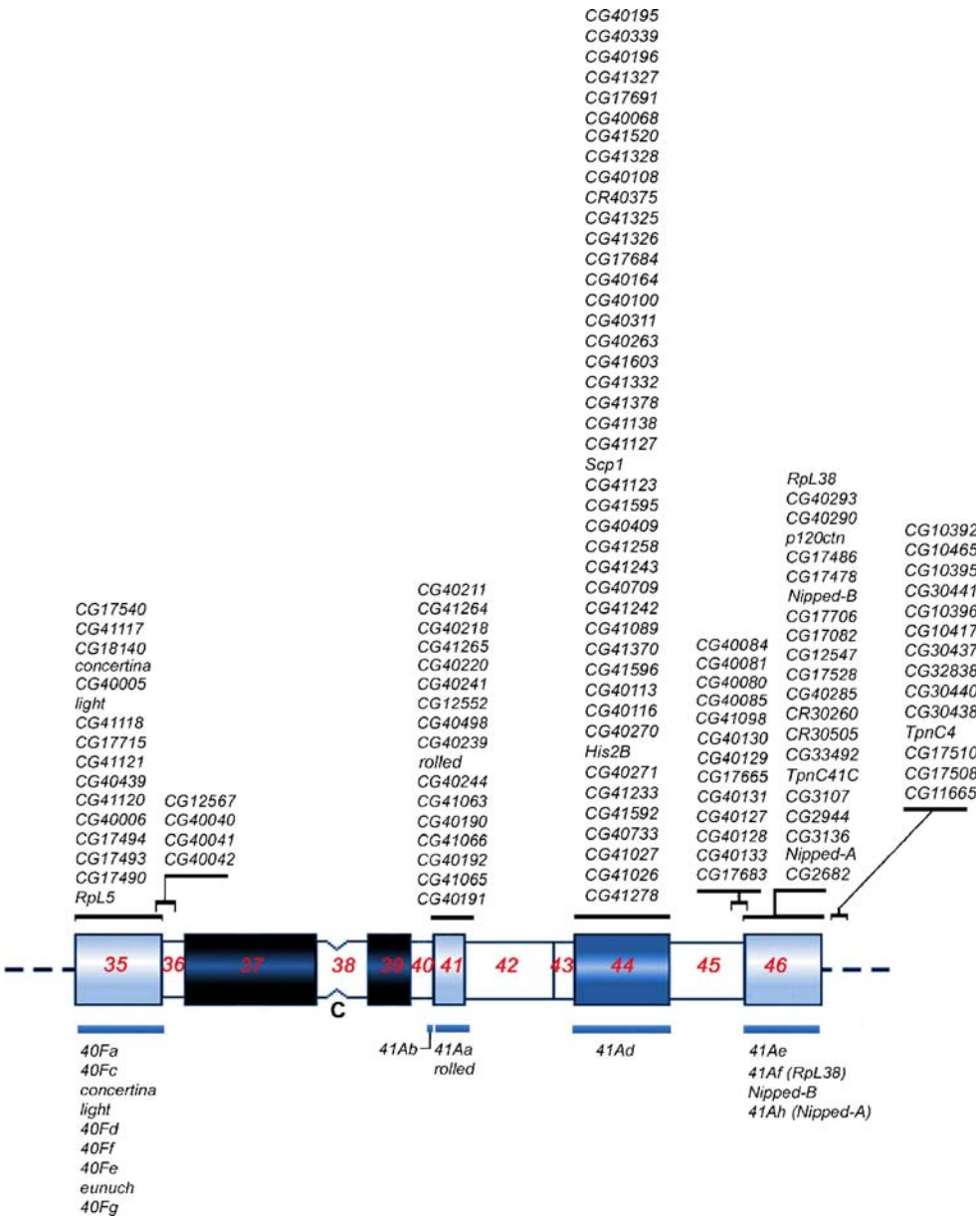

Fig. 1 Mapping of essential and putative genes to the heterochromatin of mitotic chromosome 2. Cytogenetic mapping of both essential genes defined by mutational analyses (*below*) and of putative genes (*above*) defined by computational analyses. *Shades of blue* correspond to the intensity of DAPI staining, with the *darkest blue blocks* representing regions with strong fluorescence intensity and *open blocks* representing non-fluorescent regions. The different cytological regions are *numbered in red*. Scaffold designation is shown at the *top* of each gene model list. Additional 2Rh lethal genes defined by Myster et al. (2004) are not included in the map, as it is presently unclear whether these lethals correspond to new vital genes

release1 were found to correspond to sequences originated from distal heterochromatin regions (Adams et al. 2000). More recently, an improved whole genome shotgun assembly (heterochomatic-WGS3; Hoskins et al. 2002) has been produced, which includes 20.7 Mb of draft-quality heterochromatin sequence. In the last year, 15 Mb of this sequence has been further improved or completed (Hoskins et al. 2007), and a BAC-based physical map of 13 Mb of pericentric heterochromatin, together with the cytogenetic map that locates some 11 Mb to specific heterochromatin regions, has been constructed (Hoskins et al. 2007). About 450 predicted genes were initially identified by the annotation of the heterochromatin sequence (Hoskins et al. 2002). More recently, about 250 protein-coding genes were defined in the release 5.1 annotation of the currently sequenced heterochromatin DNA (Smith et al. 2007). According to these results, the number of active genes in constitutive heterochromatin of *D. melanogaster* appears to be higher than that defined by genetic analysis.

Several studies have concentrated on an effort to map predicted genes to the mitotic heterochromatin of *D. melanogaster* using fluorescent in situ hybridization (FISH) with BACs, cDNAs, and P-elements (Hoskins et al. 2002; Corradini et al. 2003; Yasuhara et al. 2003; Rossi et al. 2007). For example, about 161 predicted genes have been assigned to specific regions of the mitotic heterochromatin of chromosome 2 (Rossi et al. 2007; Hoskins et al. 2007; Figs. 1 and 2; Table 1) in which genetic analyses defined 17 essential genes. Essential and putative genes are grouped within regions h35 and h46 in the constitutive heterochromatin of chromosome *2*, which represent the most distal portions of mitotic heterochromatin. Most of these genes are located in weakly DAPI-fluorescent chromosomal regions, which harbor clusters of transposable element-homologous sequences and lack highly repetitive satellite DNAs. The high number of predicted genes found in heterochromatin can be explained by assuming that these regions contain an excess of non-essential coding genes that escape mutational analysis. For example, *CG40293, p120*, and *CG17486* of 2Rh were found to be non-essential (Myster et al. 2004). It may be also possible that single coding sequences are fragmented due to assembly artifacts, thus giving rise to multiple shorter CGs.

PiRNAs and esiRNA clusters: a special class of heterochromatin genes

The *flamenco*/*COM* locus, involved in the regulation of *gypsy*, *Idefix*, and *ZAM* retrotransposons, has been mapped to the distal portions of the X heterochromatin (Prud'homme et al. 1995; Desset et al. 2003). Molecularly, *flamenco* was located proximally to the *DIP-1* gene and

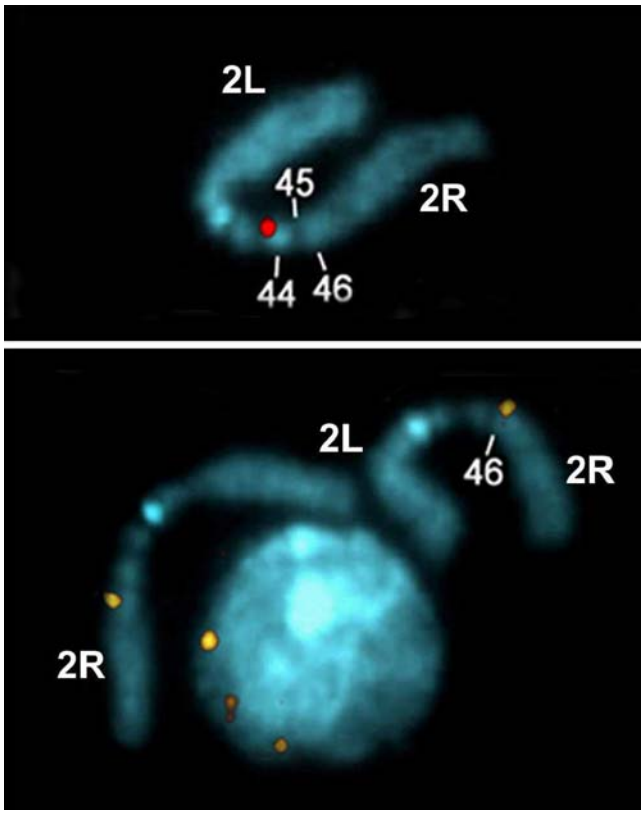

Fig. 2 FISH mapping of cDNA to mitotic heterochromatin. FISH mapping of two different cDNA clones from predicted genes on heterochromatin of the right arm of chromosome 2

proposed to span a region that corresponds to a Piwi-interacting RNA (piRNA) cluster recently identified by Brennecke et al. (2007). This piRNA cluster mainly consists of nested transposable elements (TEs) spanning a total length of 179 kb and includes numerous fragments of *gypsy*, *Idefix*, and *ZAM* retrotransposons. Brennecke et al. (2007) found about 130 piRNA loci in pericentromeric and telomeric heterochromatin, which display a high content of defective and nested TEs. Those piRNAs are restricted to gonads and at least a set of them arise through Piwi-mediated cleavage of single-stranded RNAs (Brennecke et al. 2007). Maternally transmitted I-element piRNAs originated from 42AB polytene chromosome region are involved in the control of I element transposition (Brennecke et al. (2008). Endogenous small interfering RNA (esiRNA) originated from heterochromatin TE clusters, and dependent on *Dicer-2* and *Argonaute-2*, were recently detected in somatic and gonad cells (Ghildiyal et al. 2008; Czech et al. 2008). Both piRNA and esiRNA sequence clusters present in heterochromatin are found to be involved in TE silencing. It is tempting to speculate, however, that piRNA and esiRNA clusters, and possibly other non-protein-coding RNAs originating from high TE density

Table 1 Heterochromatin genes of *D. melanogaster* chromosome 2 and evolutionary conservation of their protein products in humans

Scaffold	Location	Genes	Length (bp)	Introns	*D. melanogaster* molecular function	Closest BLASTp human hit
2Lh	h35	CG17540	1,580	2	pre-mRNA splicing factor	RNA binding motif protein 17 (2e-53)
		CG41117	310	n.a.	Unknown	No significant similarity
		CG18140	26,358	8	Chitin binding; chitinase	Acidic mammalian chitinase precursor (8e-75)
		Concertina	10,547	5	GTPase; GTP binding; signal transducer	Guanine nucleotide binding protein (6e-108)
		CG40005	988	4	GTPase	Guanine nucleotide regulatory protein alpha 13 (3e-26)
		Light	15,974	15	Ubiquitin-protein ligase; zinc ion binding	P49754, vacuolar assembly protein VPS41 homolog (S53) (3e-147)
		CG41118	2,901	n.a.	unknown	No significant similarity
		CG17715	6,015	13	Unknown	LOC157378 hypotetical protein (8e-30)
		CG41121	1,172	n.a.	Unknown	No significant similarity
		CG40439	717	2	Unknown	No significant similarity
		CG41120	451	n.a.	Unknown	No significant similarity
		CG40006	133,933	9	Serine-type endopeptidase inhibitor; receptor	Scavenger receptor class B memeber 1 (8e-49)
		CG17494	7,056	6	Unknown	NP_009090.2, sarcolemma associated protein (3e-66)
		CG17493	1,056	1	Calmodulin binding; calcium ion binding	Centrin EF hand protein 2 (7e-62); Caltractin 1e-62
		CG17490	10,227	7	Unknown	No significant similarity
		RpL5	1,905	9	Ribosomal protein L5	Ribosomal protein L5 (1e-119)
CP000215	h35-h36	CG12567	13,373	4	Thiamin diphosphokinase	No significant similarity
		CG40040	107,081	8	Unknown	No significant similarity
		CG40041	732	1	Hormone	Glycoprotein hormone beta subunit (7e-10)
		CG40042	1,141	5	Carrier; protein transporter	Mitochondrial inner membrane translocase 23 (5e-36)
CP000188	h41	CG40211	3,954	4	Unknown	No significant similarity
		CG40218	963	1	Kinesin binding	Craniofacial development protein 1 (4e-13)
		CG41265	94,547	8	Nucleic acid binding; damaged DNA binding	Hypothetical protein FLJ20753 (6e-25)
		CG40220/ CG17702	n.a.	n.a.	Unknown	No significant similarity
		CG40241	n.a.	n.a.	Unknown	No significant similarity
		CG12552	513	0	Unknown	No significant similarity
		CG40498	2,107	7	Unknown	No significant similarity
		CG40239	408	0	Unknown	No significant similarity
		Rolled	49,634	15	MAP kinase	AAH17832.1, mitogen activated protein kinase 1 (3e-171)
		CG40244	499	0	Unknown	No significant similarity
CP000188	h41-h42	CG41063	n.a.	n.a.	Unknown	No significant similarity
		CG40190	n.a.	n.a.	Protein kinase	CAD97888.1 hypothetical protein (2e-25)
		CG41066	288	0	Unknown	No significant similarity
		CG40192	n.a.	n.a.	Unknown	No significant similarity
		CG41065	n.a.	n.a.	Unknown	No significant similarity
		CG40191	2,213	9	Unknown	CGI-57 protein (6e-22)

Table 1 (continued)

Scaffold	Location	Genes	Length (bp)	Introns	*D. melanogaster* molecular function	Closest BLASTp human hit
CP000218	h44	CG40195	482	1	Unknown	No significant similarity
		CG40339	775	1	Unknown	No significant similarity
		CG40196	11,502	10	Transcriptional regulator activity	MAF-1 regulator protein (5e-73)
		CG41327	285	0	Unknown	No significant similarity
		CG17691	5,994	9	3-Methyl-2-oxobutanoate dehydrogenase	Branched chain keto acid dehydrogenase E1, (2e-140)
		CG40068	414	0	Nucleic acid binding; translation factor	Translation initiation factor 2 (5e-33)
		CG41520	126,868	9	Receptor binding	FReD superfamily; angiopoietin-2 (1e-45)
		CG41328	6,055	3	Unknown	No significant similarity
		CG40108	186	0	Unknown	No significant similarity
		CR40375	13,729	5	Unknown	
CP000223	h44	CG41325	3,880	3	Unknown	No significant similarity
		CG41326	21,338	1	Unknown	No significant similarity
		CG17684	395,988	13	Peptidase activity	Dipeptidyl peptidase 10 (4e-105)
		CG40164	598	1	Unknown	No significant similarity
		CG40100	369	1	Unknown	Ethylmalonic encephalopathy 1 protein (7e-20)
		CG40311	13,346	3	Unknown	No significant similarity
		CG40263	70,652	14	Unknown	Sialin, membrane transporter protein (8e-50)
		CG41603	177	0	Unknown	No significant similarity
		CG41332	11,482	3	Unknown	No significant similarity
		CG41378	375	0	Unknown	Legumaturain, thiol reductase, GILT superfamily (1e-11)
		CG41138	24,232	3	Unknown	No significant similarity
		CG41127	3,257	1	Unknown	No significant similarity
		Scp1	34,524	5	Sarcoplasmic calcium binding	No significant similarity
		CG41123	6,48	0	Unknown	No significant similarity
		CG41595	1395	1	Unknown	No significant similarity
		CG40409	273	0	Unknown	No significant similarity
		CG41258	369	1	Unknown	No significant similarity
		CG41243	422	0	Unknown	No significant similarity
		CG40709	394	0	Unknown	No significant similarity
		CG41242	24,621	1	Unknown	No significant similarity
		CG41089	2,605	1	Unknown	No significant similarity
		CG41370	751	1	Unknown	No significant similarity
		CG41596	15,369	3	Unknown	No significant similarity
		CG40113	13,665	6	Unknown	No significant similarity
		CG40116	303	0	Unknown	No significant similarity
CP000163	h44	CG40270	32,013	4	Unknown	No significant similarity

Scaffold	Location	Genes	Length (bp)	Introns	*D. melanogaster* molecular function	Closest BLASTp human hit
		His2B:CG40461	1,756	1	DNA binding	H2Bc (9e-24)
		CG40271	1,931	2	Unknown	No significant similarity
		CG41233	157	0	Unknown	No significant similarity
		CG41592	568	1	Unknown	No significant similarity
CP000344	h44	CG40733	11,224	1	Unknown	No significant similarity
		CG41027	267	0	Unknown	No significant similarity
		CG41026	308	0	Unknown	No significant similarity
		CG41278	14,056	1	Unknown	No significant similarity
CP000219	h45-h46	CG40084	73,978	16	Unknown	cyclin M2 isoform 2 (3e-124)
		CG40081	n.a.	n.a.	Unknown	No significant similarity
		CG40080	35,270	4	Protein serine/threonine kinase	serine/threonine protein kinase Haspin (4e-69)
		CG40085	613	0	Unknown	No significant similarity
		CG41098	n.a.	n.a.	Unknown	No significant similarity
2Rh	h45-h46	CG40130	n.a.	n.a.	Unknown	No significant similarity
		CG40129	148,560	14	G-protein coupled receptor kinase	Beta adrenergic receptor kinase 2 (0.0)
		CG17665	7,177	12	Unknown	Integrator complex subunit 3; 3' end processing of small nuclear RNAs U1 and U2 (6e-152)
		CG40131	265	n.a.	Unknown	No significant similarity
		CG4012	671	2	Unknown	hypothetical protein LOC440400 (2e-13)
		CG40128	n.a.	n.a.	Unknown	No significant similarity
		CG40133	8,325	n.a.	Unknown	No significant similarity
		CG17683	2,027	9	Oxidoreductase; ferredoxin hydrogenase	nuclear prelamin A recognition factor-like (1e-117)
2Rh	h46	*RpL38*	460	1	Ribosomal protein 38	Ribosomal protein L38 (7e-15)
		CG40293	18,578	4	Protein serine/threonine kinase	Breast cancer antigen NY-BR-96 (8e-43)
		CG40290	265	n.a.	Unknown	No significant similarity
		p120ctn	14,198	5	Adherens junction protein	Arm-repeat protein NPRAP/neurojungin (2e-131; 1e-126)
		CG17486	1,805	n.a.	Ligase; asparagine synthase	AAX88843.1 unknown (7e-76)
		CG17478	1,395	n.a.	Unknown	No significant similarity
		Nipped-B	37,323	23	Transcriptional activator; chromatid cohesion	PA_exp: transcriptional regulator (0.0)
2Rh	h46	CG40282	718	0	Unknown	No significant similarity
		CG17082	13713	14	Unknown	Rho GTPase activating protein 18 (1e-31)
		CG12547	2,341	2	Unknown	Novel NHL repeat domain containing protein (1e-134)
		CG17528	7,148	10	Microtubule binding; ATP binding;	Doublecortin and CaM kinase-like 1 (5e-132)
		CG40285/CG14464	715	1	Unknown	Chromosome 11 open reading frame 46 (2e-13)
		CR30260	71	0	tRNA	No significant similarity
		CR30505	71	0	tRNA	No significant similarity

Table 1 (continued)

Scaffold	Location	Genes	Length (bp)	Introns	*D. melanogaster* molecular function	Closest BLASTp human hit
2Rh	h46	CG33492	72,289	4	Ionotropic glutamate receptor	Glutamate receptor, ionotropic, delta 1 (2e-07)
		TpnC41C	3,920	3	Calcium ion binding	Calmodulin 2 (phosphorylase kinase, delta) (4e-25)
		CG3107	4,504	4	Metalloendopeptidase	Metalloprotease 1 (0.0)
		CG2944	11,103	11	Oocyte anterior/posterior axis determination	SPRY domain-containing SOCS box protein SSB-1 variant (7e-117)
		CG3136	10065 bp	5	DNA binding;protein homodimerization	cAMP response element binding protein-related (3e-13)
		Nipped-A	73,048	29	Transcription regulator; cytokinesis	Transformation/transcription domain-associated protein variant (0.0)
		CG2682	35,992	8	Transcription factor; ubiquitin-protein ligase	D4, zinc and double PHD fingers family 2 (5e-49)
hetero-euchromatin		CG10392	22,231	12	Transferring glycosyl groups	O-linked GlcNAc transferase isoform 2 (0.0)
transition region		CG10465	1,290	1	Voltage-gated potassium channel;pr.binding	Unnamed protein product (9e-100)
		CG10395	1,480	1	HIT Zn-finger protein domain	High mobility group AT-hook 1-like 4 (5e-14)
		CG30441	409	n. a	Unknown	Intraflagellar transport protein 20-like protein (9e-12)
		CG10396	733	1	Cytochrome-c oxidase	Cytochrome c oxidase subunit IV isoform 1 (1e-23)
		CG10417	2,778	6	Protein serine/threonine phosphatase	Protein phosphatase 1G variant (1e-61)
		CG30440	27,523	7	Guanyl-nucleotide exchange factor	MCF.2 cell line derived transforming sequence (3e-77)
		CG30438	52,876	9	Transferring glycosyl groups	Ceramide UDPgalactosyltransferase (5e-68)
		TpnC4	4,431	4	Calcium ion binding	Calmodulin 2 (7e-25)
		CG17510	1,243	9	Unknown	Tetratricopeptide repeat domain 11 (5e-12)
		CG17508	2,966	3	Unknown	C20orf108 (7e-24)
		CG11665	11,129	4	Monocarboxylic acid transporter	Solute carrier family 16 (3e-33)
		CG32838/CG42345	41,278	11	Laccase; copper ion binding	No significant similarity

Location refers to the mapping of scaffolds and genes on mitotic heterochromatin map; CG indicate the annotated genes; length means the physical size of the genomic region of a given gene. 2Lh corresponds to release 5 assembly of 2L arm that incorporates release 3 heterochromatin scaffolds (AABU1002637 and AABU1002768); the genes mapping to h35 belong to AABU1002768; 2Rh corresponds to release 5 assembly of 2R arm that incorporates release 3 heterochromatin scaffolds (AABU01002711, AABU01002752 and 2R.wgs3_extension). In release 5 sequence, release 3 scaffolds were also assembled in larger scaffolds designated with the CP acronym (Hoskins et al 2007). On 2L heterochromatin, the scaffold CP000215 contains the release 3 scaffold AABU01002756. On 2R heterochromatin, CP000188 contains the release 3 scaffolds AABU01001947, AABU01002199 and AABU01002549, while CP000218 and CP000219 contain AABU01002750 and AABU01002748, respectively. The cytological border of hetero-euchromatin transition region was established by FISH mapping of BACs (Corradini et al. 2003) and is approximate; some of the genes assigned to this region may be actually located in heterochromatin. Gene annotations were according to release 5 sequence (http://flybase.org/; www.dhgp.org) and to Smith et al (2007). Only BLASTp hits with $e < 1^5$ were selected. In Flybase, the annotation of genomic region with exon–intron structure was not available for a number of genes; we indicated these cases with n.a.=not available

heterochromatin regions, can be in fact endowed with still unidentified genetic functions.

Gene density in *D. melanogaster* heterochromatin vs euchromatin

It has been previously estimated that the density of single-copy genes in heterochromatin is some100-fold lower than that found in euchromatin (Hilliker et al. 1980). In light of the recent annotation of release 5.1 *Drosophila* heterochromatin sequence, ten to 11 genes per Megabase have been found in sequenced heterochromatin that correspond to transposon-rich regions compared with 127 genes per Megabase in euchromatin; in other words, gene density would appear to be only one order of magnitude lower compared to euchromatin. This estimate, however, does not include the satellite DNA-rich regions, within which the gene density is likely to be still very low. In this context, the Y-chromosome heterochromatin represents an interesting exception because combined cytogenetic and molecular analyses suggested it to be an almost continuous array of physically large functional genetic elements (Pimpinelli et al. 1985).

Functional and structural aspects of single-copy heterochromatin genes in *Drosophila*

The single-copy heterochromatin genes of *D. melanogaster* encode proteins involved in important cellular processes. The *light* gene product is required for cellular protein trafficking (Warner et al. 1998), while *concertina* encodes a maternal α-like subunit of a G-protein essential for gastrulation (Parks and Wieschaus 1991). The *rolled* gene was shown to be essential for imaginal disc development and suggested to be involved in cell proliferation (Hilliker 1976; Dimitri 1991); indeed, its encoded product is a mitogen-activated protein kinase required in the signal transduction pathway of the *sevenless* gene (Biggs et al. 1994) and may also be implicated in the spindle integrity checkpoint (Inoue and Glover 1998). The *Nipped-A* product facilitates assembly of the *Notch* activator complex and targets gene transcription (Gause et al. 2006), while the *Nipped-B* protein is required for both transcriptional regulation and sister chromatid cohesion (Misulovin et al. 2008). The *l(2)41Af* gene corresponds to the predicted gene *CG18001* which encodes the RpL38 ribosomal protein (Marygold et al. 2005). Two more ribosomal protein genes, *RpL5 and RpL15*, are also found on 2L and 3L heterochromatin, respectively (Marygold et al. 2005; Schulze et al. 2005). The *Parp* gene on 3Rh encodes a poly(ADP-ribose) polymerase, a major NAD-dependent modifying enzyme that mediates important steps in DNA repair, transcription, and apoptosis (Tulin et al. 2002). A significant number of essential genes located in chromosome 2 heterochromatin (Fig. 1) still need to be identified molecularly. Among those genes, *l(2)41Aa* and *l(2)41Ad* in the heterochromatin of the right arm of chromosome 2 (2Rh) are thought to be required for chromosome condensation (Cenci et al. 2003) and for proper leg and wing morphogenesis (Dimitri et al. 2005a), respectively. In particular, *l(2)41Ad* is the only known vital gene mapping to region h44 (Dimitri 1991) that contains 44 predicted genes (Fig. 1 and Table 1). Interestingly, *l(2)41Ad* is a highly mutable gene in the I-R dysgenesis system (Dimitri et al. 1997) and most of its I-R induced lethal alleles were found to be associated with cytologically visible deletions of regions of h44 spanning roughly up to 1 Mb of DNA (Dimitri et al. 2005b). In light of these genetic and cytological features, *l(2)41Ad* was suggested to be a large gene (Rossi et al. 2007). A good putative gene candidate to be *l(2)41Ad* is CG17684, the largest found in h44 and in all autosomal heterochromatin; CG17684 is about 400 kb and encodes a putative protein sharing high identity with the human dipeptidyl peptidase enzyme. Although this correspondence is suggestive, additional genetic and functional genomic studies are needed to establish the molecular identity and function of *l(2)41Ad*.

In general, therefore, based on molecular and bioinformatic analyses, both predicted and known genes resident in heterochromatin do not apparently have molecular functions that would distinguish them from genes located in euchromatin (Hoskins et al. 2002; reviewed by Dimitri et al. 2005a; Flybase 2009). In other words, the heterochromatin genome does not seem to encode a distinctive proteome. However, according to Smith et al. (2007), some classes of genes appear to be overrepresented in heterochromatin, relative to euchromatin. This is the case of putative membrane cation transporter domains and of DNA- and protein-binding domains.

A difference between heterochromatin and euchromatin genes lies in their size and molecular structure. The example of the "giant" Y-chromosome fertility factors of *D. melanogaster* mentioned above is paradigmatic in this respect (Gatti and Pimpinelli 1992). Some of the heterochromatin essential genes of chromosomes 2 and 3 are also large due to the presence of long introns made up of TE remnants (Devlin et al. 1990a, b; Tulin et al. 2002; Dimitri et al. 2003). On average, heterochromatin gene introns are five times longer than those present in euchromatin genes (Smith et al. 2007). There are, however, some exceptions: For example, *RpL38*, *RpL5*, and *RpL15*, three essential protein-coding genes on chromosome 2 and 3, are all of short size (Marygold et al. 2005; Schulze et al. 2005). How might these observations be explained? One may imagine that during evolution, older genes in heterochromatin have

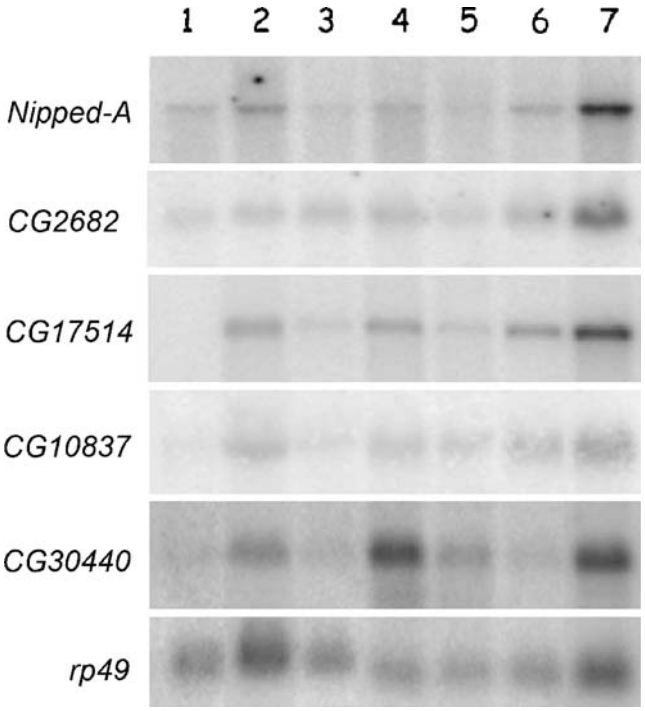

Fig. 3 Developmental Northern analysis of heterochromatic putative genes. The rp49 ribosomal protein gene was used as a loading control. The expression of the tested heterochromatin putative gene is not limited to specific stages, but is present throughout development, similarly to the vital gene *Nipped-A*. *Lane 1* embryos; *lane 2* first-instar larvae; *lane 3* third-instar larvae, *lane 4* early pupae; *lane 5* late pupae; *lane 6* adult males; *lane 7* adult females

increased their size by becoming targets for reiterated transposable-element insertions in the intronic regions. If that is true, short genes in heterochromatin should represent a set of genes recently "moved" to heterochromatin. Alternatively, genes in heterochromatin might be differentially targeted by transposable elements, with some genes being more refractory than others. Finally, there might be selective pressure to maintain some genes of short size in heterochromatin owing to particular functional properties. Interestingly, in that respect, highly expressed genes have been shown to harbor substantially shorter introns than genes expressed at low levels (Castillo-Davis et al. 2002). This is the case of *RpL38*, *RpL5*, and *RpL15* genes are that highly expressed and are all indeed short genes carrying short introns (Marygold et al. 2005; Schulze et al. 2005).

The paradox of active heterochromatin genes

The expression of heterochomatin genes such as *light*, *rolled*, *RpL5*, *RpL38*, *RpL15*, *Nipped-B*, *Nipped A*, and *Parp* is detectable throughout Drosophila developmental stages (Biggs et al. 1994; Rollins et al. 1999; Tulin et al. 2002; Marygold et al. 2005; Schulze et al. 2005; Rossi et al. 2007). Rossi et al. (2007) found a similar transcriptional profile for a group of 15 predicted genes belonging to chromosome 2 heterochromatin (see the example in Fig. 3). An exception to this pattern of widespread expression is represented by the Y-chromosome fertility factors which show tissue-limited and sex-specific expression. In recent reviews of existing data, it has been pointed out that the presence of "islands" of active genes within heterochromatin would be somewhat paradoxical (Dimitri et al. 2005a; Yasuhara and Wakimoto 2006; Huisinga et al. 2006): These genes are resident in regions that approach 90% repeat content and do not seem to be merely euchromatic sequences embedded in a repetitive environment. In fact, it is well known that heterochromatin genes, such as *light* and *rolled* for example, are repressed when moved to distal euchromatin by chromosomal rearrangements: This indicates thet proximity to heterochromatin is an important regulatory requirement for the function of heterochromatin genes (Wakimoto and Hearn 1990; Eberl et al. 1993). How might the expression of coding genes be compatible with the known silencing properties of heterochromatin and which factors account for the difference between functional and silent heterochromatin?

Transposable elements and chromosomal proteins such as heterochromatin protein 1 (HP1), known to be required for the establishment of the heterochromatin state, may contribute in ways that are still poorly understood to proper expression of heterochromatin genes in Drosophila (Weiler and Wakimoto 1995; Dimitri and Junakovic 1999; Eissenberg and Hilliker 2000; for an overview of the different models of heterochromatin gene expression, see Yasuhara and Wakimoto 2006). A large-scale mapping analysis in *D. melanogaster* Kc embryonic cells has shown that HP1 is distributed throughout the *concertina*, *light*, *rolled* heterochromatin gene regions (de Wit et al. 2005, 2007) and binds both unique and repetitive sequences in exonic and intronic portions of the gene, respectively. However, experimental evidence on HP1 roles in heterochromatin gene transcription in *D. melanogaster* are conflicting (Clegg et al. 1998; Lu et al. 2000; Greil et al. 2003; Schulze et al. 2005; Cryderman et al. 2005; Fanti et al. 2008). Notably, mutations in genes of the trx group, such as *trx* and *ash-1*, appear to reduce light and rolled gene transcription (Fanti et al. 2008).

Experimental evidence support a role of naturally occurring RNA interference (RNAi) in the formation of heterochromatin in different organisms (Volpe et al. 2002; Hall et al. 2002; Reinhart and Bartel 2002; Verdel et al. 2004; Fukagawa et al. 2004; Bernstein and Allis 2005;). A clear link between RNAi and heterochromatin in *D. melanogaster* is still debated; moreover, experimental

evidence on rasiRNA pathway involvement in heterochromatin formation in somatic tissues are conflicting (reviewed by Huisinga and Elgin 2009). Interestingly, piwi was found to be required for the expression of subtelomeric TAS repeats in both soma and germ line of *D. melanogaster* (Yin and Lin 2007). In light of this result, it may be interesting to test the effects of piwi mutations on transcription of *D. melanogaster* single-copy genes located in pericentromeric heterochromatin.

Histone modifications are also likely to play roles in the control of heterochromatin gene expression. The distribution of modified histones in heterochromatin genes has recently been studied by Yasuhara and Wakimoto (2008). They found that H3-di-methylated-at-lysine 9 (H3K9me2) is depleted at the 5′ end, but enriched throughout the transcribed portion, of heterochromatin genes, a profile different from that found in euchromatic genes. The authors suggest that heterochromatin genes are integrated into, rather than insulated from, the H3K9me2-enriched domain.

The presence of coding genes in heterochromatin is a conserved trait in the evolution of eukaryotic genomes

Recent studies have investigated the origin of *D. melanogaster* heterochromatin genes by comparing putative orthologous genes in different species of the Drosophila lineage. The first study analyzed a cluster of genes spanning 594 kb of DNA around the *light* gene, which maps to heterochromatin in *D. melanogaster* but has a euchromatic location in both *Drosophila pseudobscura* and *Drosophila virilis* (Yasuhara et al. 2005). In another study, the entire heterochomatic chromosome 4 of *D. melanogaster* (4–5 Mb of DNA) was compared to the homologous D. virilis "dot" chromosome, which is instead euchromatic (Slawson et al. 2006). Together, the results of these studies indicate that promoter regions of euchromatin and heterochromatin genes are per se essentially similar and that transposable elements play a fundamental role in the formation of heterochromatin domains.

An interesting approach designed to understand whether genes have moved into, or out of, heterochromatin regions in other species has been developed by Smith et al. (2005) and is based on the analysis of repeat content of orthologous introns and scaffolds. The location was confirmed for over 80% of the predicted orthologous genes by FISH mapping analysis on polytene chromosomes in different Drosophila species. The results indicate that a significant portion of *D. melanogaster* heterochromatin genes are likely to descend from euchromatin progenitors (C. Smith, F. Rossi, S. Celniker, P. Dimitri and Gary H. Karpen, unpublished). Thus, it would appear that during evolution, some genes have "jumped" between the two genomic compartments.

The presence of transcribed sequences in heterochromatin, far from being a peculiarity of Drosophila species, appears to be a conserved trait in the evolution of eukaryotic genomes. Single-copy protein coding genes are indeed found in *Schizosaccharomyces pombe*, rice, *A. thaliana*, and humans (reviewed by Dimitri et al. 2005a; Yasuhara and Wakimoto 2006). In particular, mapping and sequencing of the human genome indicates that pericentromeric heterochromatin is characterized by several blocks of duplicated sequences, probably generated by transposition (Eichler et al. 1996; Horvath et al. 2000; Brun et al. 2003). Fragments of genes, complete genes, and repeats are duplicated in pericentromeric regions. Generally, the pericentromeric duplications are non-functional pseudogenes, but some mRNAs and expressed sequence tags from pericentromeric sequences have been identified. Genes coding for growth factors, immunoglobins K, λ and D, plasminogen, and others have been found in these paralogous sequences (listed in Horvath et al. 2000). Moreover, many pericentromeric paralogous sequences are transcribed in germ line, fetal, or cancerous tissues (Horvath et al. 2000; Brun et al. 2003), suggesting that they are involved in fundamental biological processes. In mouse, pericentric heterochromatin is not transcriptionally inert and can give rise to transcripts spanning the major satellite repeats (Lehnertz et al. 2003).

Drosophila heterochromatin genes related to human disease genes

Developmental defects, diseases, and mechanisms underlying the onset of tumorigenesis can be investigated using Drosophila as a model system. Systematic searches for human disease-causing genes in Drosophila have shown that about 75% of human disease genes match unique Drosophila sequences (Reiter et al. 2001). Orthologs of essential and putative heterochromatin genes of *D. melanogaster* (e.g., *rolled*, *Parp*, *Nipped-A*, *Nipped-B*, *RpL38*, and others) have been found in several organisms, including yeast, mouse, and humans, and are all located in euchromatin. Table 1 shows the evolutionary conservation of *D. melanogaster* heterochromatin gene protein products in humans. In particular, among 161 predicted genes mapped to heterochromatin of chromosome 2, 47 (30%) encode protein products sharing significant conservation. Notably, the human orthologs of some of these genes are involved in human genetic diseases. For example, mutations in NIPBL, the human ortholog of the Drosophila *Nipped-B* gene, are responsible for the Cornelia de Lange syndrome, a multiple malformation disorder (Krantz et al. 2004; Tonkin et al.

2004). Another interesting example is given by *CG17528*, a putative Drosophila heterochromatin gene that encodes an evolutionarily conserved microtubule-binding protein. The human orthologs of *CG17528*, *DCX*, *DCKL1*, and *DCKL2* are implicated in lissencephaly, a genetic disorder characterized by severe mental retardation. Moreover, the Drosophila *CG40218* gene encodes a protein belonging to the evolutionarily conserved family of BCNT found in several animals and plants (A. thaliana, Oryza sativa, Neurospora, Saccharomyces cerevisiae, Caenorhabditis elegans, mosquito, flies, mouse, and humans). Little is known about the function of BCNT-like family. Craniofacial development protein 1, the human ortholog of CG40218, encodes a protein phosphorylated by casein kinase II, the function of which is still unknown. Intriguingly, it maps to chromosome 16 in 16q22.2-q22.3, in proximity to several loci associated with inherited craniofacial diseases, such as Fanconi anemia type A (Diekwisch et al. 1999).

Our preliminary data using RNAi provide some hints about the functions of *CG40218* and *CG17528*. RNAi-treated cells revealed that chromosome condensation was highly defective upon depletion of the *CG40218* gene product compared to non-treated control cells. This result supports the view that the *CG40218* protein plays a key role in chromosome organization. After inactivation of *CG17528*, several defects were found to occur with higher frequency in RNAi-treated compared to control cells: (1) aberrant anaphases, (2) binucleate cells, and (3) abnormally shaped cells (F. Rossi,P. Dimitri and G. Karpen, unpublished). These data are compatible with a role of *CG17528* in spindle and cytoskeleton organization. In vivo depletion of *CG17528* product by RNAi also causes the loss of wing margins and severe wing-to-notum transformation, suggesting that the CG17528 protein may be a new component of the wingless (wg) pathway (E. Giordano and P. Dimitri, unpublished). These observations suggest an intriguing link between the cytoskeleton dynamics and wg-mediated morphogenesis during development (Ciani et al. 2004; Shimada et al. 2006).

Heterochromatin in humans

Centromeres

Previous studies have highlighted a conserved organization of centromeric heterochromatin in Drosophila and humans (Blower et al. 2002). Constitutive heterochromatin in centromeric regions is typically associated with (1) specific histone methylation patterns, (2) high levels of DNA methylation, (3) low recombination frequency, and (4) repression of transcription. Human centromeres are genomically defined by tandem arrays of 171-bp monomeric α-satellite repeats. They are flanked by pericentromeric heterochromatin domains with a complex structure in which arrays of different repetitive elements, including satellite II and III, are interspersed with unique sequence elements. Although the size and repetitive nature of these regions have hampered the assembly of molecular maps and limited comprehensive functional analyses, it appears that the general organization of centromeric regions is highly conserved in mammals (Partridge et al. 2000). Both CEN chromatin and flanking heterochromatin are required for chromosome segregation and de novo chromosome assembly. CEN chromatin and constitutive pericentromeric heterochromatin in humans are distinct epigenetic entities (Sullivan and Karpen 2004). CEN chromatin is continuous and contains the histone variant CENP-A as well as histone H3 dimethylated on lysine 4 (H3K4me2). The flanking heterochromatin is defined by H3-K9 dimethylation and trimethylation (H3K9me2 and H3K9me3) and, contrarily to the CEN domain, exerts a repressive effect on gene transcription. This inhibitory effect appears to be relevant for the activity of the centromere (Lam et al. 2006).

Duplications, genes, and pseudogenes

In the course of evolution, most human pericentromeric regions have been subjected to a complex series of duplications, which account for at least 5% of the genome. A total of 8,343 pericentromeric duplications have been identified in the human genome, which are likely to derive from the duplication of 271 ancestral segmental duplications to 43 pericentromeric regions (She et al. 2004). This biased distribution of genome duplications within juxtacentromeric heterochromatin may reflect a higher tolerance for new insertions into these regions, as both ectopic recombination between duplicated blocks and transcription of genes in the new copy would be repressed. These duplications may have played a pivotal role in the evolution of the architecture of the human genome, in the emergence of new genes, and in the adaptation to the environment. Moreover, they contribute to large-scale structural polymorphisms and to genomic diseases (Stankiewicz and Lupski 2002). Notably, only a few juxtapositions of ancestral cassettes have created new transcripts. It has been estimated that a novel or mosaic transcript may have emerged through pericentromeric duplication once every million years. The fate and function of such evolutionary novelties remain to be determined. An example of segmental duplication has been elucidated in analyzing the pericentromeric heterochromatin region of human chromosome 9. This region is highly polymorphic in both size and orientation and contains several duplicons in which genes and pseudo-

genes are embedded. One of them, the *CNTNAP3* gene, is the first documented example of amplification for a gene in the euchromatin region bordering a pericentric heterochromatin block (Boyadjiev et al. 2005). Similar pericentromeric heterochromatin regions exist in chromosomes 1 and 16 and may also be implicated in the amplification of neighboring genes (Neglia et al. 2003).

Detailed transcriptional maps of duplication-rich regions are still rare; some features, however, emerge, indicating that genes in duplication-rich regions generally have methylated promoters (Grunau et al. 2005). Remarkably, these genes are usually silent in normal cells, yet become expressed in some tumors and in the testis (Brun et al. 2003). Microarray data on the transcription profiles of pericentromeric sequences of all human chromosomes in different tissues have been inspected in silico (Mudge and Jackson 2005). This analysis has revealed an approximate fivefold excess of transcripts specific to cancer and/or testis in pericentromeric duplications compared to the surrounding single-copy sequences, with the expression of >50% of all transcripts in duplications being restricted to these tissues. This transcriptional activation probably reflects the physiological reprogramming of the epigenome that takes place in cancer and/or testis, which is characterized by demethylation of CpG islands.

Activation of SatIII DNA transcription

As mentioned above, the ability to repress transcription of genes embedded in pericentromeric heterochromatin appears to be critical both for the centromeric function and for the evolution of novel genes. On the other hand, "euchromatinization" of these regions, which occurs under particular circumstances, offers the opportunity to test the activity of genes embedded in heterochromatin regions. It is still unknown whether the reorganization of heterochromatin domains is part of a physiological gene expression program or whether it is an undesirable product in pathological situations. In this light, it is noteworthy that the "euchromatinization" of specific blocks of pericentromeric heterochromatin is elicited by heat shock and other stress treatments and can be part of a general stress response program activated in human cells to cope with harmful conditions (Valgardsdottir et al. 2008). The critical sequence in this process is satellite III DNA, a human-specific repetitive element that forms long tandem arrays in a few pericentromeric heterochromatin bands, among which is 9q12. Heat shock and other stress treatments induce "euchromatinization" and transcription of SatIII DNA without affecting the organization of other centromeric repetitive sequences, such as α-satellite DNA. This phenomenon depends on a few transcription factors involved in stress response (e.g., heat-shock factor 1 and tonicity element-binding protein) that bind SatIII sequences and recruit chromatin-modifying activities and RNA polymerase II (Jolly et al. 2004; Rizzi et al. 2004). SatIII RNAs remain associated with sites of transcription where they contribute to the assembly of large heterochromatin transcription factories, called nuclear stress bodies (nSBs) (Biamonti 2004), which contain RNA polII, chromatin modifiers, and transcription factors along with a specific subset of pre-mRNA processing factors. The function of these structures is still a matter of investigation. We have speculated that the formation of nSBs can affect nuclear function and gene expression programs because the sequestration of transcription and RNA processing factors in nSBs can reduce the expression of genes in other nuclear districts. At the same time (as schematically proposed in Fig. 4), it may control the expression of genes adjacent to arrays of SatIII repeats. Notably, a small fraction of the SatIII sequences is physiologically in an open chromatin conformation even in unstressed cells (Gilbert et al. 2004).

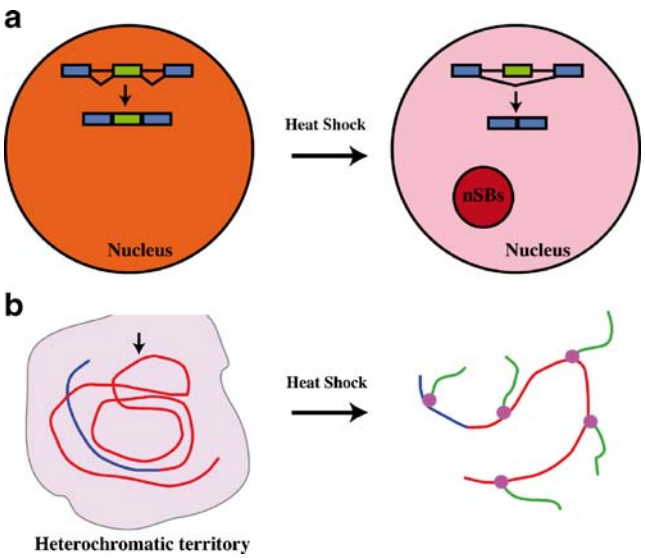

Fig. 4 Transcriptional activation of tandem arrays of satellite III DNA sequences can affect gene expression profiles. According to the model in **a**, heat shock triggers the recruitment of splicing factors to nuclear stress bodies assembled on SatIII arrays (nSBs in the right nucleus on the *right*). This results in a drop of the concentration of these factors in the nucleoplasm, as exemplified by the *color code* of the two nuclei (*heavy orange* before heat shock and *light pink* afterwards). The decreased level of splicing factors can modify the splicing program of genes: exon inclusion in the nucleus on the *left* and exon skipping in the nucleus on the *right*. Model **b**: Under normal conditions, protein coding genes (*blue line*) adjacent to SatIII arrays (*red line*) on the chromosome are embedded into heterochromatin territories. Heat shock induces the transcriptional activation of SatIII DNA and opens the chromatin structure. This results in the activation of the blue gene. *Green lines*, transcripts. *Pink ovals*, transcriptional machineries

Intriguingly, the expression of SatIII RNAs increases in progeroid laminopathies (Shumaker et al. 2006). Lamin A and B are structural components of a protein meshwork, the nuclear lamina, which underlies the inner nuclear membrane. More than 12 human diseases arise from mutations in the lamin A/C genes, among which the premature aging disorders Hutchinson–Gilford progeria syndrome (HGPS). A distinctive feature of progeroid laminopathies is the loss of peripheral heterochromatin, which is accompanied by loss of heterochromatin markers such as H3K9me3 and an altered transcription profile (Scaffidi and Misteli 2005; Columbaro et al. 2005).

Lamins are implicated in the structural integrity of the nucleus; nuclei from mouse LmnA-null cells are mechanically weak (Lammerding et al. 2004), and cells that lack A-type lamins have mechanotransduction defects that lead to misregulation of mechanosensitive genes (Stewart et al. 2007). This is probably linked to increased sensitivity to stress of HGPS cells (Caron et al. 2007). In this light, the activation of SatIII arrays and adjacent genes in pericentromeric domains may be relevant for the clinical manifestation of laminopathies.

Conclusions

In this paper, we draw the attention to recent evidence on genes found in constitutive heterochromatin in Drosophila and other organisms. Constitutive heterochromatin forms a significant fraction of metazoan genomes, which suggests an evolutionary conserved function of this distinctive genomic component. Despite persisting fragmentary knowledge, accumulating data, summarized in this review, confirm that idea and begin to unveil novel aspects of eukaryotic genome organization with relevant implications for function and evolution of constitutive heterochromatin. It is now clear that this peculiar genomic compartment contains a large variety of genetics elements. Essential and putative single-copy genes were identified in constitutive heterochromatin of *D. melanogaster*, yeast, *Arabidopsis*, rice, and human genomes. Rather than mere euchromatin sequences embedded in a "junk DNA", genes actively transcribed in heterochromatin may turn out to be an aspect of a relevant evolutionary process where a given sequence might have established positive interactions with heterochromatin environment. TE remnants, heterochromatin proteins, and specific histone modifications may have played important roles in this phenomenon. In addition to single-copy genes, Drosophila heterochromatin is known for a long time to contain repetitive loci-like ribosomal genes and a special class of "criptic" genetic elements such as *Su (Ste)* and *ABO*. More recently, a novel class of unconventional loci is given by the multiple PiRNAs and esiRNA heterochromatin clusters, some of which are involved in transposable element silencing and heterochromatin formation. These data, together with the notion that the size of heterochromatin genes can be very large, converge to conclude that the density of genetic functions in constitutive heterochromatin is not as low as previously claimed. The more we shed light on heterochromatin in Drosophila and in higher eukaryotes, the more we will be surprised by its peculiar functions and positive role on the evolution of the eukaryotic genomes. The next years will undoubtedly witness progress in this highly intriguing genomic component.

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