

# Feminizing chicks: a model for avian sex determination based on titration of Hint enzyme activity and the predicted structure of an Asw-Hint heterodimer

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## Abstract

**Background:** In birds and some lizards, females are heterogametic with a ZW karyotype, while males are ZZ homogametes. The molecular basis for sexual differentiation in birds is unknown: arguments exist for doses of Z masculinizing chicks and for W information feminizing. ASW was identified as a tandemly repeated gene conserved on avian W chromosomes that is expressed in early female development and appears to be an inactive form of avian Z-encoded HINT. Hint is a dimeric enzyme that hydrolyzes AMP linked to lysine, whose enzyme activity is required for regulation of the Cdk7 homologous Kin28 kinase in yeast. Of 16 residues most conserved across all life forms for AMP interactions, 15 are sexually dimorphic in birds, that is, altered in the female-specific Asw protein. Genomic and expression data suggest that Asw may feminize chicks, dominantly interfering with Hint function by heterodimerization.

**Results:** We consider whether positive cooperativity could explain how Hint heterodimerization with an inert enzyme might reduce specific activity by more than 50% and provide data sufficient to reject this model. Instead, we hypothesize that Asw carries a signal for mislocalization and/or proteolysis, and/or dominantly suppresses the remaining Hint active site to function as a dominant negative.

**Conclusions:** Molecular modeling suggests that Asw and Hint can heterodimerize and that Gln127, an Asw-specific alteration for Trp123, dominantly interferes with the Hint active site. An extra dose of HINT in ZZW chicks, and thus more Hint homodimer, may partially overcome the feminizing influence of ASW and lead to the observed intersexual characteristics of ZZW triploids.

## Background

Dimorphic sexes are the norm in animals, although there are rotifer species that consist solely of parthenogenetic females [1], corals that produce long-lived clones that reproduce by fragmentation [2], and some lizards and fish that spin-off parthenogenetic lines that survive for multiple generations [3]. Among the vast number of animal species that

reproduce with males and females, sexual differentiation is controlled either chromosomally or environmentally. Flies and worms are among the invertebrates that determine sex by doses of X-chromosomal information. Within the vertebrates, there are several mechanisms that account for sexually dimorphic development. Because temperature controls sexual development in crocodiles, many turtles and some

lizards, environmental sex determination (ESD) has been proposed to be the primordial vertebrate mechanism for sexual differentiation [4]. Some reptiles and most birds and mammals have chromosomal sex determination (CSD) systems and these systems involve remarkably different chromosomes and genes [4]. CSD strategies, then, may have evolved to program genetically the developmental routines that occur in response to temperature in the vertebrates that use ESD. The natural history of vertebrates suggests that there were multiple solutions to the problem of CSD.

Nearly all mammals have the XX female and XY male system, with XXY individuals being male and Xo individuals female. These observations led to a search for the testis-determining factor on the Y chromosome culminating in isolation of the *SRY* gene [5], which is sex-determining in humans [6,7] and mice [8]. Beyond *SRY*, there are several autosomal genes including the *SRY*-related *SOX9* gene [9,10], *MIS* [11,12], *SF1* [13], *WT1* [14] and *DMRT1* [15] that contribute to sexual organ formation, alterations of which can cause male-to-female sex reversal in XY males. Because there are rodent species with no *SRY* gene and no Y chromosome [16], any of these genes or the X-linked *DAX1* gene [17] might be considered candidates for a sex-determining gene in the XX rodents such as mole voles whose body plan is not so different from that of mice.

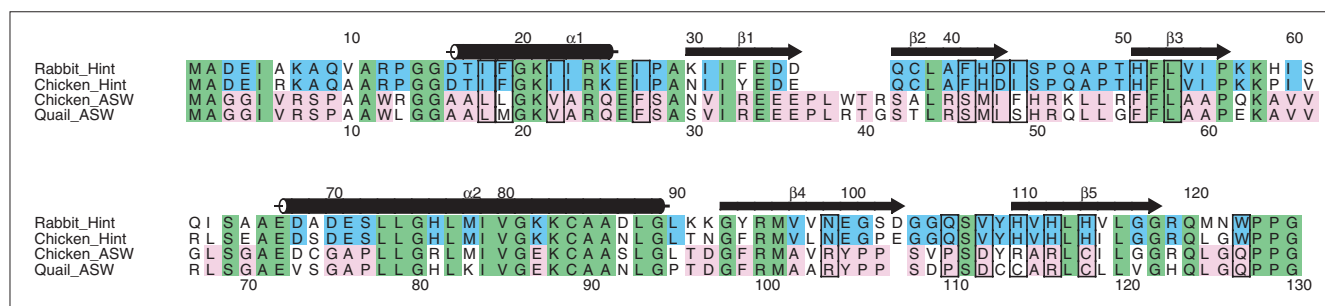
In birds, males are the homogametic sex, with two Z chromosomes, and females the heterogametic sex, with one Z and one W chromosome. Z and W are not related to mammalian X or Y chromosomes and, furthermore, it is not known if the W chromosome confers femininity and/or if doses of the Z chromosome confer masculinity [18]. In mammals and invertebrates, diploid animals of genotype Xo and XXY were crucial in establishing that doses of X confer femininity in invertebrates and that presence of Y confers masculinity in mammals. Diploid birds with one or three sex chromosomes were last reported 70 years ago [19], but triploid birds with ZZZ and ZZW karyotypes have been observed. While ZZZ triploids seemed like normal males that produced abnormal sperm, ZZW animals appeared female on hatching and then developed some male gonadal and behavioral characteristics as they matured [20]. These studies suggest that the dosage versus determining factor argument is a false dichotomy in birds: doses of Z chromosome appear to masculinize while presence of the W appears to feminize.

According to theory, a pair of autosomes can evolve into sex chromosomes by mutation of a control gene [4]. If a control gene confers sexual development by dosage, it might become lost from the alternative sex chromosome. *DMRT1* is a candidate dosage-dependent gene for masculinity in birds and alligators on the basis of Z-linkage [21], conservation, and gene-expression patterns [22]. Alternatively, if a control gene confers sexual development as a dominant determining factor, it may have evolved as an allele of a gene on the opposite sex

chromosome. This latter mechanism is thought to relate *SRY* to the X-linked *SOX3* gene in mammals [23].

Histidine triad (HIT) enzymes are a superfamily of nucleotide hydrolases and transferases that contain a catalytic motif related to the sequence His $\phi$ His $\phi$ His $\phi$  (where  $\phi$  represents a hydrophobic amino acid) and act on substrates containing a nucleoside monophosphate [24]. Branch 1 of the HIT superfamily includes the ubiquitous Hint enzymes [25] plus two enzymes with a more phylogenetically restricted distribution, namely Aprataxin, which is lost in humans with ataxia-oculomotor apraxia [26,27] and the scavenger mRNA decapping enzyme Dcps [28,29]. While Dcps enzymes are specific for hydrolysis of cap structures such as 7meGpppG [28,29], prototypical Hint enzymes such as rabbit Hint and yeast Hnt1 hydrolyze adenosine 5'-monophosphoramidate substrates such as AMP-lysine to AMP plus lysine [30]. Loss of this enzymatic activity renders yeast cells temperature-sensitive for growth on galactose medium and hypersensitive to mild mutations in the yeast homolog of mammalian Cdk7, that is, Kin28, the kinase component of general transcription factor TFIIF [30]. Loss of Hnt1 enzymatic activity also renders cells hypersensitive to mutations in the cyclin H homolog Ccl1, the MAT1 homolog Tfb3, and to Cak1, the activating kinase for Kin28, all of which lead to destabilized Kin28 complexes and a likely increase in concentration of Kin28 monomers [30]. Consequently, it was suggested that a Kin28 monomer is the likely target of Hint regulation, potentially because it is post-translationally adenylylated and is a protein substrate of the lysine-deadenylylating activity of Hint [24]. Indeed, two-dimensional electrophoretic analysis of Kin28 is consistent with Kin28 being subject to a post-translational modification in addition to phosphorylation [31,32] that appears to be controlled by *HNT1* genotype (A. Krakowiak and C.B., unpublished results). Finally, it is important to note that Hint is a dimer with two identical purine nucleoside-monophosphate-binding sites per dimer [25]. The amino-acid residues that have remained most constant throughout evolution are those that form the dimer interface and make direct contact with AMP [25].

Chickens and many other orders of birds are dimorphic for *HINT*-related genes on their sex chromosomes. The chicken Z chromosome contains the locus for a typical *HINT* gene, predicted to encode a polypeptide 83% identical to rabbit Hint [33]. However, on the gene-poor, female-specific W chromosome, the ASW (avian sex-specific W-linked) gene was identified [33,34], encoding a predicted Asw protein with striking similarity to Hint with the specific exception of residues involved in AMP recognition. As shown in Figure 1, the degree to which the nucleotide-binding site was altered is remarkable. Although human biochemists can depress activity more than 10<sup>5</sup>-fold in Fhit [35,36] and Hint [30] with a single active-site His mutation, hens apparently continued to peck at the sequence of Hint, altering all four of



**Figure 1**

Sequence alignment of Hint and Asw. Secondary structural elements are from the crystal structure of rabbit Hint [25]. The primary sequences of rabbit [40] and chicken [33] Hint are aligned with chicken [34] and quail [33] Asw. Green denotes identity among all sequences. Blue denotes identity between Hint sequences at residues not identical in Asw. Pink denotes identity between Asw sequences at residues not identical in Hint. Sixteen residues involved in AMP interactions, including 14 identified from Hint-nucleotide co-crystal structures [25] plus His110 and Trp123, are boxed. Note that 15 of 16 nucleotide-interacting (boxed) residues are chromosomally dimorphic (pink/blue) and that the dimer interface, which is located primarily in helix  $\alpha 2$  and strand  $\beta 4$ , is highly conserved.

the absolutely conserved His residues. In all, 15 of 16 normally conserved residues identified in immediate proximity to the adenine base, the ribose and the 5' phosphate [25] are altered in Asw.

ASW is absent in the ratites, emu and ostrich [34], which have indistinguishable sex chromosomes, but is tandemly repeated approximately 40 times on the W chromosome of all the non-ratite birds examined [33]. Confirming the subtractive manner in which ASW was cloned, both groups found that ASW mRNA is highly expressed in the female urogenital ridge at the stages preceding and during sexual differentiation [33,34]. Additionally, HINT mRNA is expressed at levels about two-fold greater in males than in females, in developing chicks more than in adult chickens, and at a message level one-seventh to one-tenth the level of ASW in stage-29 females [33]. ASW was cloned a third time as a message that is increased in cells lacking the splicing factor ASF/SF2 [37]. Surprisingly, ASF/SF2 did not affect splicing of the ASW message but rather destabilized the mRNA [37]. Involvement of ASF/SF2 in the regulation of ASW mRNA is interesting because sex-specific splicing of *doublesex* mRNA in *Drosophila*, a sex-determining gene for which the mammalian *DMRT* genes were named, is mediated in part by ASF/SF2 [38]. Although the initial cloning of ASW pre-dated knowledge that Hint is an enzyme, both groups suggested that Asw might function by a dominant-negative mechanism through heterodimerization with Hint, thereby inducing female development [33,34]. Heterodimerization was also discussed in the review literature [18], also without awareness that Hint function depends on enzymatic activity [30].

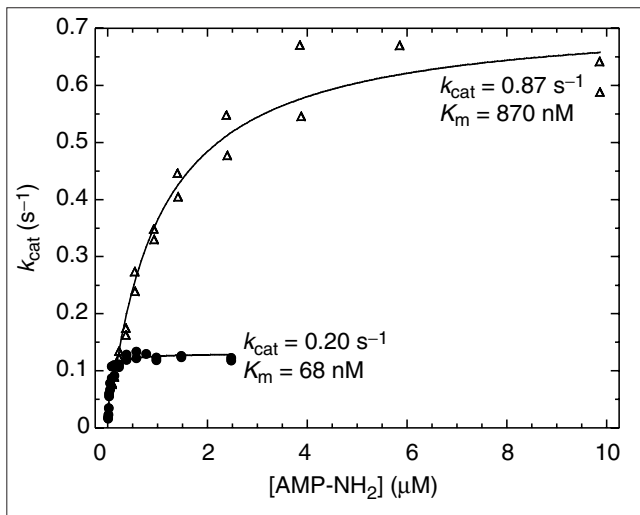
## Results and discussion

Genomic and expression data suggest that the purpose of Asw is titration of Hint function. How might this work? In the simplest case, a normally dimeric enzyme with two active

sites that is produced as a heterodimer with one good and one bad active site would be expected to have 50% of the specific activity of the homodimer. This is not a scenario for dominant negativity and, in fact, such a scenario does not explain why Asw is produced at all. If Asw were simply an inert dimerization partner for Hint, a 50% reduction in Hint cellular specific activity could be obtained if there were no ASW genes on the W chromosome. Males would have two doses of HINT and females one dose, such that twice as much Hint dimer could be made in males as in females. Furthermore, if ASW were simply a loss-of-function allele, given the paucity of genes on the W chromosome, sex-chromosome theory [4] suggests that such a gene would be lost. The repeated and highly expressed nature of the gene suggests that it has evolved to be dominantly interfering - the challenge is to determine the mechanism of dominance over the Z-encoded HINT.

One might expect a Hint-Asw heterodimer to have substantially less than 50% of the activity of a Hint homodimer if Hint homodimers showed cooperativity with respect to substrate binding and/or hydrolysis. For example, if the first nucleotide substrate were to bind weakly but the presence of the first bound substrate promoted efficient binding of a second substrate, then a heterodimer containing one functional and one nonfunctional active site would retain only one low-affinity binding site and be severely defective. This mechanism can be largely excluded, however, because sigmoidal substrate saturation kinetics were not observed with AMP-NH<sub>2</sub> [30]. The data for both rabbit and yeast Hint enzymes indicate that the dimer's two crystallographically defined nucleotide-binding sites [25] bind nucleotide independently and fit well to a single submicromolar K<sub>m</sub> for each enzyme (Figure 2) [30].

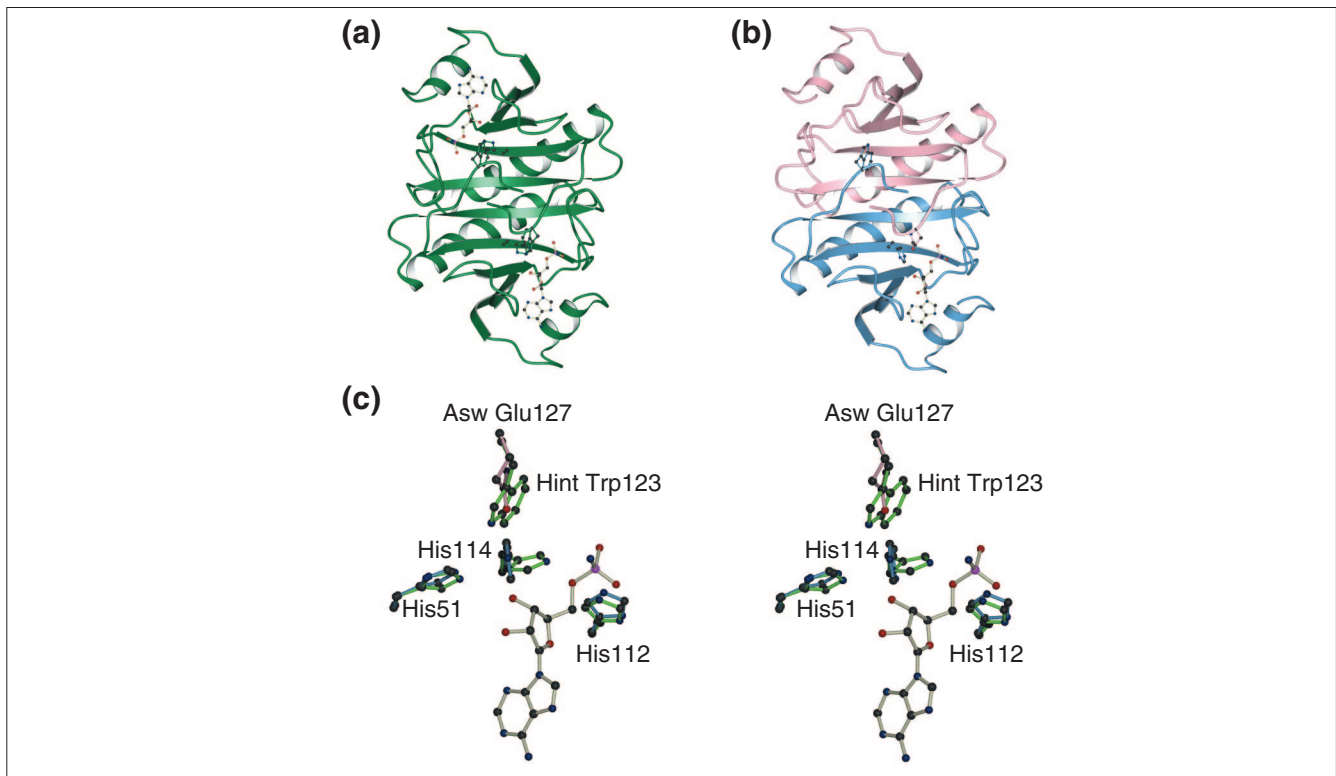
As cooperativity is difficult to invoke in this system, we consider that for Asw to titrate Hint enzyme activity by



**Figure 2**  
Hint hydrolases are not cooperative for AMP-NH<sub>2</sub> hydrolysis. Substrate concentration-dependent hydrolytic rates (per monomer) for rabbit Hint (filled circles) and yeast HntI (open triangles) indicate that Hint hydrolases, although dimeric, have a single K<sub>m</sub> for substrate. Reproduced with permission from [30].

heterodimerization, Asw must carry a signal for mislocalization and/or proteolysis, and/or somehow alter the Hint active site. We therefore constructed a molecular model of the proposed Hint-Asw heterodimer by superimposing the chicken Hint sequence on the determined X-ray structure of rabbit Hint, and threading and minimizing the Asw sequence on the opposing monomer. This analysis suggested: first, that Hint and Asw do retain sufficient sequence identity at the dimer interface to form a heterodimer; second, that Asw has an insertion sequence at the bottom of the dimer that could be a site of alternative localization or proteolysis; and third, that Gln127, a residue that Asw has substituted for Trp123 of Hint, may interfere with the function of His114 in Hint across the dimer interface. Although altered specificity of a putative Hint-Asw heterodimer is conceivable, the simplest enzymatic mechanism for dominant interference is that Gln127 from Asw depresses activity from the Hint active site.

The dimer interface of Hint is formed by antiparallel interactions between helix  $\alpha 2$  and its symmetry mate and strand  $\beta 4$  and its symmetry mate [25]. These sequences are contiguous in the primary sequence of Hint and form the region of



**Figure 3**  
Proposed structural basis for dominant negativity by Asw. (a) Hint dimer with conformations of His114 and Trp123 as determined crystallographically [25] and a model of adenosine 5'-monophosphoramidate substrates in ball-and-stick representation. (b) Proposed structure of an Asw (pink)-Hint (blue) heterodimer with predicted conformation of Asw Gln127 extending into the vicinity of Hint His114 and the bound Hint substrate. (c) Stereo view of a close-up superposition of the Hint homodimer (green) depicted in (a) with the Asw-Hint heterodimer (pink and blue) depicted in (b). The amino acids in green are in the active Hint homodimer conformation. Note that Asw Gln127 (in pink) is proposed to alter the conformation of Hint His114 (in blue) such that catalysis from the Hint active site is depressed.

greatest identity with Asw (Figure 1). Apart from the curious substitution of 15 of 16 nucleotide-proximal residues in Asw, the most dissimilar region of Asw consists of a five amino-acid insertion between strands  $\beta 1$  and  $\beta 2$  and substitution of Gly-Ala-Pro (Asw) for Asp-Glu-Ser (Hint) at the amino-terminal end of helix  $\alpha 2$ . Both these changes are located on the bottom surface of the dimer [25]. The bulky amino acids in the Asw insertion (Pro-Leu-Trp-Thr-Arg), which in Hint is an extremely tight  $\beta$  turn, may be a handle for altered localization or proteolysis. Cellular localization of green fluorescent protein (GFP) fusions to Hint and Asw was investigated in male chick embryo fibroblasts [33]. GFP-Hint was found to be distributed in the cytoplasm and the nuclei, though somewhat concentrated in the nuclei with respect to the GFP control [33]. GFP-Asw was found to be essentially confined to nuclei [33]. Though localization of Hint in Asw-overexpressing cells was not examined, if Hint has extranuclear (that is, Cdk7-exclusive) functions, then excluding Hint from the cytoplasm may be an important function of Asw.

The crystal structure of nucleotide-bound forms of rabbit Hint showed that the carboxy-terminal amino acids of each Hint monomer extend across the dimer interface and are buried near the opposing nucleotide. The Trp residue in the extreme carboxy-terminal Trp-Pro-Pro-Gly motif is buried near His114, the final His of the HIT motif, of the opposing monomer [25]. His114 corresponds to His98 of Fhit, which was localized in low barrier hydrogen-bonding distance to the  $\alpha$ - $\beta$  bridging oxygen of a bound ApppA analog in co-crystal structure with Fhit His96Asn [35]. On the basis of mutagenesis of the conserved His residues in Fhit [39] and the location of these His residues in crystal structures of Hint and Fhit [25,35], a catalytic role was proposed for His114 [24]. The structures show that His51 is in the same plane as His114, with the  $\epsilon$ N of His 51 positioned to accept a hydrogen bond from the  $\delta$ N of His114. This geometry was considered critical for the enzymatic activity of Hint and Fhit. As shown in Figure 1, Asw sequences contain a substitution of Gln127 for Trp123 of Hint. As shown in Figure 3, Gln127 of Asw is predicted to alter the conformation of Hint His114 such that His114 is not positioned for productive hydrogen bonding, dominantly depressing hydrolase activity from the Hint active site. In short, loss of the AMP-binding site is predicted to account for negativity of Asw. Gln127 and possibly insertion sequences that may have a role in stability or localization are predicted to make Asw dominant.

## Conclusions

Identification of sex-linked *ASW* and *HINT* genes in birds has raised new experimental questions. It will be interesting to learn whether Asw and Hint heteromultimerize and what the *in vitro* and *in vivo* stabilities of heterooligomers are with respect to the homodimers. It will be interesting to determine whether Asw homodimers display any binding to Hint substrates, what degree of Hint enzyme activity is retained by the

putative Hint-Asw heterodimer, and whether Gln127 is required for depression of Hint enzymatic activity in Hint-Asw heterodimers. Turning to genetic analysis, if *ASW* has a significant role in feminization of birds, then viruses that increase expression of Asw may promote female development in ZZ eggs, potentially in a manner that requires Gln127. If *HINT* is part of the Z chromosome that works by gene dosage, then viruses that direct expression of Hint may promote male or intersexual development in ZW eggs as was seen with ZZW triploids [20]. Finally, if it is true that Hint enzyme activity is important in making ZZ chicks male or if Hint inhibition is important in making ZW chicks female, then it will be interesting to learn whether Hint is involved in establishment or maintenance of sex in other animals.

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