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Delta–Notch—and then? Protein interactions and proposed modes of repression by Hes and Hey bHLH factors

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ABSTRACT

Hes and _Hey_ genes are the mammalian counterparts of the _Hairy_ and _Enhancer-of-split_ type of genes in _Drosophila_ and they represent the primary targets of the Delta–Notch signaling pathway. Hairy-related factors control multiple steps of embryonic development and misregulation is associated with various defects. _Hes_ and _Hey_ genes (also called Hesr, Chf, Hrt, Herp or gridlock) encode transcriptional regulators of the basic helix-loop-helix class that mainly act as repressors. The molecular details of how Hes and Hey proteins control transcription are still poorly understood, however.

Proposed modes of action include direct binding to N- or E-box DNA sequences of target promoters as well as indirect binding through other sequence-specific transcription factors or sequestration of transcriptional activators. Repression may rely on recruitment of corepressors and induction of histone modifications, or even interference with the general transcriptional machinery. All of these models require extensive protein–protein interactions. Here we review data published on protein–protein and protein–DNA interactions of Hairy-related factors and discuss their implications for transcriptional regulation. In addition, we summarize recent progress on the identification of potential target genes and the analysis of mouse models.

INTRODUCTION

Precise temporal and spatial control of gene expression is accomplished by a broad array of sequence-specific transcription factors. Many of these are inefficient transcriptional activators or repressors on their own, but they recruit potent coactivators or corepressors that cannot bind directly to DNA in turn (1). Regulatory mechanisms include chromatin-remodeling factors that mobilize nucleosomes and histone-modifying enzymes. The expression of such regulatory factors is controlled by diverse signaling pathways, other transcription factors and regulatory RNAs, building up a highly complex transcriptional network.

The Notch signaling pathway represents a central regulator of gene expression. This cascade controls cell fate determination and differentiation, making it essential for many aspects of embryonic development as exemplified by a variety of mouse knockout studies. In humans, mutations of Notch ligands or receptors are responsible for a number of diseases like Alagille syndrome, CADASIL, T-cell leukemia, aortic valve calcification and other cardiovascular disorders (2–4).

Notch receptors are single-pass transmembrane proteins that become activated upon ligand binding. This leads to two consecutive cleavage events releasing the intracellular domain (NICD), which then translocates to the nucleus. There NICD interacts with the DNA-binding protein RBPJκ (also known as CBF1, Rbpsuh or Su(H) in *Drosophila*), which is associated with corepressors (e.g. N-CoR, SHARP, CtBP). Interaction with NICD replaces these corepressors and allows recruitment of coactivators like Mastermind/MAML and p300/CBP leading to transcription of target genes (Figure 1) (5). The most extensively studied and best understood targets are *Hairy* and *Enhancer-of-split* [*E(spl)*] genes in *Drosophila* and the related *Hes* and *Hey* genes in mammals (6). Interestingly, there is a crosstalk between Notch and the BMP/TGF-beta, JAK-STAT, Ras and HIF signaling pathways to enhance activation of Hey/Hes expression (7–11), suggesting that these factors transduce and integrate signals from multiple pathways.

Besides the activation of target genes via RBPJκ, referred to as the canonical pathway, additional, non-canonical functions of Notch have been described that are less well characterized. These include e.g. regulation of the actin cytoskeleton, interaction with the wingless pathway,

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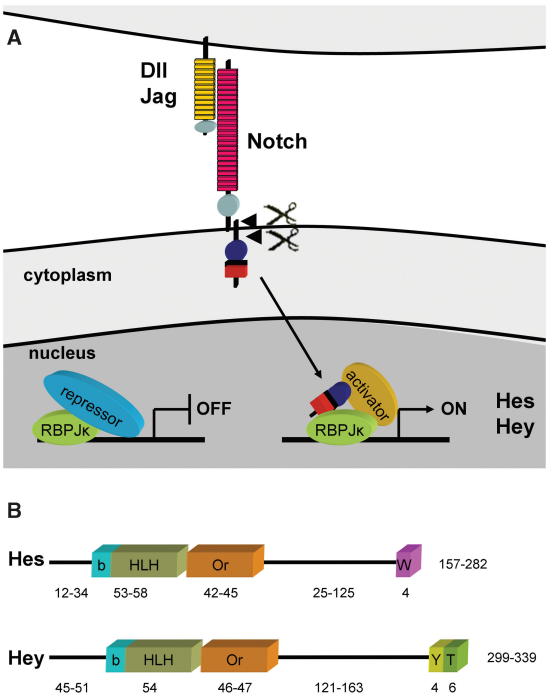

Figure 1. Scheme of Notch signaling. (**A**) Ligands of the Delta (Dll) or Jagged (Jag) family induce intramembrane cleavage of the Notch receptor. The intracellular domain replaces transcriptional corepressors with activators enabling transcription of Hes and Hey genes by RBPJκ. (**B**) Domain organization of Hes and Hey proteins. Numbers indicate the amino acid content of the individual protein domains.

RBPJκ-independent activation of target genes (12), or activation of the RNA-binding protein Musashi (13).

HAIRY AND E(spl) IN *DROSOPHILA*

In the fruitfly *Drosophila melanogaster*, *Hairy* and seven clustered *E(spl)* genes (*m8*, *m7*, *m5*, *m3*, *mβ*, *mγ* and *mδ*) control crucial developmental processes like segmentation, myogenesis or neurogenesis. All of these genes encode basic helix-loop-helix (bHLH) proteins (14). The DNA-binding basic domain (b) is contiguous with one of two amphipathic α-helices separated by a loop (HLH) that serve as a dimerization domain and as a platform for additional protein interactions (15). The HLH region is followed by two additional α-helical stretches (helix3/4), called the Orange domain. This domain is thought to serve as an additional interface for protein interactions and it acts as a transcriptional repressor when fused to a DNA-binding domain (16). A further characteristic of Hairy and E(spl) proteins is the invariant proline residue in the basic domain and a highly conserved carboxyterminal tetrapeptide motif WRPW that recruits the corepressor Groucho (14).

E(spl) genes are activated by Notch signaling and their protein products, as well as Hairy, block neuronal differentiation by inhibiting proneural bHLH activators like Atonal, Daughterless and those of the Achaete–Scute complex (14). The molecular details of how this is achieved are diverse and in part still controversial. Proposed models include sequestering of activator complexes away from DNA (17), direct binding to promoters of target genes and recruitment to promoters without direct DNA binding (18).

MAMMALIAN HES AND HEY PROTEINS

In the mouse and rat genomes, seven *Hes* (*Hes1-7*) (19–25) and three *Hey* genes (*Hey1,2,L;* also published as *Hrt1,2,3; Hesr1,2; Herp2,1 or Chf2,1*) have been identified (26–30). Hes proteins are highly similar to Hairy and E(spl), especially within the bHLH, Orange and WRPW domains. Similar to their *Drosophila* ancestors, Hes proteins are supposed to bind N- and E-box DNA sequences (CACNAG, CANNTG) and they can recruit TLE1-4 corepressors (the orthologs of Groucho) through their WRPW tetrapeptide (31). While *Hes1*, *Hes5* and *Hes7* can be induced by the Notch pathway (25,32–35), *Hes2*, *Hes3* (34) and *Hes6* (24) appear to be independent of Notch signaling, and further data on *Hes4* are lacking.

All members of the *Hey* gene family can be induced by Notch (26,27,29,30,36–39), they are strongly conserved during evolution (40) and there is also a *Drosophila Hey* gene with hitherto unknown function (26,27). Especially the bHLH and Orange domains are similar to those of Hes proteins, but the invariant proline residue in their basic domains is replaced by glycine and they do not bind to N-box sequences (41). Hey proteins preferentially bind to an E-box sequence that is also recognized by Hes1, Hes6 and E(spl) proteins (29,41–43). The most striking difference between Hes and Hey proteins is the lack of the WRPW tetrapeptide in the latter. Instead a related YRPW peptide or a further degenerated YXXW (HeyL) sequence can be found, which cannot bind TLE corepressors (41,42). The YXXW motif is followed by a conserved TE(I/V)GAF peptide with presently unknown function (Figure 1B).

There are several additional mammalian proteins that exhibit strong homologies to Hairy and E(spl). Examples are Helt, DEC1 (also known as Stra13, SHARP-2 or BHLHB2) and DEC2. They generally lack WRPW/YRPW motif sequences and there is no evidence for a Notch-dependent expression thus far.

BIOLOGICAL FUNCTIONS OF *HES* AND *HEY* GENES

Mammalian Hairy-related proteins are specifically expressed in various tissues and they fulfill important roles during development and adulthood. It is beyond the scope of this manuscript to review all these functions in detail, instead Table 1 provides a short overview of the phenotypes seen in gene targeting experiments in mice.

Hes1 plays an essential role in the development of the nervous system, sensory organs (eye, inner ear), pancreas and endocrine cells, as well as lymphocytes. Loss of *Hes5* or *Hes3* is less severe, but combined with *Hes1* deficiency leads to more profound pathologies, as there is partial redundancy among these genes. *Hes7* is important for somitogenesis. In contrast, *Hey* genes play critical roles in

Table 1. Phenotypes of *Hes* or *Hey* gene deficient mice

	Notch regulated	Mouse knockout phenotype	References
Hes1	Yes	Neurulation defects, premature differentiation of neural progenitor cells	(120–122)
		Eye and inner ear defects	(123–125)
		Pancreas defects and defective endocrine differentiation	(126)
		Disturbed T-cell differentiation, lack of thymus	(127)
Hes3	No	Viable, fertile	(128)
Hes5	Yes	Viable, fertile	(35,121)
		Eye and inner ear defects	(129,104)
		Elevated myelin levels in central nervous system	(117)
Hes6	No	Viable, fertile	(24)
Hes7	Yes	Somitogenesis defects	(108)
Hes1/5		Enhancement of $Hes1^{-/-}$ phenotype	(35,121,125,130)
		Defects in cranial and spinal nerves	(131)
Hes1/3		Missing midbrain and anterior hindbrain due to premature neuronal differentiation	(128)
Hes1/3/5		More severe than Hes1/5 loss	(125)
Hey1	Yes	Viable, fertile	(132,133)
Hey2	Yes	Congenital heart defects	(134–138)
		Decreased arterial neointima formation	(87)
HeyL	Yes	Viable, fertile	(47)
Hey1/2		Angiogenesis and arterial differentiation defects	(132,133)
Hey1/L		Congenital heart defects	(47)

the cardiovascular system. $Hey2^{-/-}$ mice and those with a combined *Hey1/L* loss suffer from severe congenital heart defects. While $Hey1^{-/-}$ mutants are viable, a combined *Hey1/2* deficiency phenocopies the vascular defects of $Notch1^{-/-}$ embryos, including impaired angiogenic remodeling and a lack of arterial differentiation. The known overlap in expression sites (31) suggests that there may be additional genetic interactions to be uncovered in compound *Hes* and *Hey* deficient mutants.

PROTEIN INTERACTIONS AND MODES OF TRANSCRIPTIONAL REPRESSION

Hairy-related proteins can interact with a large number of HLH proteins, but they also recruit transcriptional corepressors like histone deacetylases. Furthermore, Hes and Hey proteins can form complexes with other transcription factors, which often turns these into transcriptional repressors. Currently known interacting proteins are summarized in Table 2 and a schematic overview of the functional roles of such complexes is presented in Figure 2.

Hes and Hey factors form homo- and heterodimers

Drosophila E(spl) proteins form homodimers and heterodimers with each other via their HLH domains (17) and this has also been found for Hes and Hey proteins (41,44–47). Heterodimers between Hes and Hey family members appear to be even more stable than the corresponding homodimers. The Orange domain significantly improves interaction strength (44) and such heterodimers bind to DNA target sequences with even higher affinity than the corresponding homodimers (41). As there is overlapping expression between *Hes* and *Hey* genes in several tissues (31), it is conceivable that such heterodimers are formed *in vivo*. Indeed, affinity purification of Hes1 from preadipocytes led to copurification of Hey1 as well as Helt, a new Hey-related protein that associates with Hey2 and Hes5 (46). Surprisingly, Helt appears to use its bHLH domain to bind Hey2, but its Orange domain to interact with Hes5 (48). As Hes and Hey factors differ in the recruitment of corepressors as discussed below, heterodimers broaden the respective repression capacity (Figure 2D). In rare cases heterodimerization may even be antagonistic, e.g. during neural differentiation, where Hes6 counteracts Hes1 repression activity by forming Hes1/Hes6 heterodimers (22).

Interaction with other bHLH proteins

The bHLH family consists of about 125 members (49). Hes and Hey proteins have been shown to interact specifically with some of the ubiquituosly expressed E-proteins (21,44,50,51). The latter tend to form homodimers or heterodimers with lineage-specific bHLH factors and they activate transcription by binding to E-box DNA sequences (CANNTG). Interaction with Hes1, 5 or 7, however, strongly reduces transcriptional activity of E-proteins, presumably in a squelching type of action (19,21,25,50).

Hairy-related proteins also interact with lineage-specific bHLH factors. Hey1 forms dimers with the muscle-specific factor MyoD and prevents its activity during myogenic differentiation of 10T1/2 cells. Here, Hey1 is supposed to counteract formation of a critical MyoD/E47 heterodimer (52). Hey2 was shown to antagonize activation of the VEGF promoter by the ARNT/EPAS (HIF2) complex (28) and the activity of Ptf1-p48, a bHLH protein important for pancreas development, is likewise strongly decreased by Hes1, Hey1 or Hey2 (53) (Figure 2G and H). Finally, interaction of Hey proteins with Hand1 and Hand2, two important bHLH regulators of heart development, has also been described (54), but functional data are still lacking.

Table 2. Summary of protein-protein interactions of Hes (A) and Hey (B) proteins

Interaction partner	Hes/Hey protein	Interacting Hes/Hey domain	Method	Comments	References
(A)					
Homo/Heterodimers					
Hes1	Hes1	bHLH-Or	GST, IP, Y2H		(41,44)
Hey1,2	Hes1	bHLH (Or stabilizes)	GST, IP, Y2H	Stronger than homodimers	(41,44–46)
Hes6	Hes1	ND	IP	Repression of Hes1 activity	(22)
Helt	Hes5 (not Hes1)	(Orange of Helt)	IP		(48)
HLH factors					
E47 E2-2	Hes1,5	ND	IP, M2H	Repression of transcriptional activity	(21,51)
Id1,2,3,4	Hes1	ND	IP*, M2H	Sequestration	(51)
ITF1,2	Hes1	bHLH	GST, Y2H		(44)
Mash1 (Ascl1)	Hes5	ND	IP	Repression, sequestration	(21,117)
Ptf1-p48	Hes1	ND	GST, IP, Y2H	Repression of transcriptional activity	(53)
Other transcription fators					
c-myb	Hes1	ND	IP*	Repression of transcriptional activation of CD4 promoter	(94)
GATA1	Hes1	ND	GST, IP	Represses GATA1 activity, but not DNA-binding capacity.	(85)
RBPJκ	Hes1	bHLH (H1)	IP	Repression of transcriptional activity	(75)
Runx2 (Cbfa1)	Hes1	C-terminus (not WRPW)	GST, IP, Y2H	Enhances Runx2 activity, interferes with TLE1 and HDAC1 recruitment	(63,92,93)
Runx1 (Cbfa2)	Hes1	ND	GST, IP		(92)
Sox10	Hes5	ND	IP	Repression, sequestration	(116)
STAT3 JAK2	Hes1,5	bHLH-Or	IP*	Promotes STAT3 phosphorylation and nuclear translocation	(11)
Transcriptional cofactors					
TLE1,2,3,4	Hes1,5,6	WRPW	GST, IP*, Y2H	Function as a corepressor	(46,59–65,68)
SIRT1	Hes1	bHLH	GST, IP	Augments repression capacity	(77)
HDAC1	Hes1	ND	IP		(93)
CBP	Hes1	ND	IP	Turns Hes1 into transcriptional activator	(68)
Others					
pRB	Hes1	ND	IP	Enhances Runx2/Hes1 activity	(139)
Ubiquilin 1	Hes1	ND	M2H		(140)
(B)					
Homo/Heterodimers					
Hey1,2,L	Hey1,2,L	bHLH (Or stabilizes)	GST, IP, Y2H		(41,44,47)
Hes1	Hey1,2	bHLH (Or stabilizes)	GST, IP, Y2H	Stronger than homodimers	(41,44–46)
Helt	Hey2	(bHLH of Helt)	IP		(48)
HLH factors					
ARNT	Hey1,2	ND	Y2H	Repression of ARNT/EPAS induction of VEGF promoter	(28)
HAND1,2	Hey1,2,L	ND	GST		(54)
Id1	Hey1	ND	IP	Reduced half-life of Id1	(119)
ITF1,2	Hey1,2	bHLH	GST, Y2H		(44)
MyoD	Hey1,2	ND	IP	Repression of MyoD activity, sequestration	(52)
Ptf1-p48	Hey1,2	ND	IP	Repression of Ptf1-p48/E47-induced gene expression	(53)
Other transcription fators					
AR SRC	Hey1	ND	GST, IP*	Repression of AR/SCR-induced gene expression	(74)
GATA1,2	Hey1,2	bHLH	GST, IP*	Repression of transcriptional activity	(83)
GATA4,6	Hey1,2,L	bHLH	IP	Repression of transcriptional activity	(72,73,76,88)
RBPJκ	Hey1,2,L	bHLH (H1)	IP	Repression of transcriptional activity	(75)
Runx2 (Cbfa1)	Hey2	ND	GST	Repression of transcriptional activity	(4)
SRF	Hey2	bHLH	GST, IP	Prevents SRF interaction with CArG box	(89) Contradictory result: (84)
STAT3	Hey1,2	ND	IP	Enhances transcriptional activity	(11)
Transcriptional cofactors					
Sin3A N-CoR	Hey1,2	bHLH	GST, IP	Augments repression capacity, recruitment of HDAC1	(41)
SIRT1	Hey2	bHLH	GST, IP	Augments repression capacity	(77)
Others					
BOIP	Hey1 (not Hey2)	Or	IP, Y2H		(45)

Abbreviations: H1, helix 1; IP, co-immunoprecipitation, IP*; co-immunoprecipitation with endogenous proteins; GST, GST pull-down assay; M2H, mammalian two-hybrid assay; Or, Orange domain; Y2H, yeast two-hybrid assay.

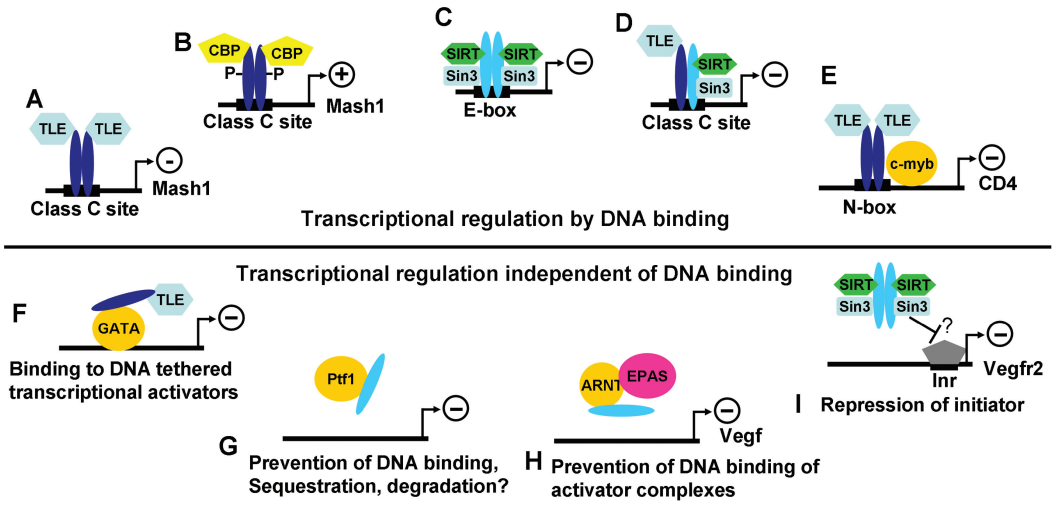

Figure 2. Proposed models of how Hairy-related factors affect gene expression. (**A** and **B**) Binding of Hes1 (dark blue) to a class C E-box can repress or activate the *Mash1* promoter depending on the recruited cofactors. (**C**) Hey proteins (light blue) recruit cofactors different than Hes1 and bind to E-box sequences *in vitro*. (**D**) DNA binding of a Hes/Hey heterodimer. (**E**) Combined DNA and protein binding turning a transcriptional activator into a repressor. (**F**–**I**) Transcriptional regulation independent of DNA binding includes turning activators into repressors (F), prevention of DNA binding, sequestration, degradation (**G** and **H**) or interference with the basal transcriptional machinery (I).

How can Hairy-related factors alter the functional properties of many different bHLH proteins? The promiscuity of HLH heterodimerization already generates a spectrum of pairings with varying affinities that may indirectly reduce the amount of specific dimers. Work in *Drosophila* further suggests that inhibition of bHLH proteins like Scute does not rely on formation of compromised or novel HLH dimers but may be due to binding of E(spl) repressors to the Scute transactivation domain. Thus, even within the bHLH family of proteins multiple modes of interaction are possible (17,18,55).

Recruitment of Groucho/TLE

The WRPW motif of Hairy and E(spl) proteins has been assumed to mediate transcriptional repression in *Drosophila* as it was only found in repressor proteins (56). Its functional importance was underscored by the fact that two mutant *Hairy* alleles carry mutations of the WPRW sequence (57). In *Drosophila*, the WRPW motif of Hairy and E(spl) proteins binds the corepressor Groucho (58) and in mammals Hes proteins recruit the Groucho homologs TLE1-4 to generate a transcriptional repressor complex (46,59–65). Groucho/TLE is supposed to attract further corepressors like histone deacetylases and members of the Sin3 complex, suggesting that such interactions lead to strong transcriptional repression (66,67). The interaction with TLE proteins appears to be quite stable, as affinity purification of Hes1 containing nuclear complexes from mouse preadipocytes yielded only a small number of interacting proteins that include TLE1, 3 and 4 (46).

A recent report on the control of Mash1 expression in differentiating neural stem cells suggested that the Hes1/TLE interaction can be dissociated by cellular signaling pathways that may even convert Hes1 into a transcriptional activator (68). In this case the corepressor complex disassembles, but Hes1 remains bound to the Mash1 promoter and after CaMKIIdelta-dependent phosphorylation recruits coactivators including p300/CBP (Figure 2A and B). It remains to be seen, however, to what extend this scenario can be generalized.

Another surprise came from a genome-wide chromatin profiling analysis in *Drosophila*, where 59 putative target genes were detected for Hairy and 155 for Groucho. Quite unexpectedly, only a single gene was targeted by both proteins, while there was a strong overlap of binding sites for Hairy and other corepressors like CtBP and Sir2 (69). As the screen for Groucho targets was only performed in Kc cells, this may not necessarily be representative for other cell types and differentiation states, but decoration of larval polytene chromosomes rather supported the conclusions of little overlap in targeted genes. This clearly suggests that Groucho is not the primary cofactor for Hairy and perhaps for E(spl) proteins alike, but this may well depend on the cellular context.

In contrast to Hes proteins, the Hey proteins cannot bind to TLE proteins (41,42). Hey1 and Hey2 lack a WRPW motif, but harbor a related YRPW (YQPW) motif. The crystal structure of the WRPW–TLE1 interaction revealed that the N-terminal tryptophan residue binds in a hydrophobic pocket (70), where the tyrosine residue of Hey proteins cannot efficiently interact. Indeed, a corresponding tyrosine to tryptophan (Y→W) exchange in Hey1 allows TLE1 binding (42). Consistent with these data deletion of the YXXW-TE(I/V)GAF motif of Hey proteins was found to have no effect on repression capacity and the reason for its strong conservation remains to be established (4,52,71–76).

Interaction with other corepressors—localization of repression domains

Despite the lack of TLE recruitment, Hey proteins are strong transcriptional repressors, and this prompted searches for interacting corepressors other than TLE.

Strong repression activity could be mapped within the bHLH domain of Hey1 and Hey2, which directly interacts with N-CoR and mSin3A. These corepressors then indirectly recruit histone deacetylase-1 (HDAC1) (41).

The bHLH domains of Hey2 and Hes1 can recruit yet another histone deacetylase, SIRT1 (77). This direct interaction is evolutionary conserved as *Drosophila* Hairy interacts with the SIRT1 homolog Sir2, a NAD^+-dependent histone deacetylase associated with gene silencing, control of metabolism and aging (78). Loss of Sir2 function leads to reduced activity of Hairy repressor activity (79).

The region between the Orange domain and the WRPW/YXXW motif also possesses some repression capacity, but its mode of action is unclear (4,41,52, 72–74,76). In *Drosophila*, Hairy and E(spl)mδ interact with the C-terminal binding protein (CtBP) via the pentapeptides PLSLV or PVNLA that are located close to the WRPW motif, but the physiological relevance of this interaction has been questioned (80,81). CtBP is assumed to again recruit chromatin-modifying enzymes like histone deacetylases to mediate repression (82). The fact that there was a striking overlap of genomic targets for Hairy, Sir2 and CtBP in the above-mentioned screen of *Drosophila* Kc cell chromatin strongly argues for a common molecular pathway and perhaps physical interaction of the proteins (69).

Interaction with distinct HDAC classes

Studies using the histone deacetylase inhibitor trichostatin A (TSA) that inhibits HDACs, but not sirtuins like Sir2/SIRT1, revealed that recruitment of both types of histone deacetylases is necessary for full Hes and Hey repression activity. A plausible scenario suggests that Hey and Hes factors can recruit HDAC1 using the bHLH domain and the C-terminus, while SIRT1 is only bound by the bHLH domain. If this model is correct, TSA treatment would only partially block repression by inhibiting HDAC1 without affecting SIRT1. Indeed, many reports describe only moderate effects of TSA on modulation of Hes or Hey repression activity (29,73,74,77,83,84) with one exception (85). In contrast, repression capacity of the carboxyterminal half of the Hes1 and Hey2 proteins, which does not bind SIRT1, is almost abolished by TSA treatment (74,77).

Taken together, these observations suggest that Hey and Hes factors use combinations of both, TSA sensitive and insensitive histone deacetylases to mediate repression by histone modification.

Repression of GATA factors

The zinc finger transcription factors GATA1, 2 and 3 play crucial roles e.g. in the hematopoietic system, whereas GATA4, 5 and 6 are important regulators in the cardiovascular system. GATA factor activity is tightly regulated by interaction with cofactors (86). There is convincing evidence that Hes and Hey proteins interact with GATA factors (Figure 2F) to strongly repress GATA transcriptional activity (72,73,76,83,85,87,88). In hematopoietic progenitor cells there seems to be competition between Hes1 and the coactivator p300 for GATA1 interaction (85), but the molecular mode of repression is not clear, as studies on the influence on DNA binding by GATA are controversial (72,83,85). In the developing heart, Hey2 appears to limit GATA4/6 activity and this is consistent with an elevated expression of GATA target genes in *Hey2* knockout mice (73).

Interference with the Myocardin/SRF complex

Hey2 does not only interfere with GATA4/6, it also seems to interfere with another master regulator in vascular smooth muscle cells (VSMCs), Myocardin. Hey2 is coexpressed with Myocardin in VSMCs of arteriosclerotic lesions as well as after vascular wall injury, where it represses Myocardin-induced upregulation of the *smooth muscle myosin heavy chain* promoter (84,89). Myocardin forms a ternary complex with serum response factor (SRF) on CArG-boxes (90), but the question of how Hey2 interferes with the function of this Myocardin/SRF complex is open. While one study found a direct Hey2/SRF interaction that inhibited DNA binding of the ternary complex, another found no evidence for this and invoked independent parallel pathways acting on smooth muscle genes (84,89).

Other protein interactions

Besides the more classical interaction partners that were seen in multiple organisms, there are several additional interactions that may be of functional relevance. Runx2 (Cbfa1), a central regulator of bone development, physically interacts with Hairy-related factors, but Hes and Hey proteins seem to have opposing effects in this case. While Hey1 and Hey2 strongly inhibit Runx2 activity (4,91), Hes1 cooperates with Runx2 to stimulate the *Osteopontin* or *Osteocalcin* promoters (92,93). In the latter case, interaction of Runx2 or Runx1 (AML1, Cbfa2) with Hes1 inhibits formation of the Hes1/TLE complex and thus blocks Hes1 repression capacity (63,92).

Hey1 was also shown to interact with the androgen receptor (AR) that binds the coactivator SRC1 to activate transcription of androgen-responsive promoters. Recruitment of Hey1 to the AR/SRC1 complex, however, prevents activation of AR (74). Similarly, there is evidence that c-myb is turned from a transcriptional activator into a repressor of the CD4 enhancer in the presence of Hes1 and both proteins form a stable nuclear complex in T-cells (94) (Figure 2E).

Finally, there is compelling evidence that the Notch pathway interacts with other important signaling cascades like BMP/TGF-beta (7) or hypoxia-induced signaling (8,9). It appears that Notch enhances signaling through the JAK-STAT pathway since Hes1, Hes5, Hey1 and Hey2 bind to STAT3, which enhances phosphorylation and nuclear translocation of STAT3 (11).

Hey1 was even postulated to directly act on the basal transcriptional machinery to repress promoters that contain an initiator element, but lack a TATA box, where introduction of a TATA box would relieve repression by Hey1 (95) (Figure 2I). This hypothesis is based on repression by Hey1 and Hey2 of the minimal

Vegfr2 promoter (96–98) that lacks E-box sequences and it has precedence in the binding of the Hairy-related factor Stra13 to the basal transcription factor TFIIB (99). Nevertheless, it remains to be seen whether these findings hold up to further scrutiny and can be generalized.

In summary, there is a plethora of interactions based on classical HLH dimerization as well as interactions with corepressors or histone deacetylases. The exact target sites of such bHLH heterodimers are currently unknown. In several cases, Hes and Hey proteins appear to bind to other DNA-binding transcription factors to modulate their activity. Quite surprisingly they often seem to rely on the DNA-binding capacity of their respective partners and do not need E- or N-box type target sequences. With probably rare exceptions, Hes and Hey proteins act as genuine transcriptional repressors.

POTENTIAL TARGET GENES OF HAIRY-RELATED PROTEINS

The expression levels of multiple genes are affected by Hairy-related transcription factors. Here we present a short summary of potential target genes that have at least been verified using promoter-reporter assays (Table 3). These data suggest that Hes factors use both, DNA-binding dependent and independent functions to regulate transcription, whereas Hey factors seem to primarily repress the function of activating transcription factors by forming protein–protein complexes with them. However, this is not simply a tritrating-out mechanism or prevention of DNA binding by activators. It appears more likely that DNA-bound activators are turned into repressors by recruitment of Hey factors associated with corepressors.

Potential DNA-binding sites

Studies in *Drosophila* have shown that Hairy and E(spl) are capable of DNA binding. While initial studies suggest that these factors bind to N-box (CACNAG) sequences and a variant thereof, the class C E-box site (CACGCG), later studies revealed that the class B E-box sequence CACGTG is the preferred binding site for E(spl) proteins (56,100–102). The flanking nucleotides of such a core sequence may also influence binding as shown by site selection experiments, which revealed tggCACGTGcca as the optimal binding site (103).

In mammals Hes1, 2, 3, 5 and 7 can bind to N-box sequences (19–21,25,41). Hes1 also interacts strongly with class C sites, but the binding affinities of Hes1, 2 and 7 towards class B E-box sequences appears to be somewhat lower (20,25,41). Interestingly, it was proposed that Hes6, an antagonist of Hes1, does not bind to standard N- or E-boxes (22), but to the E(spl) specific class B site (43). In the case of Hes1, DNA binding can be inhibited by PKC-mediated phosphorylation of serine residues in the basic domain (118), but it is unclear if this is physiologically relevant and the site is not conserved in other Hes and Hey proteins.

The preferred binding site for Hey1 and Hey2 is the same class B site as described for the E(spl) proteins (29,41–43,52,71), and even the preference for the flanking nucleotides is conserved (42). N-box binding of Hey1 and Hey2 is very weak, but binding to class C sites can be enhanced by formation of a Hey2/Hes1 heterodimer (41) (Figure 2A–D). Thus it is still difficult to reconcile the diverse biological functions with the apparent overlaps in potential genomic targets as identified by binding-site selection schemes. Selectivity may thus depend on additional cofactors, unappreciated differences in *in vivo* affinities and spatio-temporal expression patterns.

Autoregulation and oscillating gene expression

During formation of the somites in the embryo several components of the Notch signaling system are expressed in a cyclical fashion. Waves of expression move through the presomitic mesoderm and finally arrest in a newly formed somite, which then buds off. This repeated process, referred to as the somite segmentation clock (105), was first discovered in chicken, where an oscillatory expression of the *Hes1* ortholog *c-Hairy1* (106) and of *c-Hey2* (44) was observed. In mice, *Hes1* and *Hes7* are critical cycling genes (107,108) and loss of *Hes7* causes a loss of somite segmentation (108).

Mathematical models suggest that Hes proteins are translated and subsequently repress their own transcription. Due to the short half-life of Hes proteins, autorepression is relieved allowing a new wave of Hes transcription and translation every 90–120 min. Indeed there are reports showing that Hes1, 7 and Hey1, 2 and L proteins can repress their own promoters (29,38,108,109). Elegant work from Ryoichiro Kageyama's group showed that the short half-life of Hes7 (~22 min) is indeed crucial for oscillating gene expression during somitogenesis, since mutant mice expressing a more stable Hes7 protein (half-life ~30 min) lose synchronization of the clock (110).

There is some evidence that cycling *Hes1* expression is an even more general process, since it was observed after serum stimulation in cultured cell lines (111), however, it seems that exact synchronization and stabilization of the oscillation is dependent upon sufficient cell–cell contacts to keep neighbors in phase (112). Proposed mechanisms how Hes and Hey proteins repress their own promoters include direct DNA binding (109) and interaction with RBPJk (75), but none of those has been fully verified.

Regulation of endogenous Hes and Hey target genes

In recent years, a few dozen potential target genes of Hes and Hey proteins have been described in the literature (Table 3). Most of these were identified by candidate gene approaches. In future studies whole transcriptome analyses should reveal a more global view of the complex regulatory network. First attempts have been undertaken to elucidate targets of Hey2 in endothelial cells or Hes1 in preadipocytes by microarray analyses (46,113). However, such experiments will have to be extended to differentiate direct targets from indirectly regulated genes. This may have to rely on techniques like chromatin-IP followed by large-scale tag-sequencing or whole genome microarray analyses.

Table 3. Summary of known promoters and DNA-binding sites of Hes (A) and Hey (B) transcription factors

Target gene	Hes/Hey protein	Method	Mode of action/binding site	Comment	References
(A)					
			Direct		
Acid-α-Glucosidase	Hes1	CAT EMSA	Class C E-box binding in first intron	**Repression or activation** depending on cell type	(141,142)
Calcipressin	Hes1 (not Hey1,2)	ChIP	DNA binding	Repression of endogenous gene expression	(143)
CD4	Hes1	EMSA Luc	N-box binding in CD4 silencer, interference with c-myb	Repression of endogenous gene in T-cells	(94,144)
E2F1	Hes1	EMSA Luc	N-box binding (homodimer and Hes1/Hey1)	Repression of endogenous gene	(145)
Hes1	Hes1	CAT FP ChIP EMSA	Direct: N-box binding Indirect: Binding to RBPJκ	Mutation of N-boxes prevents inhibition	(75,109,146,147)
Mash1 (Ascl1)	Hes1	ChIP EMSA Luc	Class C E-box binding	**Repression** of endogenous gene. **Activator** by CBP binding instead of TLE1	(68,114,115)
MBP	Hes5	ChIP Luc	Direct: DNA binding and HDAC1 recruitment; Indirect: repression of Mash1 and Sox10	Elevated myelin levels in *Hes5*$^{-/-}$ mice	(117)
Neurogenin3	Hes1	EMSA Luc	N-box binding		(148)
p27(KIP1)	Hes1,6	ChIP EMSA Luc	Class C E-box binding	Higher p27(KIP1) levels in *Hes1*$^{-/-}$ mice	(43,116)
PGDS	Hes1	ChIP EMSA Luc	E-box binding		(149)
			Indirect		
APRE (acute phase resp. element)	Hes1,5	EMSA Luc	Promotion of STAT3 phosphorylation by interaction with JAK2/STAT3	Suppression of endogenous Hes1 reduces STAT3 phosphorylation	(11)
ATH5	Hes1	CAT	Competition with Ngn2 and ATH5	Dominant negative regulator of ATH5 in retina	(150)
Fatty acid synthase	Hes1	Luc	Repression of SREBP transcriptional activity	Blocks adipogenesis in preadipocytes	(46)
E-box promoter	Hes1	CAT	Binding to MyoD inhibits MyoD-driven transcription	Diminished myogenic conversion of C3H10T1/2 cells induced by MyoD	(19)
E-box promoter	Hes1,5,7	CAT FP Luc	Binding to E47 inhibits transcriptional activity and DNA binding	Interference with B-cell differentiation	(19,21,25,50)
E-box promoter	Hes1	CAT Luc	Binding to Mash1 inhibits transcriptional activity; Rapid degradation of Mash1	Neuronal differentiation is dependent of Hes1 downregulation	(19,115,151)
GATA-binding elements	Hes1	ChIP, EMSA, Luc	Binding to GATA1 does not interfere with DNA binding, but recruitment of p300 is inhibited	Inhibition of erythroid and megakaryocytic differentiation	(85)
Osteopontin Osteocalcin	Hes1	Luc	Binding to Runx2 interferes with recruitment of TLE1/HDAC1; enhanced by Vit.-D3 and pRB	Potentiation of Runx2-induced Osteopontin expression (**Activation**!)	(63,92,93,139)
p21	Hes1	Luc	Repression of MASH/E47-driven p21 expression	Suppression of endogenous gene; inhibition of proliferation	(147)
Ptf1-binding elements	Hes1	Luc	Binding to Ptf1-p48 interferes with Ptf1-p48 DNA binding	Ectopic Hes1 represses acinar cell differentiation	(53,152)
			Unknown		
DLK-Pref1	Hes1	Luc	ND	Repression of endogenous gene	(153)
Mdm2	Hes1	Luc	ND	Hes1 causes p53 upregulation	(154)
p57	Hes1	CAT	ND	Higher p57 levels in *Hes1*$^{-/-}$ mice	(155)
(B)					
			Direct		
DAT1	Hey1	Luc, Y1H	Binding 3′ UTR	Repression of endogenous gene; upregulation in *Hey1*$^{-/-}$ mice	(156,157)
Vegfr2	Hey1	ChIP, Luc	Interference with initiator element	Repression of endogenous gene in endothelial cells	(95,96)

Continued

Table 3. Continued

Target gene	Hes/Hey protein	Method	Mode of action/binding site	Comment	References
			Indirect		
αMyHC	Hey1,2,L	Luc	Binding to GATA4 represses transcriptional activity		(72)
ANF	Hey1,2,L	EMSA, Luc	Binding to GATA4/6 represses transcriptional activity. DNA binding of GATA can occur (72), or is prevented (88)	Repression of endogenous gene in cardiomyocytes. Ectopic expression in *Hey2*$^{-/-}$ hearts	(72,73,88)
APRE (acute phase resp. element)	Hey1,2	Luc	Promotion of STAT3 phosphorylation by interaction with JAK2/STAT3	**Activation**	(11)
E-box promoter	Hey1,2	Luc	Prevent dimer formation of Mash1/E47 and Math3/E47	Interference with neural differentiation	(158)
GATA-binding elements	Hey1	EMSA, Luc	Binding to GATA1. No interference with DNA binding of GATA1	Inhibition of erythroid differentiation of K562 cells	(83)
Myogenin	Hey1,2	EMSA, Luc	Binding to MyoD prevents MyoD/E47 complex formation	Hey1 inhibits myogenic conversion of 10T1/2 cells induced by MyoD	(52)
Nkx2.5	Hey1,2,L	Luc	Binding to GATA4 represses transcriptional activity		(72)
Osteocalcin	Hey1	Luc	Binding to Runx2 represses transcriptional activity	Hey1 downregulation enhances mineralization of MC3T3 cells	(4,91)
Probasin	Hey1,2	Luc	Binding to AR and SRC1 interferes with AR/SRC1-transcriptional activity	Downregulation of Hey1 inhibits repression of AR-driven gene expression	(74)
Ptf1-binding elements	Hey1	EMSA, Luc	Binding to Ptf1-p48 interferes with Ptf1-p48 DNA binding	Repression of acinar cell differentiation	(53,152)
SM22α SM-MHC	Hey1,2	Luc	Binding to Myocardin or GATA6 represses transcript. activity or prevents SRF binding to CArG box	Repression of endogenous gene in smooth muscle cells and 10T1/2 cells	(76,84,89)
VEGF	Hey2	EMSA, Luc	Binding to ARNT prevents ARNT/EPAS DNA binding		(28)
			Unknown		
Coup-TFII	Hey1,2	Luc		Downregulation under hypoxia (Hey induction)	(9)
GATA4,6	Hey1,2,L	Luc			(73)
Hey1,2,L	Hey1,2,L	Luc	Indirect: Binding to RBPJκ		(29,38,75)
Hypoxia response element	Hey1,2	Luc			(9,159)
Mdm2	Hey1	Luc		Hey1 causes p53 upregulation	(154)
RTA (virus)	Hey1	ChIP, Luc	Independent of DNA binding		(160)
Tbx2	Hey1	Luc	ND	Tbx2 repression in *Hey1* or *Hey2* overexpressing mice	(161)

Abbreviations: CAT, chloramphenicol acetyl transferase assay; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; FP, DNA footprint; Luc, luciferase assay.

At least some studies have already employed more rigorous tests to show that e.g. Hes1 binds to the *Mash1 and p27(KIP1)* promoters to regulate their activity (see Table 3). Importantly, these functions are well in line with the phenotypes observed in *Hes1* or *Hes5* knockout mice. Loss of *Hes1* leads to premature neuroendocrine differentiation due to absence of Hes1-mediated inhibition of the promoter of the neurogenic gene Mash1 (68,114,115). In the context of this promoter Hes1 may even function as an integrator of additional signals and become the core of an activator complex in later developmental steps (68). Another link to explain gene knockout phenotypes comes from the reduced inhibition of the cyclin-dependent kinase (CDK) inhibitor p27(KIP1), which seems to be a key factor leading to strongly reduced thymocyte proliferation and reduced thymus size in *Hes1*$^{-/-}$ embryos (43,116).

For Hes5, the direct and indirect repressive effects on the *myelin basic protein (MBP)* promoter correlate well with the upregulation of MBP in *Hes5*$^{-/-}$ brains. This on the other hand is consistent with the limited remyelination in patients with multiple sclerosis, where *Hes5* is highly expressed in immature oligodendrocytes of lesions (117). These examples clearly suggest that a set of several direct Hes or Hey target genes will be needed to fully explain the phenotypes of gain or loss of function alleles in various tissues.

CONCLUDING REMARKS

Almost thirty years of research on Hairy and related factors, lead to the publication of a few hundred papers,

providing a wealth of information. This survey gathers data on the progress on mouse models, protein–protein and protein–DNA interactions to faciliate future studies and to highlight questions that are still unresolved.

Although quite a number of cofactors are now known that are recruited by Hairy-related proteins, little is known about the dynamics of these interactions. While the Hes1/TLE1 interaction is necessary for neural stem cell maintenance, it dissociates during neuronal differentiation and Hes1 is turned from a repressor into an activator, which is at least partly mediated by post-translational modifications like phosphorylation (68). In addition, the repressive functions of Hes6 can be blocked by phosphorylation of a specific serine residue by CK2 (65). It appears obvious that such strategies might also be employed for tightly regulated complex formation and dissociation of Hes and Hey proteins with other factors dependent on the cellular context.

Furthermore, DNA-binding affinities of Hes and Hey factors may be regulated by kinases. Phosphorylation of a residue in the basic domain of Hes1 decreases DNA-binding affinity (118), implying that Hairy-related factors can be influenced by growth factor signaling cascades. During the last years it already became clear that there is a crosstalk between Notch and the BMP/TGF-beta, JAK/STAT, Ras and hypoxia signaling cascades in the regulation of Hes and Hey genes (7–11). Elucidation of the biological effects as well as the associated post-translational modifications of those interactions will be a challenge in the future.

The question of how Hes and Hey factors act as repressors when they bind to other transcription factors is also not fully answered. We have schematically summarized the current models in Figure 2. Another possible mode of repression, which was not explicitly mentioned so far, is Hes/Hey-mediated protein degradation of transcriptional activators. It has been proposed that the WRWP peptide of Hes6 mediates proteins degradation and Hes1 seems to induce rapid degradation of the Mash1 transcription factor (68,114,115). Furthermore, the half-life of BMP-induced Id1 is reduced upon Hey1 binding (119). Again, knowledge of protein modifications like ubiquitinylation or SUMOylation is elusive.

Finally, it will be challenging to analyze the cellular localization of these factors in more detail. It already became clear that Hey1 is located in the cytoplasm and the nucleus of benign prostate hyperplasia, but is excluded from the nucleus in many malignant prostate cancers (74). The cytoplasmic functions of bHLH transcription factors are unknown. It will be interesting to see, if these proteins possess additional functions besides transcriptional regulation.

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